The Endocannabinoid Anandamide
Metabolism & Neuroprotection

Het Endocannabinoïde Anandamide
Metabolisme & Neuroprotectie

(met een samenvatting in het Nederlands)

Proefschrift

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Logic will get you from A to B.
Imagination will take you everywhere.
- Einstein -

Voor mijn ouders

Contents

Abbreviations:

AA, arachidonic acid

2-AG, 2-arachidonoylglycerol

ADC, apparent diffusion constant

AEA, anandamide (arachidonoylethanolamide)

BSA, bovine serum albumine,

cAMP, cyclic adenosinemonophosphate

CB receptor, cannabinoid receptor

CBD, cannabidiol

CCCP, carbonyl cyanide *m*-chlorophenylhydrazone

CD, circular dichroism

CNS, central nervous system

(CR)EAE, chronic relapsing experimental allergic encephalomyelitis

DSE, depolarization-induced suppression of excitation

DSI, depolarizition-induced suppression of inhibition

DW, diffusion weighted,

ELISA, enzyme-linked immunosorbent assay

EPSC, excitatory postsynaptic currents

FAAH, fatty acid amide hydrolase

GABA, γ-aminobutyric acid

GAR-AP, goat anti-rabbit alkaline phosphatase conjugate

GC/MS, gas chromatography / mass spectrometry

GFAP, glial fibrillary acidic protein

IEF, isoelectric focusing;

IP$_3$, inositoltriphosphate

IPSC, inhibitory postsynaptic currents

LC-MS, liquid chromatography mass spectrometry

LSC, liquid scintillation cocktail

LTXy, leukotriene X_y

MD, molecular dynamics,

MEM, (Eagle's) minimal essential medium (plus Earle's salts)

MRI, magnetic resonance imaging

mRNA, messenger RNA

NAE, N-acylethanolamine

NAPE, N-acylphosphatidylethanolamine

NMDA, N-methyl-D-Aspartate

NMR, nuclear magnetic resonance,

NO, nitric oxide

NOESY, nuclear overhauser effect spectroscopy

NONOate, (*Z*–1-{N–[3–aminopropyl]–N–[4–(3–aminopropyl–ammonio)butyl]–amino}–diazen–1–ium–1,2–diolate);

PBS, phosphate buffered saline

PGXy, prostaglandin Xy

pI, isoelectric point

PLD, phospholipase D

PMSF, phenylmethylsulfonyl fluoride

PND, post natal day

QSAR, quantitative structure activity relation

RP/CP–HPLC, reversed phase / chiral phase high performance liquid chromatography

RT–PCR, reverse transcriptase polymerase chain reaction

SD, standard deviation

SEM, standard error of the mean

SNAP, S–nitroso–N–acetylpenicillamine

SNP, sodium nitroprusside

SPE, solid phase extraction

SPER–NO, spermine

TE, time of echo

THC, tetrahydrocannabinol

TMS, trimethylsilyl

TNF, tumor necrosis factor

TOCSY, total correlation spectroscopy

TR, time of repetition

VR$_1$, vanilloid receptor

x–H(P)AEA, x–hydro(pero)xy anandamide

Anandamide, a lipid with cannabinoid activity

1.1 Historical background

Marijuana is the most widely used illegal drug throughout the world and has aroused great controversies. In the Netherlands, public debate centres upon the possible legalization of marijuana for recreational and therapeutic uses. Nowadays, marijuana is used by multiple sclerosis patients to alleviate tremors and by aids patients as well as cancer patients with chemotherapy to stimulate appetite and to relieve nausea. However, in 1996, the Health Council of the Netherlands came to the conclusion, based on a literature survey, that the scientific evidence to justify the medical use of marijuana was insufficient[1]. The Council concluded that physicians cannot accept responsibility for a product of unknown composition that has not been subjected to a quality control. The Committee was unable to comment on the use of any other preparation of the hemp plant, and its active ingredient Δ^9-tetrahydrocannabinol (THC, Fig. 1 and 2), or other components, since there were no published reports[1]. Despite this advice the Dutch government has recently approved the use of marijuana for medicinal purposes. In this introduction we will give an overview of the latest developments in cannabinoid research, which may give rise to a new assessment of this issue.

Marijuana (cannabis, bangh, hashish) is an extract from the plant *cannabis sativa* and contains at least 400 chemical components of which 60 belong to the cannabinoid class[2]. Marijuana and its main psychoactive compound THC, have been used for centuries. A first description of its use came from the Chinese nearly 5000 years ago. Cannabis was cultivated for its fibre and oil of the seeds. It was never widely used as a psychoactive substance in China, because it disturbed the equilibrium of the brain and made one "see devils"[3]. Ancient Hindus hailed the medicinal properties of the plant, which was credited with curing all major ills and creating "vital energy"[3].

It took until 1964 before THC was isolated and characterized[4]. The structure elucidation of THC led to the design of a series of analogs. More than 300 cannabinoid derivatives were synthesized and used to study the structure activity relationships for psychotropic activity by 1986[5]. In addition, a completely different class of molecules, termed aminoalkylindoles, was discovered which could elicit strong cannabimimetic effects (Fig. 2). The main representative of this class is WIN55.212-2 .

The mechanism of action of THC has also been a subject of discussion. At first it was thought that cannabinoids due to their lipophilic nature perturbed the membrane, thereby producing cellular effects. However, the requirement of a specific enantiomer and stringent structural features of THC to produce pharmacological responses raised the question whether cannabinoids could act via membrane receptors. The development of the potent bicyclic cannabinoid CP55.940 (Fig. 2), which could be radio-labelled, allowed Devane *et al.* to demonstrate selective and specific binding sites in the brain for the first time in 1988[6]. The identification and cloning of a receptor responsible for this binding followed within two years[7]. This receptor, which is highly expressed in the central nervous

2-Arachidonoylglycerol

Δ^9-Tetrahydrocannabinol

Anandamide

CB$_1$

cellular effects
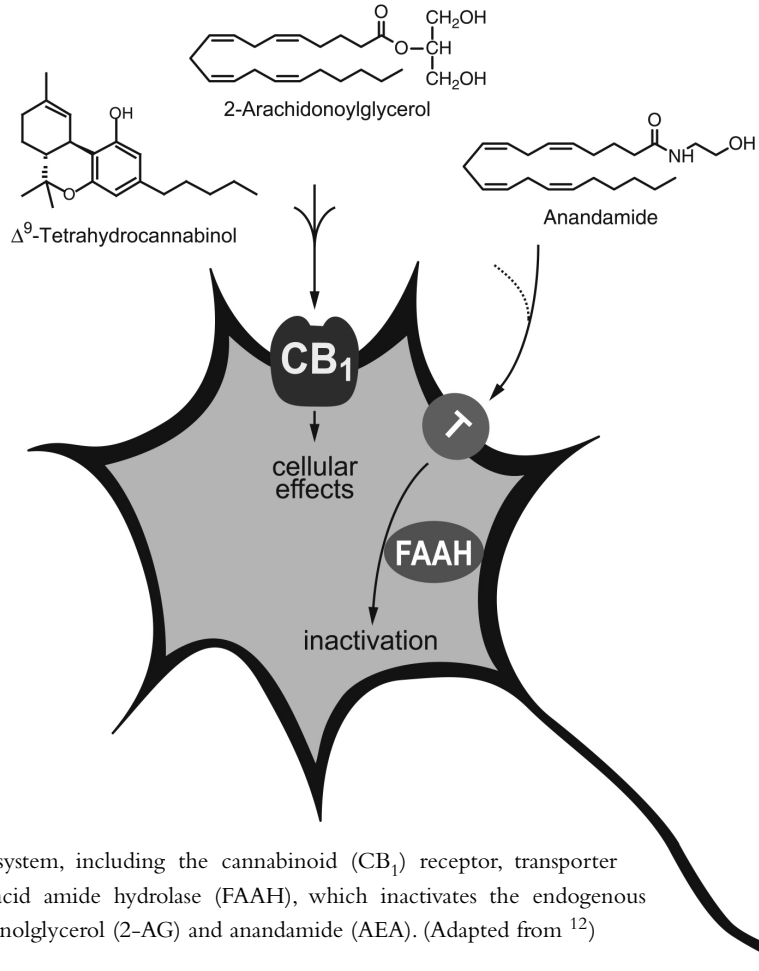
FAAH

inactivation

The endocannabinoid system, including the cannabinoid (CB$_1$) receptor, transporter protein (T) and fatty acid amide hydrolase (FAAH), which inactivates the endogenous cannabinoids 2-arachidonolglycerol (2–AG) and anandamide (AEA). (Adapted from [12])

system (CNS), was termed cannabinoid (CB$_1$) receptor. The existence of another subtype cannabinoid receptor(CB$_2$) was demonstrated in 1993[8]. The CB$_2$ receptor is primarily found in cells of the immune system[8,9].

The presence of cannabinoid receptors in mammalians suggested the existence of endogenous ligands for these receptors. In 1992, the first endogenous ligand for these receptors was isolated. The chemical structure of this component proved to be an amide of ethanolamine and arachidonate, and named anandamide (ananda is Sanskrit for internal

bliss, Fig. 1 and 2)[10]. Anandamide (AEA; $5Z,8Z,11Z,14Z$-eicosatetraenoyl–N–(2-hydroxy-ethyl)-amine was able to inhibit the *vas deferens* twitch-response, a typical cannabinoid action. Furthermore, AEA could be synthesized by neurons upon stimulation and it mimicked many biochemical, pharmacological and behavioural properties of classical cannabinoids (Fig. 1 and 2)[11,12].

Three years after the discovery of AEA, 2-arachidonoylglycerol (2-AG) was found as a second endogenous ligand for the cannabinoid receptors[13,14]. AEA and 2-AG are termed endocannabinoids. Very recently a third endogenous compound, 2-arachidonoyl glyceryl ether, was isolated from porcine brain, which could activate CB_1 receptors[15].

In order to function as a neuromodulator or as a neurotransmitter the concentrations of endocannabinoids have to be regulated in a strict manner. A reuptake process into cells and a rapid enzymatic inactivation have been reported (Fig. 3). In 1996 the enzyme responsible for the degradation of AEA by hydrolysis of the amide bond was purified, cloned and termed fatty acid amide hydrolase (FAAH)[16]. The CB receptors, endocannabinoids and the proteins of the inactivation process constitute the endogenous cannabinoid system. To date, the proteins responsible for the biosynthesis of AEA and its facilitated diffusion into cells have not been purified or cloned. The discovery of the cannabinoid system together with the development of the selective antagonists SR141716 and SR144528 for CB_1 and CB_2 receptors, respectively, have led to an explosion of knowledge on the molecular mode of action of cannabinoids[17,18]. Its (patho)physiological role is beginning to be unravelled. It is thought that the endocannabinoid system is involved amongst others in physiological processes, such as the regulation of pain, motor activity, blood pressure, appetite and tumour cell growth[19-22].

This introduction will focus on the biosynthesis, metabolism and function of endocannabinoids, in particularly AEA, and CB receptors in relation to neuronal cell injury and the current knowledge of (endo)cannabinoids in neuroprotection.

1.2 Biosynthesis of anandamide

In contrast to most other neurotransmitters, AEA is not stored in vesicles, but is released from cells upon demand by stimulus-dependent cleavage of membrane phospholipid precursors[11]. The biosynthesis of AEA has been demonstrated to occur in several cell-types, *e.g.* N18 neuroblastoma cells, J774 mouse macrophages, RBL-2H3 basophiles and in mesenteric arteries[23]. Astrocytes do not seem to be able to synthesize AEA. Stimulation of striatal neurons and J774 macrophages with the calcium ionophore ionomycin increased AEA content. Importantly, AEA, but not 2-AG, has been shown to be released in the striatum of freely moving rats upon stimulation of dopamine D_2-like receptors[21]. However, stimulation of glutamate-releasing fibers in the Schaffer collaterals in the hippocampus did not increase AEA concentrations, while 2-AG content was

AEA

2–AG

Noladin–ether

THC

CBD

CP55940

WIN55.212–2

SR141716A

SR144528

15S–HAEA

12S–HAEA
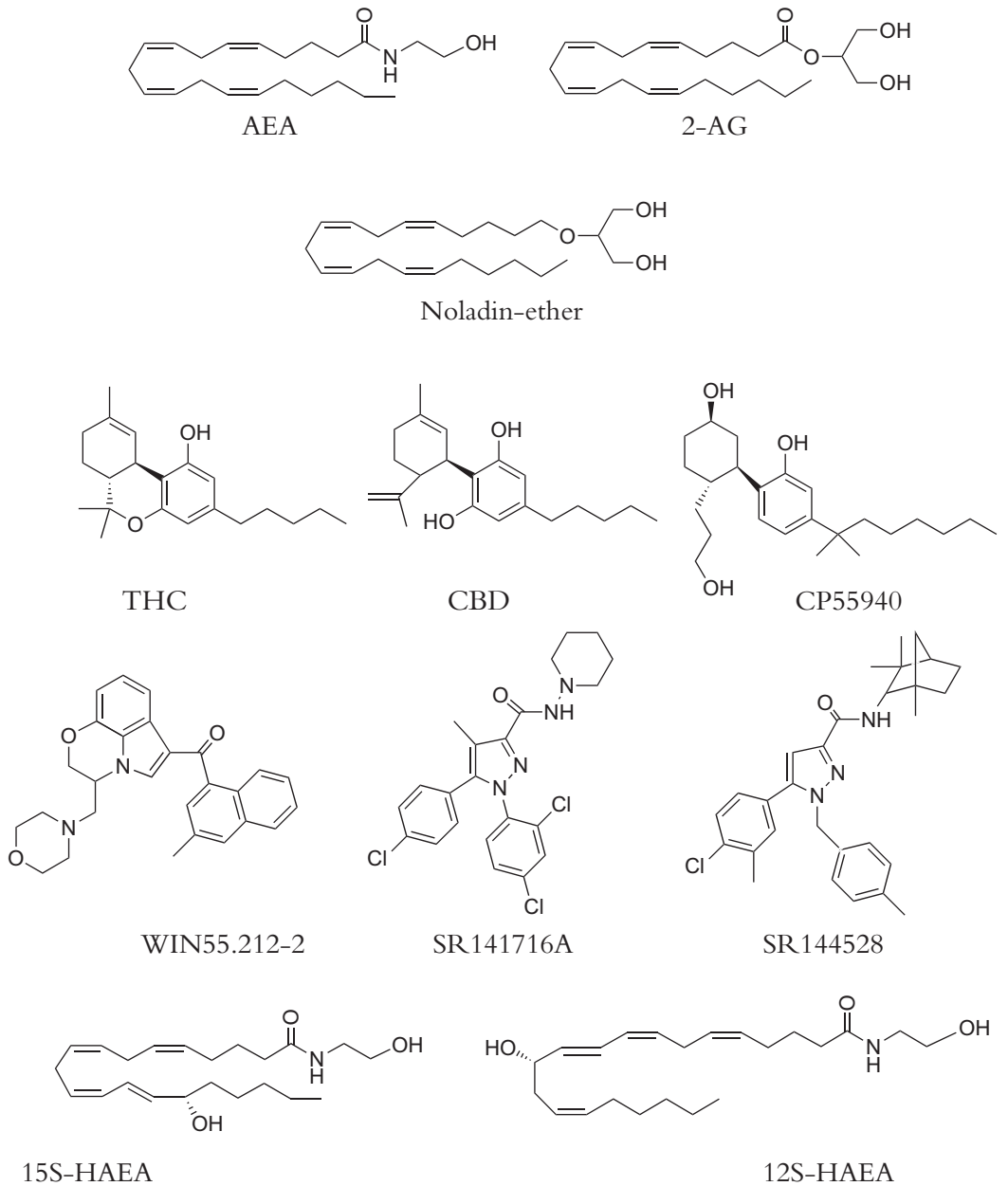
increased [24].

Already in the early 80's Schmid et al. had characterized a biosynthetic pathway for N-acylethanolamines. According to this scheme acylethanolamides, including AEA and linoleoylethanolamide (18:2), are formed through sequential N-acylation and phosphodiesterase activities (Fig. 3). A membrane-bound calcium-dependent transacylase catalyses the transfer of fatty acids from the sn-1 position of various 1,2-diacyl glycerophospolipids and 1-acyl-lysophospholipids to yield N-acyl(lyso)phosphatidyl-ethanolamines (NAPEs). As yet, the N-acyltransferase is not purified or cloned, but several properties of this enzyme are known. The N-acyl transferase activity is membrane-associated and energy-independent; neither free fatty acids nor acyl-CoA derivatives serve as the acyl donor. The N-acyltransferase activity in brain is calcium-dependent at concentrations between 0.5 and 2 mM $CaCl_2$. This suggests that the N-acyltransferase is quiescent at resting concentrations of calcium in the cell and is only activated when cellular concentrations rise to very high values. NAPE synthesis is inhibited by the calcium chelator EGTA, but not by inhibitors of calcium/calmodulin-dependent protein kinases or protein kinase C. Activators of adenylyl cyclase, such as forskolin, have no effect alone, but enhance ionomycin-induced NAPE synthesis in intact cells. The activity of N-acyltransferase changes considerably during development of rat brain being high in infant rats and several fold lower in adult rats (reviews[25,26]).

The formation of NAPEs seems to be rate limiting. Enzymatic hydrolysis of the corresponding NAPEs by a phosphodiesterase of the phospholipase D-type, which seems to be an unregulated enzyme, generates the corresponding acylethanolamide (Fig.3). In rat brain NAPE phospholipase D activity increases with development being very low at birth and increasing to a 15-fold higher level in the adult rat. It is important to consider that so far no fatty acid selectivity for either the enzymes involved in the biosynthesis or hydrolysis of N-acyl-PE could be demonstrated. Furthermore, very low amounts of arachidonic acid are found at the sn-1 position of phospholipids. It is almost exclusively in the sn-2 position of brain phospholipids. (reviewed by Schmid et al.[25,26]). Any selectivity in AEA generation must therefore rely on other mechanisms. For example, physiological stimuli might increase arachidonic acid content at the sn-1 position. Recently, it has been shown that the simultaneous application of glutamate and carbachol (an acetylcholine receptor agonist), but not of either agent alone, caused a marked increase in AEA biosynthesis in cortical neurons[27]. Thus, membrane depolarization was necessary for the biosynthesis, but was insufficient per se to initiate AEA biosynthesis. Interestingly, AEA-formation was stimulated by coactivation of α_7-muscarinic receptors, while palmitoylethanolamide and oleoylethanolamide levels were increased by coactivation of muscarinic receptors[27]. However, it remains unclear where this selectivity was generated, because the distribution of fatty acids in NAPEs and at the sn-1 position of donor-phospholipids was not studied.

AEA concentrations are very low in brain homogenates and have been shown to vary

in different brain regions. Highest AEA concentrations were found in human (2h *postmortem*) hippocampus (110 pmol/g) and thalamus (75 pmol/g) and somewhat lesser amounts in striatum (55 pmol/g) and cerebellum (30 pmol/g)[28]. The levels of AEA measured in human brain were at least 10-fold lower than those reported for GABA and glutamate. In rat brain AEA concentrations varied from 0-87 pmol/g, being highest in the striatum and lowest in cerebellum and cortex. AEA accounts for a smaller part of the total NAE, usually between 1-10%. (reviewed by Hansen et al.[29])

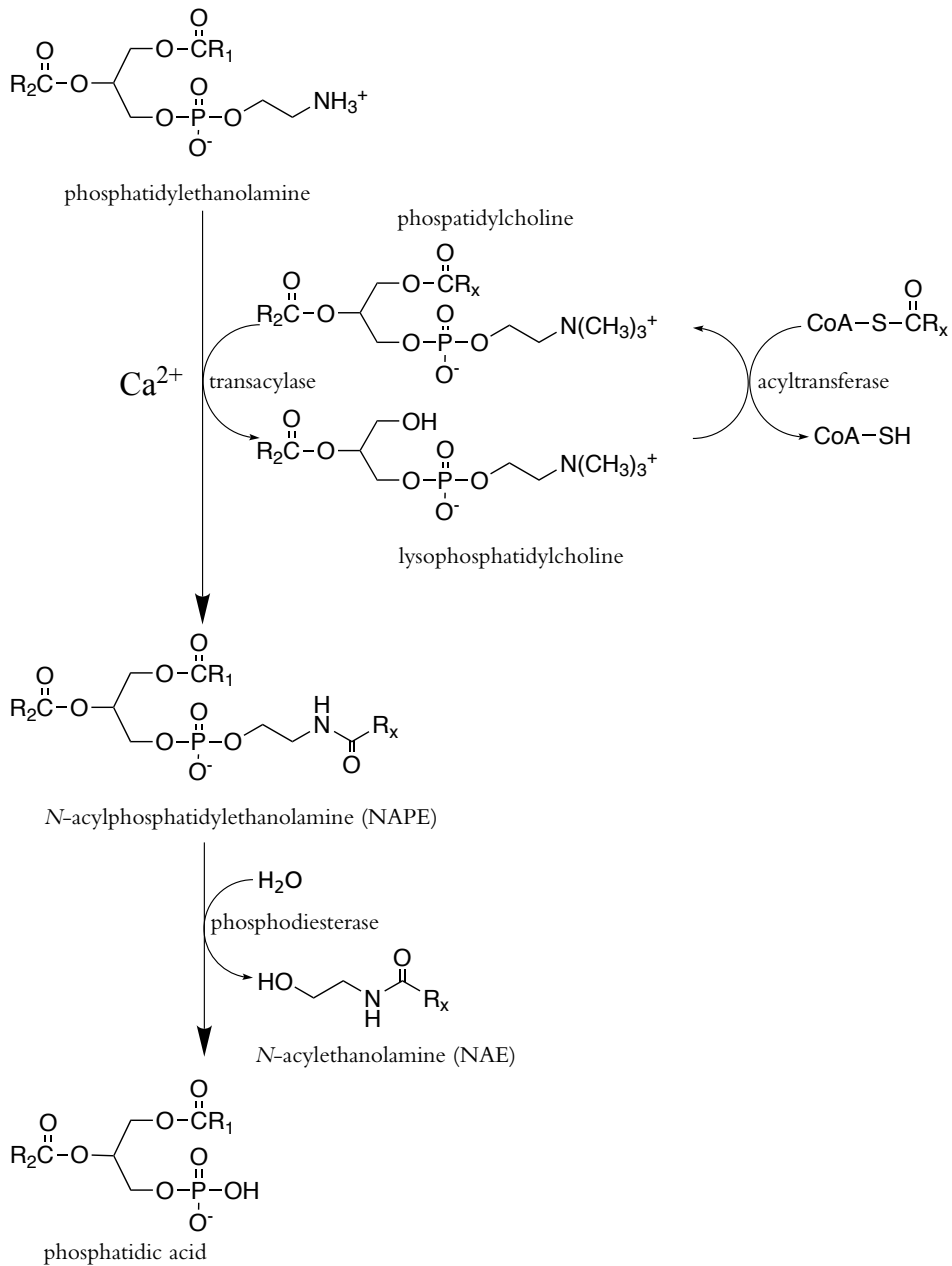## 1.3 Formation of endocannabinoids in models of neuronal injury

It should be noted that the variations of AEA levels in brain do not necessarily reflect differences in AEA content of "anandamergic" neurons. It is possible that differences in the analytical methods and tissue preparation caused the observed variations. It is known that when the time between death of animal and the lipid extraction procedure increased, AEA content increased. While *postmortem* increase in brain AEA levels may be an artifact of tissue damage, it is also possible that similar mechanisms may regulate AEA concentrations in living tissue that has been subjected to ischemic or hypoxic stress. Various studies indicate that *in vivo* and *in vitro* levels of NAPEs and NAE increased after toxic stimuli, *e.g.* in ischemic canine hearts and in inflamed testes[25,30,31]. In primary neocortical neurons in culture treated with *N*-methyl-D-aspartate (NMDA) or glutamate AEA levels were also increased[32]. Hansen *et al.* have demonstrated that the increase in NAPEs and NAEs varied in different *in vivo* models of neuronal damage and is dependent on the type of cell death[33]. High levels of NAPEs were found in neonatal rats 24 h after receiving a striatal injection of NMDA (25 nmol). In the same model, total NAE levels were increased 46-fold in cerebral cortex[34]. While AEA concentrations were elevated by a factor 13, no increase in 2-AG levels was found. Mild closed head injury or NMDA receptor blockade, which is a model for apoptotic cell death, produced a less pronounced NAE accumulation and had no effect on 2-AG levels[34]. In contrast, a large increase in 2-AG was found in a closed head injury model in mice[35] and in rats treated with picrotoxinin[36]. Differences in species, brain areas analyzed and type of injury may explain the varying results.

1.4 Metabolism of anandamide

1.4.1 Transport into cells and enzymatic degradation
After release, AEA and 2-AG may be eliminated by a two-step mechanism consisting of carrier-mediated transport into cells followed by enzymatic hydrolysis (Fig. 4). Neuronal and non-neuronal cells rapidly take up extracellular AEA through a mechanism

Figure 3   Biosynthetic pathway of N-acylethanolamines (NAE), including AEA (Rx=20:4)

phosphatidylethanolamine

phospatidylcholine

Ca^{2+} | transacylase

acyltransferase

lysophosphatidylcholine

N-acylphosphatidylethanolamine (NAPE)

H_2O

phosphodiesterase

N-acylethanolamine (NAE)

phosphatidic acid

that meets four key criteria of carrier–mediated transport: fast rate, temperature dependent, saturability, and substrate selectivity[37,38]. Importantly and in contrast with transport systems for classical neurotransmitters, [^{3}H]AEA reuptake is neither dependent on external Na^+ nor affected by metabolic inhibitors, suggesting that a process of carrier–facilitated diffusion may mediate it. This suggests that AEA movement across cellular membranes should be bi–directional. [^{3}H]AEA uptake was not affected by fatty acids, neutral lipids, neurotransmitters, biogenic amines, prostaglandins, leukotriens and was insensitive to substrates or inhibitors of fatty acid transport, organic anion transport, P–glycoproteins (reviewed by Hillard[39]). To date, the protein responsible for [^{3}H]AEA uptake has not been purified or cloned, but its activity has been demonstrated in human umbilical vein endothelial cells, RBL–2H3, cells, porcine leukocytes, a human astrocytoma cell line, primary cultures of rat cortical neurons or astrocytes and rat brain slices[38-42]. To date, no data are available about [^{3}H]AEA uptake in human neuronal and immune cells.

Once inside the cell AEA is rapidly inactivated by hydrolysis of its amide bond to arachidonic acid and ethanolamine. An amidohydrolase activity was already identified by Schmid et al. in rat liver in 1984. After the discovery of AEA, biochemical evidence suggested that the same enzyme activity could degrade AEA[11,43]. This activity has been characterized in rat neuronal and RBL–2H3 cells and in rat, porcine and dog brain homogenates. The gene responsible for the enzyme activity has recently been cloned from rat, mouse and human liver cDNAs allowing molecular mass determination and substrate specificity analysis of the enzyme[44]. The enzyme is termed fatty acid amide hydrolase (FAAH) and has been shown to degrade a broad spectrum of fatty acid amides and esters among them were oleamide (a novel sleep inducing factor), palmitoylethanolamide and 2–arachidonoylglycerol[16,45]. FAAH is an intracellular membrane-bound 64 kDA protein with a conserved amidase sequence. A consensus class II SH3 domain binding sequence was also identified suggesting that other proteins may interact with FAAH to regulate its activity and/or subcellular localization. The rank order in activity in homogenates of different rat brain areas was: globus pallidus > hippocampus > sustantia nigra > striatum > thalamus > cerebellum > cortex > brain stem > medulla[46,47]. CB$_1$ receptors are present in various brain regions that also express FAAH, but there appears to be no direct correlation between the concentrations for these two proteins[47]. Recently, FAAH knock-out mice have been bred[48]. These mice were severely impaired in their ability to degrade AEA and when treated with AEA exhibited an array of intense CB$_1$–dependent behavioural responses, including hypomotility, analgesia, catalepsy and hypothermia. FAAH$^{-/-}$ mice possessed 15-fold augmented endogenous brain levels of AEA and displayed reduced pain sensation, which was reversed via SR141716[48]. In contrast, to date no information is available on the inactivation process of AEA in human brain tissue and cells.

1.4.2 Oxidative metabolism

In mice AEA has been shown to be metabolized within a few minutes to arachidonic acid and to more polar derivatives, but the pharmacological effects lasted for a longer time period[49-52]. It is thought that some of the metabolites of AEA may produce some of the pharmacological effects. The identity of these metabolites was never investigated. It is known that AEA with its unmodified arachidonic acid backbone is a substrate for cytochrome P450s, cyclooxygenases and mammalian lipoxygenases, which produce more polar metabolites. To date very little information is available about the oxygenated AEA–derivatives.

AEA was shown to be oxidized by cytochrome P_{450} to yield at least 20 different metabolites, however, most of their structures were not identified. The P_{450}–metabolism was partly inhibited by the addition of the non–psychoactive cannabinoid CBD[53-55]. Arachidonic acid can be converted into a large array of bioactive eicosanoids such as prostaglandins, prostacyclins and thromboxanes. It is unknown whether AEA is converted into all these types of metabolites, but selective cyclooxygenase inhibitors, like ibuprofen, have been shown to inhibit the metabolism of AEA[56]. Furthermore, cyclooxygenase-2, which normally catalyzes the formation of the prostaglandin PGE_2, converted AEA into PGE_2-ethanolamide, whereas cyclooxygenase-1 was inactive[57]. The chemically synthesized putative cyclooxygenase products of AEA, *i.e.* ethanolamides of PGE_2, PGA_2, PGB_1 and PGB_2, failed to bind to the CB_1 receptor[58].

AEA has been shown to be a substrate *in vitro* for porcine leukocyte 12-lipoxygenase [59] and rat pineal gland 12-lipoxygenase[60]. Human platelets converted AEA into 12-hydroxyanandamide (12-HAEA) and human polymorphonuclear leukocytes oxygenated AEA to 12-HAEA and 15-hydroxyanandamide (15-HAEA)[61]. Furthermore, the affinities of 12-HAEA and 15-HAEA for rat CB_1 and human CB_2 receptor were reported[60,61]. The

Figure 4   Reaction scheme of inactivation of endocannabinoids
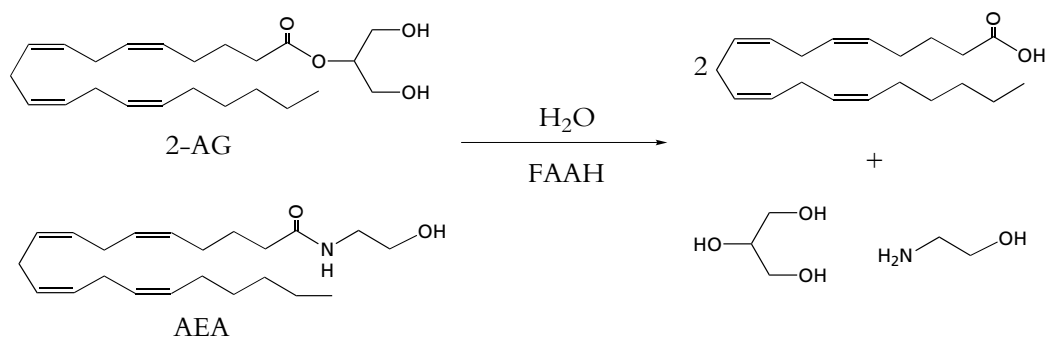
presence of endogenous lipoxygenase metabolites of AEA has never been detected, but recent data raise the possibility that the contractile action of AEA in guinea–pig bronchus may be due, at least in part, to some oxygenated metabolites of AEA[62].

1.5 CB_1 and CB_2 receptor expression

Many of the actions of AEA are mediated via either the CB_1 or CB_2 receptor. The cannabinoid receptors belong to the class of G-protein coupled receptors. CB_1 and CB_2 are the only two cannabinoid receptors so far identified, but recent pharmacological and electrophysiological data in CB_1 knock–out mice suggest that a novel CB-type receptor(s) may exist[63-65]. A splice variant of CB_1 cDNA CB_{1a} has also been isolated. CB_{1a} mRNA exists only as a minor transcript and there is no evidence for any notable differences between the pharmacology of CB_1 and CB_{1a} receptors[66]. CB_1 receptor has been detected both in the central nervous system and in certain peripheral tissues. The central distribution pattern of CB_1 receptor is heterogeneous and was found in high levels in brain regions thought to mediate typical cannabinoid agonist effects, *i.e.,* impairment of motor behaviour, memory and cognition (Fig. 5). In many brain areas specific cannabinoid binding sites density greatly exceeded that of neuropeptide receptors and is similar to the densities of cortical benzodiazepine, striatal dopamine and whole brain glutamate receptors. The CB_1 receptor is found at high or moderate levels in the cerebral cortex, hippocampus, lateral caudate putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus and the molecular layer of the cerebellum[67-69]. CB_1 transcripts are also found in peripheral tissues such as, pituitary gland, adrenal gland, heart, lung, prostate, uterus, ovary, testis, bone marrow, thymus and tonsils[70].

In situ hybridisation using labelled oligonucleotides to CB_2 have suggested that CB_2 transcripts are not present in brain tissue. CB_2 receptor mRNA has primarily been found in immune tissues, with an expression level 10-100x higher than that of CB_1 in these tissues[9]. In spleen and tonsils, the CB_2-transcript content reaches levels equivalent to that of CB_1-mRNA in the central nervous tissue. Among the human blood cell populations CB_2 receptor was found in B-cells > natural killer cells > monocytes > polymorphonuclear neutrophils > T8 cells > T4 cells[9].

Figure 5   Transversal view of human brain.
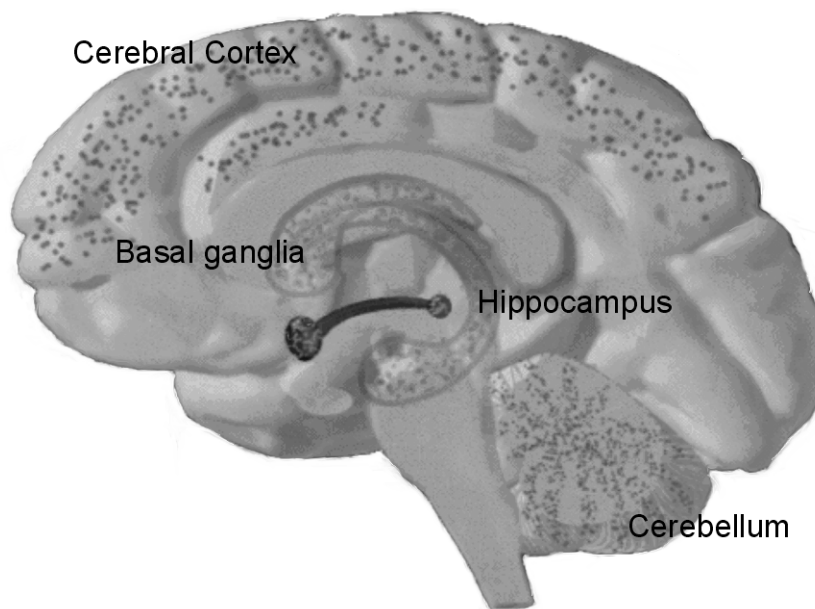
Dots indicate expression of CB_1 receptors, which is found amongst other areas in the cerebral cortex, cerebellum, hippocampus and in the basal ganglia, which consist of four nuclei (caudate nucleus, putamen (which form together the striatum), substantia nigra and globus pallidus). (Adapted from NIDA)

1.6 Signal transduction via CB receptors

The cannabinoid receptors have the structural characteristics of G-protein coupled receptors. K_i values for AEA to displace [^{3}H]CP55940, a potent synthetic cannabinoid, from the CB_1 receptor range from 27-543 nM in the presence of the FAAH inhibitor PMSF and 1-10 µM in its absence[70]. K_i values for AEA varied from 30-2000 nM for the CB_2 receptor[70]. The ligand selectivities of the CB_1 and CB_2 receptor do not differ to a large extent. The rank order of binding affinities for both receptors is HU210 > CP55940 > THC > AEA > CBD[71].

AEA has been shown via [^{35}S] GTPγS-autoradiography to induce binding of $G_{i/o}$-proteins to the cannabinoid receptors and to stimulate low K_m GTPase activities. The efficacy of cannabinoid agonists on mouse brain membranes was: CP55940 > HU 210 > AEA > THC[72].

Both CB_1 and CB_2 receptors inhibit cAMP formation via G_i–proteins and activate mitogen–activated–protein (MAP) kinases (Fig 6.)[70]. In addition, CB_1 receptors activate ion channels such as A-type and inwardly rectifying potassium channels and inhibit voltage sensitive N-type and P/Q-type calcium channels and D-type potassium channels[12,73,74]. The coupling to A and D-type potassium channels is thought to be regulated via cAMP. Activation of CB_1 receptors may also lead to arachidonic acid release and closing of $5-HT_3$ receptor ion channels. Under certain conditions the CB_1 receptor may couple to G_s–proteins, which activate adenylate cyclase and reduce outward potassium K^+ current, possibly via arachidonic acid stimulation of protein kinase C[75]. CB_1 and CB_2 receptor activation by AEA and 2–AG in N18TG2 neuroblastoma and NG108–15 neuroblastoma–glioma hybrid cells have been shown to induce a rapid transient increase in intracellular free calcium via its release from IP_3–sensitive calcium stores[76,77]. It should be noted that CHO-cells transfected with either CB_1 or CB_2 receptors failed to exhibit changes in IP_3 or phosphatidic acid in response to AEA or WIN55212–2 under conditions in which other exogenously expressed receptors coupled to phospholipases C could evoke such responses[70,71,78]. Hampson *et al.* demonstrated that AEA reduced NMDA receptor mediated calcium responses in rat cortical and cerebellar slices, which could be blocked by pertussis toxin and agatoxin (P/Q-type calcium channel blockers)[79]. It was suggested that voltage sensitive calcium channels were activated in response to the depolarization associated with NMDA-induced calcium influx, so that their inhibition by cannabinoid receptor activation reduced the overall calcium current[79]. Noteworthy, AEA potentiated NMDA-induced calcium currents in cortical, cerebellar and hippocampal slices in the presence of SR141716A. This capacity of AEA to increase calcium currents was also observed in Xenopus oocytes expressing NMDA receptors, which suggested that AEA could directly interact with NMDA receptors[79]. In the same expression system AEA was shown to directly inhibit AMPA receptor currents in a CB_1 receptor independent manner[80]. Furthermore, it was recently shown that CB_1 receptors on cultured cerebellar granule neurons could operate through a phospholipase C-sensitive mechanism to enhance NMDA-elicited calcium release from inositol-1,4,5-triphosphate–gated intracellular stores[81].

CB receptors have also been shown to couple to the generation of the lipid second messenger ceramide via two different pathways: sphingomyelin hydrolysis and ceramide synthesis *de novo*[82,83]. Ceramide in turn has been shown to control cell fate and to mediate cannabinoid–induced apoptosis.

1.7 Signal transduction via other proteins

Recent data demonstrate that AEA is capable of interacting with other molecular targets than CB receptors, such as 5-hydroxytryptamine receptors, L-type calcium channels, Shaker related K^+-channels, TASK-1 channels, a non-CB_1 G-protein coupled

Figure 6   Molecular bases of some neuromodulatory actions of AEA.
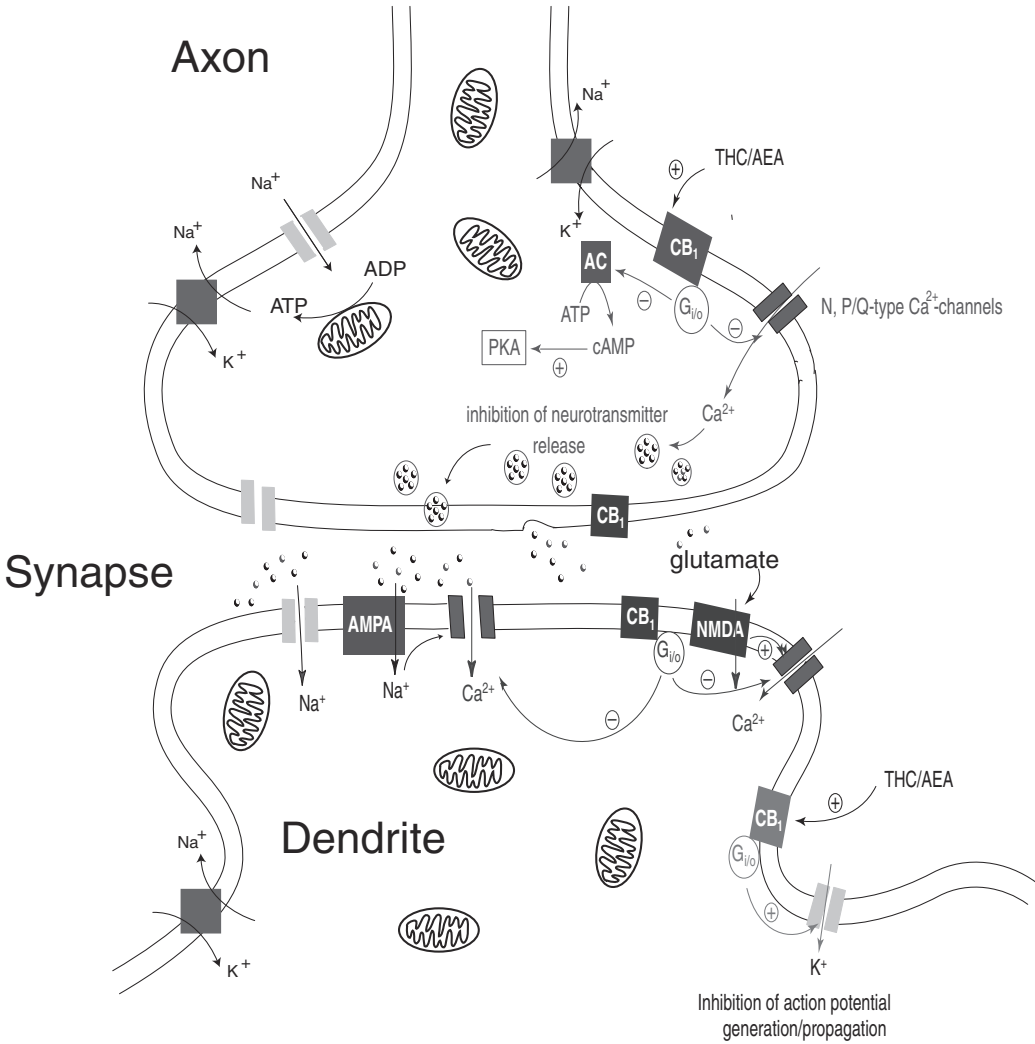
At the presynaptic and postsynaptic nerve terminal AEA and THC can activate G-protein coupled CB_1 receptors, modulating neuronal membrane permeability to Ca^{2+} and K^+ ions and the activity of adenylate cyclase (AC), thereby affecting neurotransmitter release or action, or both. (Figure adapted from [12])

AEA receptor in astrocytes and a non–CB_1 non–CB_2 G-protein coupled receptor for AEA and WIN55.212-2 in mouse brain[64,84]. Furthermore, AEA seems to be a full agonist at vanilloid type 1 receptors (VR1), the sites of action of the pungent component of 'hot' red peppers, capsaicin. Yet, in heterologous expression systems the potency of AEA to induce typical VR_1-mediated effects (*e.g.* cation currents, Ca^{2+}-influx and cell depolarization) is 5-20 fold lower than its average potency at CB_1 receptors[85]. However, recent data seems to indicate that some *in vitro* and *ex vivo* pharmacological actions of AEA are due to activation of native vanilloid receptors. It is suggested that AEA might act as an "endovanilloid" [86].

1.8 (Endo)cannabinoids modulate neurotransmission

The relation of the cannabinoid system to various neurotransmitter systems is of importance to understand its action on the central and peripheral nervous system. The cannabinergic system is involved via presynaptic and postsynaptic CB_1 receptors in regulation of the release, uptake and actions of excitatory and inhibitory transmitters such as acetylcholine, noradrenaline, dopamine, 5-hydroxytryptamine, γ-aminoburyric acid (GABA), glutamate and aspartate (Fig. 6)[12].

1.8.1 GABA-ergic transmission
According to the location and morphology, many CB_1-like neurons appeared to be GABAergic in an immunohistochemical distribution study[87]. Cannabinoids facilitate GABAergic transmission in some brain areas as reviewed by[88,89]. It is suggested that the cannabinoid–induced increase in GABAergic transmission in the globus pallidus is mainly caused by an inhibited uptake of GABA, rather than an increased release of GABA[90-92]. By contrast, cannabinoids have also been shown to suppress the release of GABA from presynaptic terminals in nucleus accumbens, hippocampus, substantia nigra and rostral ventromedial medulla neurons[22,93-97].

1.8.2 Dopaminergic transmission
Dopamine levels in the nucleus accumbens and in the vental tegmental area were increased by application of cannabinoids. It was shown that the mesolimbic system was activated by cannabinoids in a similar manner to heroin[98,99]. Furthermore, increased levels of dopamine upon application of exogenous cannabinoids excited dopaminergic neurons in the substantia nigra. However, other studies indicate that cannabinoids potentiate the behavioural effects of dopamine antagonists (neuroleptics) and reduce electrically evoked dopamine release from rat striatal slices. Injection of CB_1 receptor agonist in rat basal ganglia counteracted the motor responses of locally administered D_2 receptor agonists. Activation of dopamine D_2 receptors led to an increase in AEA-concentrations in the

striatum of freely moving rats, thereby it was demonstrated for the first time that endogenous AEA was acting as a neuromodulator *in vivo*[21].

1.8.3 Glutamatergic transmission

In the brain, glutamate is the predominant excitatory neurotransmitter and it has an important role in many brain pathologies. In rat hippocampal cultures glutamatergic transmission could be blocked by cannabinoids at subnanomolar concentrations in a receptor–mediated manner[100]. The inhibition was stereoselective with the following rank order CP55.939> CP55.940> WIN55.212-2> AEA and could be blocked by pertussis toxin. WIN55.212-2 blocked stereo selectively AMPA receptor–mediated excitatory postsynaptic currents (EPSCs) elicited by presynaptic stimulation with an extracellular electrode, but did not affect the presynaptic action potential or currents elicited by direct application of kainate[100]. It was suggested that this CB_1–mediated inhibition of excitatory synaptic transmission was developmentally regulated in rat hippocampus, being most prominent in neonatal rats[101]. Furthermore, other studies have indicated that CB_1 receptors are involved in the regulation of long term potentation in the hippocampus[24]. By contrast, histological studies could not detect CB_1 receptors on glutamatergic terminals in the hippocampus. A possible explanation was recently put forward by Hajos *et al.*[65]. Electrophysiological measurements in hippocampal slices of CB_1 knock–out mice suggested that a novel cannabinoid-sensitive receptor could be responsible for the inhibition of glutamatergic neurotransmission[65]. Nevertheless, definite proof of a new CB-type receptor in the hippocampus awaits cloning.

Recently, it was shown that WIN55.212, HU210 and AEA inhibited excitatory synaptic transmission in rat striatal neurons[102,103]. HU210 reduced the amplitude of evoked EPSCs in a dose-dependent matter. WIN55.212-2 significantly increased the paired-pulse facilitation of synaptically evoked EPSCs, while having no effect on the sensitivity of postsynaptic neurons to AMPA[102]. Superfusion of WIN55.212-2 elicited a membrane hyperpolarization accompanied by a decrease in input resistance. The WIN55.212-2-mediated synaptic inhibition was blocked by the $G_{i/o}$–protein inhibitor pertussis toxin and reversed by SR141716, but not by $GABA_A$ receptor antagonist bicuculline or $GABA_B$ receptor antagonist SCH50911.[102] Pretreatment with the N-type Ca^{2+} channel antagonist ω–conotoxin (GVIZ) selectively abolished the WIN-55.212-2 mediated synaptic inhibition. These results suggest that cannabinoids depress corticostriatal glutamatergic synaptic transmission through the activation of presynaptic CB_1 receptors to inhibit N-type Ca^{2+} channels activity, which in turn reduces glutamate release. When Ca^{2+} was replaced by Sr^{2+} in the extracellular solution, application of HU–210 significantly reduced the frequency, but not amplitude, of evoked, asynchronous quantal release events[103]. Spontaneous synaptic release events occurring between stimuli were similarly decreased in frequency but not in amplitude. Thus while CB_1-activation may lead to reduced glutamate release via reduced Ca^{2+} influx, a more direct and Ca^{2+}-

independent inhibition of the release process is also implicated by the results of Gerdeman and Lovinger[103].

CB$_1$–mediated inhibition of glutamatergic transmission has also been found in rat cerebellar slices[104], substantia nigra pars reticulata[105], in rat substantia gelatinosa neurons of the spinal cord[106], mouse nucleus accumbens[107] and in slices of the periaqueductal gray[108] as well as in rat prefrontal cortex pyrimidal neurons[109]. In the latter study it was shown that SR141617A acutely increased glutamatergic transmission and favoured Long Term Potentiation at the expense of Long Term Depression[109]. In contrast, *in vivo* microdialysis in rat prefrontal cortex revealed that *i.p.* injection of WIN55.212-2 led to a release of glutamate, which was counteracted by SR141716A[110].

1.8.4 Endocannabinoids as retrograde messengers

Importantly, the findings, as described above, were taken a step further by the results of a number recent studies in which endogenously released endocannabinoids were shown to mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals (Fig. 7;[22,111-116]). It is thought that endocannabinoids are responsible for depolarization–induced suppression of inhibition and excitation (DSI and DSE). In hippocampal neurons, depolarization of postsynaptic neurons and resultant elevation of intracellular Ca^{2+} led to transient suppression of inhibitory transmitter release, while in cerebellar purkinje cells post-synaptic elevation of intracellular Ca^{2+} concentrations caused transient suppression of excitatory transmitter release. These effects could be mimicked by cannabinoid receptor agonist WIN55.212-2 and were completely blocked by application of the CB$_1$ antagonists SR141716A and AM281[22,111-116]. DSI was not present in CB$_1$ knock–out mice, and pharmacological and kinetic data suggested that CB$_1$ activation inhibited presynaptic Ca^{2+} channels through direct G–protein inhibition[111]. Metabotropic glutamate receptor antagonists, a GABA$_B$ receptor antagonist and postsynaptically applied botulinum toxin, which inhibits vesicular glutamate release from the postsynaptic cell, were ineffective. Interestingly, flash photolysis of caged Ca^{2+} was sufficient to induce DSI. However, stimulation of metabotropic glutamate receptors, without elevation of calcium levels, could also induce cannabinoid–dependent DSE in cerebellar purkinje cells[112]. At the moment it is unclear, which endocannabinoid is responsible for DSI and DSE and what the mechanism is of its synthesis and release.

1.9 Physiological functions

The involvement of the endocannabinoid system in the regulation of three widespread cellular signalling mechanisms (calcium, cAMP, and ceramide) combined with its broad distribution pattern in both the central nervous system and in peripheral tissues may explain its implication in many physiological actions. The general idea is that the

Figure 7   Hypothetical model of retrograde AEA-action.
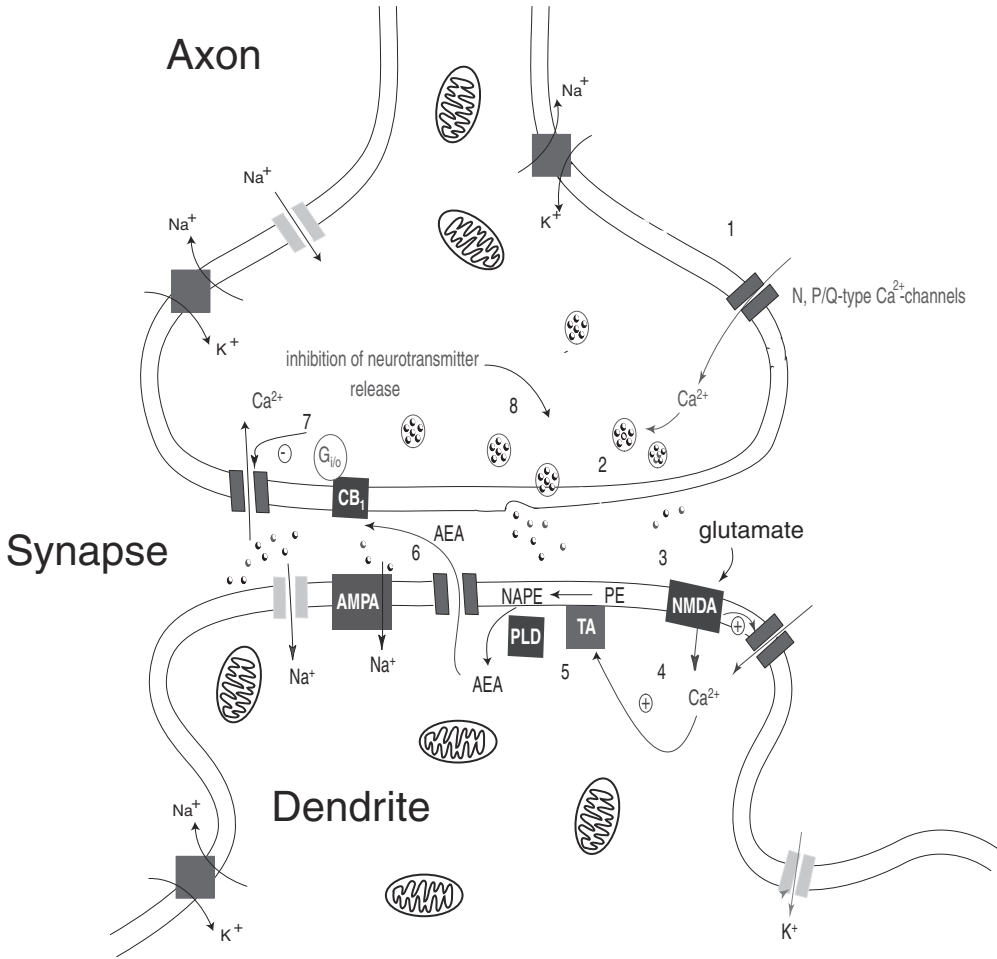
(1) Presynaptic depolarization leads to calcium influx, which in turn activates glutamate exocytosis (2). Glutamate diffuses through synaptic cleft and activates post-synaptic glutamate receptors (3). Activation of NMDA, AMPA and other glutamate-subtype receptors lead to post-synaptic depolarization and calcium influx (4). Elevated post-synaptic calcium levels activate a transacylase (TA), which converts phosphatidylethanolamine (PE) into *N*-arachidonoylphosphatidylethanolamine (NAPE) (5). NAPE is hydrolysed by a phospholipase D (PLD), which yields AEA. AEA is released from the post-synaptic cell and diffuses back to presynaptic CB_1 receptors (6). Upon activation of CB_1 receptors by AEA, $G_{i/o}$-proteins are released, which inhibit N-, and P/Q-type voltage sensitive calcium channels (7). Closing of voltage sensitive calcium channels results in a reduced release of neurotransmitters such as glutamate or GABA (8). Adapted from [12]

endogenous cannabinoid system may be a widespread tuning system of various different physiological responses. It should be noted that the physiological effects of the cannabinoid system are usually studied with the use of exogenously applied (endo)cannabinoids. Little is known of the involvement of the endogenously formed ligands of the cannabinoid system. The endocannabinoid system seems to be involved in regulation of blood pressure, secretion of pituitary and steroid hormones, embryo implantation, tumour growth and immunomodulation. The latter may depend at least in part on CB_2 receptor mediated suppression of proinflammatory cytokine release and enhancement of anti–inflammatory cytokine release from immune cells. CB_1 receptors may regulate different physiological functions by modulating neurotransmission in different parts of the brain, such as body temperature (thalamus), perception (sensorimotorcortex, olfactory bulb), cognitive functions such as sleep and memory (hippocampus, cortex), appetite (hypothalamus), pain processing (thalamus, periaqueductal grey, amygdala) and (psycho)motor functions (basal ganglia, cerebellum).[12,20,21,70,83,117-126]

Recently, two different strains of CB_1 knock–out mice have been bred[127,128]. Differences between the strains have been observed. The $CB_1^{-/-}$ mice generated by Ledent[128] were fertile and appeared healthy. The mice exhibited mild increased locomotor activity and the spontaneous nociceptive threshold of wild-type and mutant and naive mice was similar. Interestingly, in these $CB_1^{-/-}$ mice the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced. These observations suggest that the CB_1 receptor is involved in motivational properties of opiates and in the development of physical dependence.

The mice generated by Zimmer et al. appeared also healthy and fertile[127], however, in contrast to the Ledent mice, the Zimmer mice displayed reduced locomotor activity, increased ring catalepsy, and hypoalgesia in hotplate and formalin tests. Remarkably, the mice had a significantly increased mortality rate. The mice died suddenly without any obvious signs of disease, such as weight loss, dehydration, or abnormal posture. The reason for this increased mortality is currently unknown. Since cannabinoids induce pronounced hypotension and bradycardia, it is speculated that the mice succumb to latent cardiovascular problems. Alternatively, $CB1^{-/-}$ mice have an increased risk of developing neurological problems such as seizures[127].

1.10 Pathophysiological implications

1.10.1 Huntington's disease

The cannabinoid system has been implicated in a variety of neurological and neurodegenerative diseases, such as glioma growth, Parkinson's and Huntington's disease and Multiple Sclerosis[19,129]. In *postmortem* brains of patients with Huntington's disease a relative loss of CB_1 and dopamine receptors was observed through several grades of pathology in the globus pallidus and substantia nigra[130]. It is speculated that the early

down regulation of cannabinoid receptors is a compensatory mechanism in Huntington's disease. In a recent study it has been shown that CB_1 mRNA was down regulated in the lateral striatum and cortical regions of transgenetic Huntington's disease mice, prior to the development of either Huntington's disease phenotype or neuronal degeneration[131]. Furthermore, in another rat model of Huntington's disease CB_1 binding sites were lost, and the CB_1-mediated activation of GTP-binding proteins were reduced in the basal ganglia[132]. These changes were paralleled by a decrease of AEA and 2-AG levels in the striatum, whereas in the non-lesioned cerebral cortex normal endocannabinoid levels were found. At the moment it is unclear to what extent these observations contribute to the symptomology of Huntington's disease, or whether they are a side effect of toxin-induced destruction of striatal GABAergic neurons[132].

1.10.2 Parkinson's disease

It was shown that intrapallidal administration of cannabinoids reduced the uptake of GABA from striatopallidal terminals and voluntary movement, thereby reproducing Parkinson's disease-like symptoms[90,91]. Stimulation of CB receptors increased catalepsy produced by dopamine receptor antagonists[133], and it reduced the anti-parkinson-like actions of D_2 dopamine receptor agonists[134]. Furthermore, increased 2-AG levels were found in the globus pallidus of rats treated with reserpine, a rodent model of Parkinson's disease. This substantiates the hypothesis that an increased cannabinoid tone in the globus pallidus contributes to the symptoms of Parkinsons' disease[135]. Indeed, SR141716A increased the efficacy of anti-parkinson-like effects of dopamine receptor agonists in this model[135]. Moreover, SR141716A was therapeutically useful, by reducing L-dopa-induced dyskinesia in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-treated primates[136]. However, in another recent study with non-human primates SR141716A failed to alleviate the parkinson-like symptoms[137,138]. Differences in dose-regimen, methods to deplete dopamine, and species differences in endogenous cannabinoids levels might explain the differences between rodent and primate models[137].

1.10.3 Multiple Sclerosis

Cannabis use has been reported to reduce the muscle spasticity associated with multiple sclerosis or spinal cord injury (reviewed:[129]). THC has been shown to reduce spasticity and pain in a few small clinical trials[129]. The efficacy of THC in MS has also been tested in animal models. THC was able to inhibit the progression of experimental autoimmune encephalomyelititis (EAE), the most commonly used animal model of MS, in rodents. The presumed mode of protection was its immunosuppressive actions rather than a direct effect on paralysis itself[139,140] However, recent studies indicate that THC as well as synthetic cannabinoids can ameliorate both tremor and spasticity via CB_1 and/or CB_2 receptors in mice with chronic relapsing experimental allergic encephalomyelitis (CREAE)[141]. Also AEA, 2-AG and palmitoylethanolamide were demonstrated to

ameliorate the spasticity in the same model[142]. Interestingly, in brain areas associated with brain damage, increased levels of AEA, 2-AG and palmitoylethanolamide were found, whereas comparable levels of these compounds were found in normal and non-spastic mice[142]. Hydrolysis and uptake inhibitors of endocannabinoids were shown to ameliorate spasticity to an extent comparable with that observed with synthetic potent cannabinoid agonists. According to the authors the results opened new horizons to the therapy of multiple sclerosis and other neuromuscular diseases, based on agents modulating endocannabinoid levels and actions, which exhibit little psychotropic activity[142].

1.10.4 Ischemia and excitotoxicity

The central nervous system is highly vulnerable to ischemia induced by a stroke or traumatic brain injury. Neuronal death caused by ischemia is executed via a complex array of processes in which excitotoxicity plays a major role. Several lines of evidence reveal a connection between cannabinoids and excitotoxicity. In excitotoxicity, cell death is triggered by the overstimulation of excitatory amino acid receptors by glutamate. This leads to cytotoxic levels of calcium and subsequent activation of destructive pathways, involving among others caspases, calpains and the generation of reactive oxygen species[143,144]. The excitotoxicity hypothesis is also used to explain the common biochemical basis behind many chronic neurodegenerative disorders such as amyotrophic lateral sclerosis, Parkinson's, Huntington's and Alzheimer's diseases[143-145].

Several *in vitro* studies have reported neuroprotection with classical and synthetic cannabinoids (see also chapter 6). Depending on the model used, cannabinoids were shown to protect neurons via CB_1-mediated inhibition of glutamate exocytosis[146,] CB_1-mediated closing of voltage sensitive calcium channels[147,148], anti-oxidant activity[149], and suppression of the formation of tumour necrosis factor alpha[150]. AEA and 2-AG have been shown to rescue cerebral neurons from *in vitro* hypoxia and glucose deprivation via a CB_1 and CB_2-independent pathway[151]. By contrast, some *in vitro* studies do not support a neuroprotective action of cannabinoids. AEA was shown to be ineffective to protect neurons against prolonged exposure to toxic levels of glutamate[152,153]. Hampson and Grimaldi have suggested that the varying and uncontrolled levels of cAMP in *in vitro* models of neurotoxicity may explain the lack of neuroprotection by cannabinoids[154].

The therapeutic effects of cannabinoids in *in vivo* models of cerebral ischemia are described in a few studies, but are also not consistent. Chronic Δ^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[155]. 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 was protective[1]. By contrast, the CB receptor

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[1] Muthian, S. and Hillard, C.J., Symposium on the Cannabinoids, Burlington, Vermont, ICRS **2000**, p 107.

agonist WIN 55.212-2 afforded protection to hippocampal and cortical neurons in CB_1-dependent manner in rats with a permanent middle cerebral artery occlusion or global ischemia[156]. In gerbils subjected to transient global ischemia pretreatment with CP55940 reduced ischemia-induced hyperlocomotion and improved electroencephalographic (EEG) spectral power after 24h, which lasted for at least 7 days[157]. Currently, there is no explanation for the contradictory *in vivo* studies. It should be noted that in each of these stroke models different perturbations in cerebral blood pressure and flow were induced. It might be possible that cannabinoid-induced vasorelaxation[158] is affecting the extent and pathway of neuronal demise in a different manner in each of these stroke models.

In summary, exogenously applied cannabinoids can protect neurons against neuronal damage in various ways depending on dose, time of application and type of injury. However, to date most studies have been conducted *in vitro* with classical and synthetic cannabinoids. Only one *in vitro* study has demonstrated a neuroprotective effect of AEA and there are no data available from *in vivo* studies. To date it is unknown whether endogenously released AEA can protect the brain against acute neuronal injury. The role of endocannabinoids in neurodegenerative diseases may be clarified by studying the effects of both exogenous and endogenous cannabinoids in an *in vivo* model of (secondary) excitotoxicity, without directly affecting blood pressure and flow to the brain.

1.11 Aim

The developments as described above indicate that the endocannabinoid system is a potentially valuable field for drug discovery. CB_1 and CB_2 receptors, FAAH and the transporter protein may play a role as important drug targets. This will circumvent the need of a marijuana extract as a medicine, thereby avoiding marijuana's complex unknown and varying composition and possibly its psychotropic side effects. For example, the development of CB_2 receptor selective ligands may lead to therapeutic agents without activation of the central CB_1 receptor, which is responsible for the psychotropic effects. The same accounts for CB_1 receptor ligands, which cannot pass the blood brain barrier, but which can still activate the peripheral CB_1 receptors. The development of selective FAAH inhibitors or AEA-uptake inhibitors might also yield useful therapeutics. FAAH inhibitors may act as indirect agonists. It is unlikely that they will affect all parts of the endocannabinoid system at one time, like direct agonists. They will only increase the concentrations of endocannabinoids at sites where ongoing biosynthesis is already occurring, thereby preventing the sustained activation of CB receptors at other sites. Yet, if pharmaceutical agents targeted towards the cannabinoid system are going to play a role in medicine, if any, some important questions have to be answered. *i*) Is it possible to develop selective and potent molecular probes to each of the proteins of the endocannabinoid system? *ii*) Is the endocannabinoid system involved in specific pathophysiological states? If inhibitors of endocannabinoid inactivation are going to play

a role, then *iii*) does the inactivation system also limit the actions of the endocannabinoid system in humans? *iv*) What is the influence of the oxidative metabolism of endocannabinoids on their function?

The aim of this thesis is therefore to study a) the metabolism of AEA in human cells and brain tissue, b) the interaction of its oxygenated metabolites with the proteins of the endocannabinoid system, and c) the role of the endocannabinoid system in in vivo neurodegeneration.

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Anandamide Hydrolysis by Human Cells in Culture and Brain

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Abstract

Anandamide (arachidonoylethanolamide, AEA) has important neuromodulatory and immunomodulatory activities. This lipid is rapidly taken up and hydrolyzed to arachidonate and ethanolamine in many organisms. As yet, AEA inactivation has not been studied in humans. Here, a human brain fatty acid amide hydrolase (FAAH) has been characterized as a single protein of 67 kDa and pI of 7.6, showing apparent K_m and V_{max} for AEA of 2.0 ± 0.2 µM and 800 ± 75 pmol.min^{-1}.mg protein^{-1}, respectively. The optimum pH and temperature for AEA hydrolysis were 9.0 and 37°C, respectively, and the activation energy of the reaction was 43.5 ± 4.5 kJ.mol^{-1}. Hydro(pero)xides derived from AEA or its linoleoyl analogues by lipoxygenase action were competitive inhibitors of human brain FAAH, with apparent K_i values in the low micromolar range. A FAAH activity sharing several biochemical properties with the human brain enzyme was demonstrated in human neuroblastoma CHP100 and lymphoma U937 cells. Both cell lines have a high affinity transporter for AEA, which had apparent K_m and V_{max} for AEA of 0.20 ± 0.02 µM, 30 ± 3 pmol.min^{-1}.mg protein^{-1} (CHP100 cells), and 0.13 ± 0.01 µM, and 140 ± 15 pmol.min^{-1}.mg protein^{-1} (U937 cells), respectively. The AEA carrier of both cell lines was activated up to 170% of the control by nitric oxide.

Introduction

Anandamide (arachidonoylethanolamide, AEA) is an endogenous lipid that binds to cannabinoid CB_1 and CB_2 receptors, which are mainly found in the central nervous system and in peripheral immune cells. It mimics the pharmacological effects of Δ^9-tetrahydrocannabinol, the active principle of hashish and marijuana[1,2]. AEA formation occurs mainly through phosphodiesterase–mediated cleavage of *N*-arachidonoyl–phosphatidylethanolamine[3,4], though a direct synthesis from arachidonic acid and ethanolamine has also been described[5,6]. AEA can be released from depolarized neurons[3]. Upon binding to CB_1 receptors, AEA induces inhibition of forskolin–induced cAMP accumulation, inhibition of N-type Ca^{2+} channels, activation of mitogen–activated–protein (MAP) kinase signal transduction pathway[7], and increases protein tyrosine phosphorylation[8]. Activation of the CB_2 receptor leads to inhibition of adenylate cyclase and activation of the MAP-kinase signaling[9]. Interestingly, AEA binding to cannabinoid receptors is coupled to nitric oxide (NO) release in the central nervous system of invertebrates and in peripheral immune cells of both invertebrates and humans[10].

The pharmacological effects of AEA on CB_1 and CB_2 receptors depend on the life span of the lipid in the extracellular space, which is limited by a rapid and selective process

of cellular uptake, followed by intracellular degradation of AEA to ethanolamine and arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH). Both components of the inactivation process of AEA are the subject of active investigation. AEA uptake has been characterized in rat neuronal cells[3,11,12] and rat basophilic leukemia (RBL-2H3) cells[13]. FAAH has been demonstrated and partially characterized in rat, porcine and dog brains[14-16]. Furthermore, FAAH activity has been shown in one "neuronal" cell line, namely mouse neuroblastoma $N_{18}TG_2$[17], and in one "non-neuronal" cell line, namely RBL-2H3[13]. The FAAH gene has recently been cloned from rat, mouse and human liver cDNAs, allowing molecular mass determination and substrate specificity analysis of the enzyme[18,19]. As yet, no information is available on the activity of human FAAH, or on AEA uptake by human cells. This prompted us to investigate some biochemical properties of FAAH from human brain and human neuronal and immune cells, *i.e.* neuroblastoma CHP100 and lymphoma U937 cells. AEA uptake was characterized in these two cell types to gain information on the AEA inactivation process in humans. The cell lines chosen are widely used as experimental models for neuronal and immune tissues[20,21]. In these two cell types AEA uptake was demonstrated and characterized.

Taken together, the results reported here represent the first biochemical characterization of human brain FAAH. Most properties of this enzyme are shared by FAAH found in human neuronal and immune cells in culture. Remarkably, both cell lines seem to inactivate AEA in the same way, which strengthens the concept of a neuroimmune axis in humans, which is evident, for instance, in the "axon–reflex" model for neurogenic inflammation[13]. Possible implications of FAAH activity and expression in brain pathology are also discussed.

Materials and Methods

Materials

Chemicals were of the purest analytical grade. Anandamide (arachidonoylethanolamide; AEA), arachidonic acid, ethanolamine, phenylmethylsulfonyl fluoride (PMSF), iodoacetic acid, *N*-ethylmaleimide, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and sodium nitroprusside (SNP) were purchased from Sigma. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) was from Research Biochemicals International, and spermine NONOate (SPER/NO, (Z)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]-amino}-diazen-1-ium-1,2-diolate) was from Alexis Corporation (Switzerland). Leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) were from Cayman Chemical Co. Inc. [1-^{14}C]AEA was synthesized from ethanolamine and [1-^{14}C]arachidonic acid (NEN DuPont de Nemours, Germany, 52 mCi/mmol) as described[22]. Linoleoylethanolamide (9*Z*,12*Z*-octadecadienoylethanolamide, LEA), linoleoylamide (9*Z*,12*Z*-octadecadienoylamide, LA), linoleoylmethylamide (9*Z*,12*Z*-octadecadienoylmethylamide, LMA), and their 13-hydroxy derivatives 13-HLEA, 13-HLA and 13-HLMA, were synthesized and characterized (purity > 96%

by gas–liquid chromatography) as described[22] 15-Hydro(pero)xyanandamide (15-hydro-(pero)xyeicosa-5Z,8Z,11Z,13E-tetraenoyl–ethanolamide, 15-H(P)AEA, purity > 96%) and 11–hydro(pero)xyanandamide (11-H(P)AEA, a mixture of 45% 11-H(P)AEA, 24% 5-H(P)AEA, 18% 15-H(P)AEA, 9% 8-/9-H(P)AEA and 4% 12-H(P)AEA by reversed phase high performance liquid chromatography) were synthesized as described in[23].

Biological material

   Human brain specimens were obtained from five different male patients (aged 73-77), undergoing surgical operation to remove meningioma tumors. Brain tissues were removed and donated by Prof. R. Giuffrè and Dr. G. De Caro (Neurosurgery Division, University of Rome Tor Vergata, Sant'Eugenio Hospital, Rome, Italy). In four cases the perilesional white matter surrounding the tumor area was removed (1 g of fresh tissue in total) and used for fatty acid amide hydrolase (FAAH) characterization. In one case, both meningioma and perilesional white matter (0.1 g of each fresh tissue) were removed and used to compare FAAH activity and expression in meningioma and healthy brain.

   Human neuroblastoma CHP100 cells were cultured as reported[20], in a 1:1 mixture of Eagle's minimal essential medium plus Earle's salts and Ham's F-12 medium (Flow Laboratories Ltd., United Kingdom), supplemented with 15% heat-inactivated fetal bovine serum, sodium bicarbonate (1.2 g/l), 15 mM Hepes, 2 mM L-glutamine and 1% non essential amino acids. Human lymphoma U937 cells, a gift by Dr. E. Faggioli (Department of Public Health and Cell Biology, University of Rome Tor Vergata), were cultured in RPMI 1640 medium (Gibco, United Kingdom), supplemented with 25 mM Hepes, 2.5 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum[21]. Both CHP100 and U937 cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Assay of fatty acid amide hydrolase (FAAH)

   Immediately after surgical removal, human brain specimens were washed in phosphate-buffered saline and homogenized with an UltraTurrax T25 in 50 mM Tris-HCl and 1 mM EDTA, pH 7.4 (buffer A), at a 1:10 homogenization ratio (fresh weight/volume). Membranes from these tissue homogenates were then prepared as described[22]. The final pellet, containing most FAAH activity, was resuspended in ice-cold buffer A at a protein concentration of 1 mg/ml and stored at -80°C until use. Both CHP100 and U937 cells (3×10^8/sample) were collected in phosphate-buffered saline and centrifuged at 1000 xg for 10 min. The dry pellet was resuspended in 30 ml ice-cold buffer A and sonicated on ice three times for 10 s, with 10 s intervals, using a Vibracell sonifier (Sonics & Materials Inc.) with a microtip at maximum power. The homogenate was then centrifuged sequentially as described above for the human brain, and the final pellet was stored at -80°C in buffer A at a protein concentration of 1 mg ml^{-1} until use.

   The assay of FAAH (E.C. 3.5.1.4, arachidonoylethanolamide amidohydrolase) activity was performed by reversed-phase high performance liquid chromatography (HPLC) as described[22]. Thermal stability and pH dependency of FAAH activity were studied as described[17]. Activation

energy values were calculated as reported[24]. Kinetic and inhibition studies were performed using different concentrations of [1-^{14}C]AEA (in the range 0–21 μM) and two different concentrations (10 μM and 20 μM) of each inhibitor to calculate the kinetic parameters. Fitting of the experimental points to a Lineweaver–Burk plot by a linear regression programme (Kaleidagraph 3.0) yielded straight lines with r-values > 0.95.

The assay of the FAAH synthase activity was performed by measuring the formation of [1-^{14}C]AEA from [1-^{14}C]arachidonic acid and ethanolamine as reported[5]. Tissue or cell homogenates (20 μg proteins/test) were incubated for 15 min at 37°C in 200 μl 50 mM Tris-HCl, pH 9.0, containing 10 μM [1-^{14}C]arachidonic acid (52 mCi/mmol) and 2 mM ethanolamine. The reaction was stopped, and the products were extracted and analyzed by reversed-phase HPLC, following the same procedure as described above for the hydrolase activity. FAAH synthase activity is expressed as pmol AEA min^{-1}mg protein^{-1}. The effect of various compounds on the hydrolase or synthase activity of FAAH was determined by adding each substance directly to the assay buffer at the indicated concentrations.

Immunochemical analysis

SDS–polyacrylamide gel electrophoresis (12%) was performed under reducing conditions in a Mini Protean II apparatus (Bio–Rad) with 0.75-mm spacer arms. Rainbow molecular mass markers (Amersham International, United Kingdom) were phosphorylase b (97.4 kDa), bovine serum albumin (66.0 kDa) and ovalbumin (46.0 kDa). Native isoelectric focusing (IEF) was performed in the Mini Protean II apparatus using a 5% polyacrylamide gel containing ampholytes in the pH range 5.0-9.0 (Sigma) as described[25]. IEF was calibrated by running the following isoelectric point (pI) markers (Sigma): lentil (*Lens culinaris*) lectin (pI 8.8, 8.6 and 8.2), myoglobin from horse heart (pI 7.2 and 6.8), carbonic anhydrase I from human erythrocytes (pI 6.6) and carbonic anhydrase II from bovine erythrocytes (pI 5.9). Human brain homogenates (20 μg/lane), prepared as described above for FAAH assay, were subjected to either SDS–polyacrylamide gel electrophoresis or IEF and then slab gels were electroblotted onto 0.45 μm nitrocellulose filters (Bio–Rad), using a Mini Trans Blot apparatus (Bio–Rad) as reported[26]. Immunodetection of FAAH on nitrocellulose filters was performed with specific anti–FAAH polyclonal antibodies (diluted 1:200), raised in rabbits against the conserved FAAH sequence VGYYETDNYTMPSPAMR[19], conjugated to ovalbumin. This peptide antigen and the anti–FAAH polyclonal antibodies were prepared by Primm S.r.l. (Milan, Italy). Goat anti-rabbit alkaline phosphatase conjugate (GAR–AP, Bio–Rad) (diluted 1:2000) was used as secondary antibody, and immunoreactive bands were stained with the alkaline phosphatase staining solution according to the manufacturer's instructions (Bio–Rad).

Enzyme-linked immunosorbent assay (ELISA) was performed by coating the plate with human brain homogenate (20 μg/well), prepared as described above for FAAH assay. Anti–FAAH polyclonal antibodies were used as primary antibody (diluted 1:300) and GAR–AP as secondary antibody (diluted 1:2000). Color development of the alkaline phosphatase reaction was measured at 405 nm, using *p*-nitrophenylphosphate as substrate. For peptide competition experiments, the peptide antigen was preincubated with a 1000-fold molar excess of anti–FAAH polyclonal antibodies for 30

min at room temperature before adding the antibodies to the wells[18]. Controls were carried out using non–immune rabbit serum and included wells coated with different amounts of bovine serum albumin.

Reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing

2–5x10^6 cells or 20 mg tissue were used to isolate total RNA by means of the S.N.A.P.™ Total RNA Isolation Kit (Invitrogen). Control reactions were carried out to ensure complete removal of genomic DNA. RT-PCRs were performed using the EZ rTth RNA PCR kit (Perkin Elmer) following the manufacturer's instructions. The reaction conditions were carefully examined to stop the reaction during the exponential phase of amplification of each gene. Briefly, 100 ng (for the amplification of FAAH), or 0.4 ng (for 18S rRNA) of total RNA, were reversibly transcribed and amplified in the same tube in a total reaction volume of 10 μl, in the presence of 3 mCi of α-^{32}P-dCTP (Amersham International, United Kingdom, 3000 Ci/mmol). The amplification parameters were as follows: 2 min at 95°C, 45 s at 95°C, 30 s at 55°C and 30 s at 60°C. Linear amplification was observed after 20 cycles. The primers were as follows:

(+)5'-TGGAAGTCCTCCAAAAGCCCAG,(-) 5'-TGTCCATAGACACAGCCCTTCAG, for FAAH;(+)5'-AGTTGCTGCAGTTAAAAAGC, (-)5'-CCTCAGTTCCGAAAAC-CAAC, for 18S rRNA.

Five μl of the reaction mixture were electrophoresed on a 6% polyacrylamide gel, which was then dried and subjected to autoradiography. Products were validated by size determination and sequencing. For quantitation of the RT-PCR products, bands were excised from the gel and counted in a LKB1214 Rackbeta scintillation counter (Sweden). Linear amplification sequencing was performed by using Cyclist™ DNA Sequencing Kit (Stratagene), according to the manufacturer's instructions. RT-PCR products for sequencing were prepared without the α-^{32}P-dCTP and sequenced with the same primers used for amplification after labeling them with γ-^{32}P-dATP (Amersham International, United Kingdom, 3000 Ci/mmol).

Determination of anandamide uptake

The uptake of [1-^{14}C]AEA (52 mCi/mmol) by intact CHP100 or U937 cells was studied essentially as described[13]. CHP100 and U937 cells were resuspended in their serum-free culture media at a density of 1x10^6 cells/ml. Cell suspensions (2 ml/test) were incubated for different time intervals at 37°C with 100 nM [1-^{14}C]AEA, then they were washed three times in 2 ml culture medium containing 1% bovine serum albumin and were finally resuspended in 200 μl phosphate-buffered saline. Membrane lipids were then extracted[27], resuspended in 0.5 ml methanol and mixed with 3.5 ml Sigma-Fluor liquid scintillation cocktail for non-aqueous samples (Sigma), and radioactivity was measured in a LKB1214 Rackbeta scintillation counter (Sweden). To discern non-protein-mediated from protein-mediated transport of AEA into cell membranes, control experiments were carried out at 4°C[13]. Incubations (15 min) were also carried out with different concentrations of [1-^{14}C]AEA (in the range 0-750 nM) in order to determine apparent K_m and V_{max} of the uptake by Lineweaver–Burk analysis (in this case, the uptake at 4°C was subtracted from

that at 37°C). The Q_{10} value was calculated as the ratio of AEA uptake at 30°C and 20°C[11]. AEA uptake is expressed as pmol AEA taken up per min mg protein[-1]. The effect of different compounds on AEA uptake was determined by adding each substance directly to the incubation medium at the indicated concentrations. In the case of CCCP, cells were preincubated with 50 μM CCCP for 15 min at 37°C before addition of [1-^{14}C]AEA to abolish mitochondrial transmembrane potential[28]. Cell viability after each treatment was checked with Trypan blue and found to be higher than 90% in all cases. It is noteworthy that no specific binding of [^{3}H]CP55940, a potent cannabinoid, was obtained with plasma membranes of CHP100 cells, and U937 cells express hardly detectable CB_1 mRNA and very low levels of CB_2 mRNA[21], thus [1-^{14}C]AEA binding to CB receptors is not likely to interfere in the uptake experiments[11,13].

Data analysis

Data reported in this paper are the mean (± S.D.) of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by the Student's *t*-test, elaborating experimental data by means of the InStat programme (GraphPad Software).

Results

*Characterization of FAAH in human brain and human CHP100 and U937 cells*

Pilot experiments indicated that human brain FAAH activity was linearly dependent on the amount of tissue homogenate (in the range 0–30 μg protein) and the incubation time of the reaction (in the range 0–30 min), whereas it depended on AEA concentration according to Michaelis–Menten kinetics (Fig. 1A) (data not shown), yielding an apparent K_m of 2.0 ± 0.2 μM and a V_{max} of 800 ± 75 pmol.min^{-1}.mg protein[-1]. The activity of FAAH was assayed in the pH range 5.0-11.0 and in the temperature range 20-65°C, showing an optimum pH and temperature at 9.0 and 37°C, respectively. Arrhenius diagrams of AEA hydrolysis by FAAH in the temperature range 20-45°C allowed us to calculate an activation energy of 43.5 ± 4.5 kJ.mol^{-1}.

Western blotting showed that anti-FAAH polyclonal antibodies specifically recognized a single immunoreactive band in brain homogenates, corresponding to a molecular mass of ~ 67 kDa and an isoelectric point of ~ 7.6 (Fig. 2).

Hydroxylated AEA derivatives and its linoleoyl analogues were competitive inhibitors of human brain FAAH, with apparent K_i values ranging from 3.2 to 24.5 μM (Table I). The substrate specificity of FAAH from human brain resembled that of the enzyme from mouse or rat brain[18,19].

Human neuronal (CHP100) and immune (U937) cells in culture also showed FAAH activity, with pH and temperature profiles superimposable to those observed with the human brain enzyme (data not shown). Both cell lines showed a FAAH activity (Fig. 1A and B) characterized by apparent K_m and V_{max} values of 6.5 ± 0.6 μM, 32 ± 3 pmol.min^{-1}.mg

Figure 1   Dependence of fatty acid amide hydrolase (FAAH) activity on anandamide concentration.
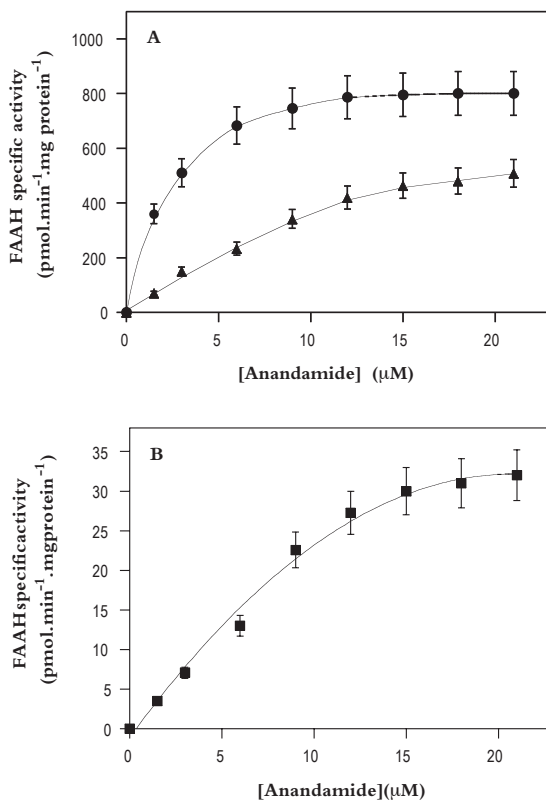
In A, FAAH activity was assayed at various anandamide concentrations in human brain (●) and human lymphoma U937 cells (▲) in culture. In B, FAAH activity was assayed in human neuroblastoma CHP100 cells (■) in culture. In both panels, FAAH activity was measured at pH 9.0 and 37°C.

protein^{-1} (CHP100) and 6.5 ± 0.6 µM, 520 ± 50 pmol.min^{-1}.mg protein^{-1} (U937), for AEA. The activation energy of AEA hydrolysis by FAAH from CHP100 or U937 cells (45.0 ± 4.5 kJ.mol^{-1} in either case) was the same as the human brain enzyme. Moreover, 15-HAEA, LEA and 13-HLEA competitively inhibited FAAH activity from both cell lines, with apparant K_i 4.5 ± 0.4, 11.1 ± 0.9, and 6.1 ± 0.5 µM (CHP100) and 3.8 ± 0.4, 10.5 ± 1.0, and 4.5 ± 0.4 µM (U937), respectively.

Excess (100 µM) arachidonic acid, but not ethanolamine, strongly inhibited FAAH activity in all human sources tested, in line with previous findings on mouse FAAH[17].

Inhibition of human brain FAAH activity by different anandamide products and analogues   **Table 1**

Apparent inhibition constant (K_i) values were calculated by Lineweaver-Burk profiles of AEA hydrolysis by FAAH. All compounds were reversible, competitive inhibitors of FAAH activity.

Compound	(K_i, µM)
AEA	None
15–HPAEA	4.8 ± 0.5
15–HAEA	3.2 ± 0.3
11–HPAEA[a]	5.2 ± 0.5
11–HAEA[b]	4.0 ± 0.4
LEA	9.0 ± 0.9
LA	14.1 ± 1.3
LMA	24.5 ± 2.1
3–HLEA	3.0 ± 0.3
13–HLA	5.3 ± 0.5
13–HLMA	9.3 ± 0.9

[a] 11–HPAEA was a mixture of 11–HPAEA (45%), 5–HPAEA (24%), 15–HPAEA (18%), 8–/9–HPAEA (9%) and 12–HPAEA (4%);

[b] 11–HAEA was the same mixture as 11–HPAEA, reduced with $NaBH_4$.

Electrophoretic properties of human brain FAAH.   **Figure 2**
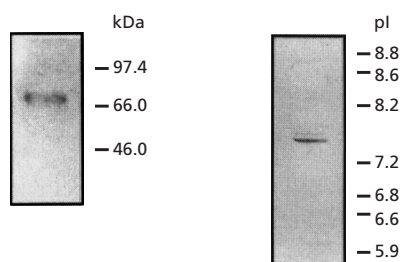
Human brain extracts (20 µg/lane) were subjected to either SDS-polyacrylamide gel electrophoresis (left panel) or isoelectric focusing (right panel). Slab gels were then electroblotted onto nitrocellulose filters and FAAH was detected as immunoreactive band with specific anti–FAAH polyclonal antibodies. Molecular mass markers and pI markers are shown.

Table 2 **Inhibition of FAAH activity and [1-14C]anandamide uptake in human brain and human CHP100 and U937 cells**

FAAH activity was determined using 10 μM AEA as substrate. For uptake experiments, cells (2x10^6) were incubated for 15 min at 37°C with 100 nM [1-^{14}C]AEA in the presence of each compound. Activity and uptake values are expressed as percentage of the untreated controls, arbitrarily set to 100 (see below for absolute values). Results on FAAH activity in CHP100 and U937 cells were superimposable, thus FAAH activity in CHP100 cells was omitted for the sake of clarity.

Compound	FAAH activity		Anandamide uptake	
	Brain	**U937**	**CHP100**	**U937**
	(%)		(%)	
None	100[a]	100[b]	100[c]	100[d]
Arachidonic acid (100 μM)	18 ± 2	16 ± 2	100 ± 10	100 ± 10
Ethanolamine (100 μM)	83 ± 8	80 ± 8	95 ± 10	88 ± 9
15-HAEA (10 μM)	33 ± 3	50 ± 5	90 ± 9	87 ± 9
LEA (10 μM)	56 ± 6	62 ± 6	89 ± 9	85 ± 9
13-HLEA (10 μM)	26 ± 3	43 ± 4	80 ± 8	82 ± 8
Leukotriene B$_4$ (1 μM)	ND	ND	105 ± 10	100 ± 10
Prostaglandin E$_2$ (10 μM)	ND	ND	105 ± 10	105 ± 10
PMSF (100 μM)	6 ± 1	8 ± 1	50 ± 5	52 ± 5
Iodoacetic acid (100 μM)	10 ± 1	12 ± 1	50 ± 5	48 ± 5
N-Ethylmaleimide (100 μM)	15 ± 2	18 ± 2	55 ± 5	50 ± 5
CCCP (50 μM)	ND	ND	85 ± 9	86 ± 9
SNP (5 mM)	87 ± 9	85 ± 9	170 ± 17	see Fig. 5B
SNAP (5 mM)	85 ± 9	87 ± 9	175 ± 18	see Fig. 5B
SPER–NO (5 mM)	88 ± 9	84 ± 9	172 ± 17	see Fig. 5B

[a]100% = 750 ± 70 pmol.min^{-1}.mg protein^{-1};
[b]100% = 390 ± 40 pmol.min^{-1}.mg protein^{-1};
[c]100% = 7.0 ± 0.7 pmol.min^{-1}.mg protein^{-1};
[d]100% = 53.0 ± 5.5 pmol.min^{-1}.mg protein^{-1}.
ND, not determined.

Alkylating agents such as PMSF, iodoacetic acid and *N*-ethylmaleimide (at 100 μM) almost abolished FAAH activity in all sources (Table 2). The NO-donors SNP, SNAP and SPER-NO (at millimolar concentrations that release nanomolar concentrations of NO in solution[29,30]) hardly affected the hydrolase activity (Table 2).

An anandamide synthase activity[31] was also present in the materials from human sources. The following maximum reaction rates were found: 70 ± 7 (human brain), 24.5 ± 2.5 (CHP100) and 40 ± 4 (U937) pmol.min^{-1}.mg protein^{-1}. These values were ~ 5-fold (CHP100 cells) to 10-fold (human brain and U937 cells) lower than the hydrolase activity under the same assay conditions (*i.e.*, 10 μM arachidonic acid and 20 μg proteins), as shown in Fig. 1. Nevertheless, the synthase was affected by 15-HAEA, LEA, 13-HLEA PMSF and SNP in the same way as the hydrolase activity (Table 2), both in human brain and human cell lines (data not shown).

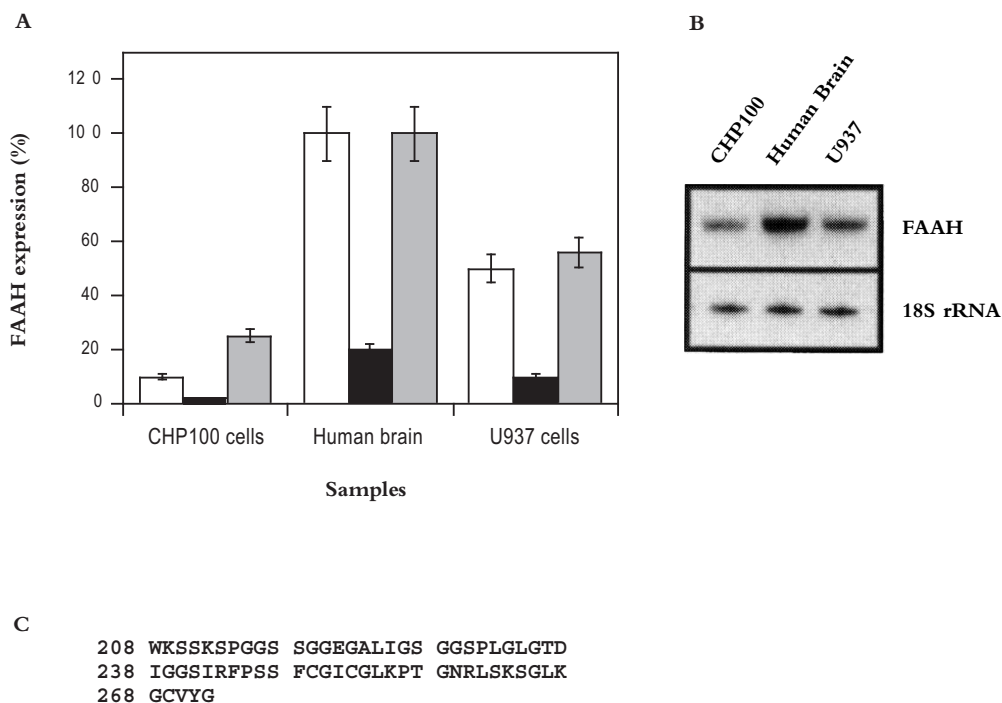*Expression of FAAH in human brain and human CHP100 and U937 cells*

The analysis of FAAH expression in human brain and human cells was performed at the protein (by ELISA) and mRNA (by RT-PCR) levels. The amount of FAAH protein in human brain was ~2- or 10-fold higher than that observed in U937 or CHP100 cells, respectively (Fig. 3A). This quantitation was validated by antigen competition experiments[18], showing that immunoreaction of the anti-FAAH polyclonal antibodies with the enzyme protein in human homogenates was specific (Fig. 3A). RT-PCR analysis showed similar differences in the mRNA levels (Fig. 3A and B). Sequencing of the FAAH mRNA, amplified by RT-PCR from human brain, human CHP100 or U937 cells, showed that human FAAH possessed a completely conserved sequence between aminoacids 208-272, which contains a typical amidase consensus sequence (Fig. 3C).

FAAH activity and expression were measured also in human meningioma and were compared to those found in the perilesional white matter (healthy brain). AEA hydrolysis by meningioma FAAH followed Michaelis–Menten kinetics, with apparent K_m and V_{max} values of 4.0 ± 0.4 μM and 370 ± 40 pmol.min^{-1}.mg protein^{-1}. Interestingly, the specific activity of FAAH in human meningioma was 50% compared with that in healthy brain, a value that was paralleled by the amount of FAAH protein in the same tissues (Fig. 4).

Characterization of AEA uptake in human CHP100 and U937 cells

Neuroblastoma CHP100 and lymphoma U937 cells were able to accumulate [1-^{14}C]AEA, a process that was temperature-dependent ($Q_{10} = 1.5$ for both cell lines), time-dependent ($t_{1/2} = 5$ min for both cell lines), and concentration-dependent (Fig. 5A) (data not shown). [1-^{14}C]AEA uptake in CHP100 and U937 cells was saturable ($K_m = 0.20 \pm 0.02$ and 0.13 ± 0.01 μM, $V_{max} = 30 \pm 3$ and 140 ± 15 pmol.min^{-1}.mg protein^{-1}, respectively) was enhanced when incubations were carried out in the presence of NO-donors SNP, SNAP or SPER-NO (Table 2 and Fig. 5B) and was reduced in the presence of PMSF, iodoacetic acid or *N*-ethylmaleimide, each used at a 100 μM final concentration


Figure 3   Quantitation of FAAH in human brain and human CHP100 and U937 cells.

A

B

C

```
208 WKSSKSPGGS SGGEGALIGS GGSPLGLGTD
238 IGGSIRFPSS FCGICGLKPT GNRLSKSGLK
268 GCVYG
```

A) tissue or cell homogenates (20 μg/well) were subjected to ELISA using specific anti-FAAH polyclonal antibodies (white bars). Antigen competition ELISA (black bars) are performed by preincubating anti-FAAH polyclonals with a 1000-fold molar excess of peptide antigen. Absorbance values are expressed as percentage of the maximum, arbitrarily set to 100 (100% corresponds to 0.760 ± 0.080 absorbance units at 405 nm). FAAH mRNA levels (grey bars) were quantitated by liquid scintillation counting and were expressed as percentage of the maximum, arbitrarily set to 100 (100% = 20000 ± 2000 cpm). The radioactivity of the bands corresponding to 18S rRNA (see panel B), was identical in all samples (5000 ± 500 cpm). B) FAAH mRNA (50 ng/lane) and 18S rRNA (0.2 ng/lane) were amplified by RT-PCR and electrophoresed on 6% polyacrylamide gels. C) Shown is the conserved amino acid sequence deduced from FAAH mRNA isolated from human brain, CHP100 or U937 cells. The sequence contains the amidase consensus sequence (aminoacids 215-246) typical of all FAAH as yet known.

Comparison of FAAH activity and expression in human healthy brain and meningioma.   **Figure 4**
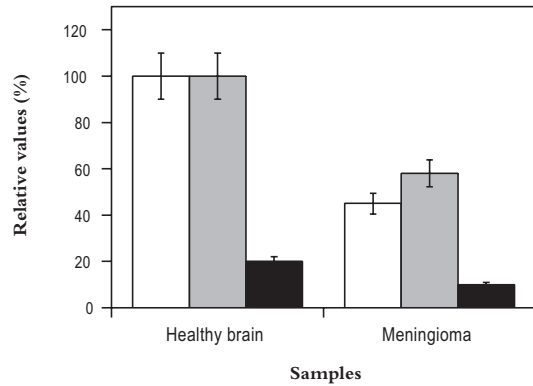
FAAH activity (white bars) was measured using 10 μM AEA as substrate. FAAH protein content (grey bars) was determined by ELISA using 20 μg proteins/well. Antigen competition ELISA (black bars) was performed by preincubating anti-FAAH polyclonals with a 1000-fold molar excess of peptide antigen. FAAH activity and content are expressed as percentage of the control (healthy brain), arbitrarily set to 100 (100% = 750 ± 70 pmol.min^{-1}.mg protein^{-1} for the activity; 100% = 0.760 ± 0.080 absorbance units at 405 nm for the protein content).

Uptake of [1-^{14}C]anandamide by intact CHP100 and U937 cells.   **Figure 5**
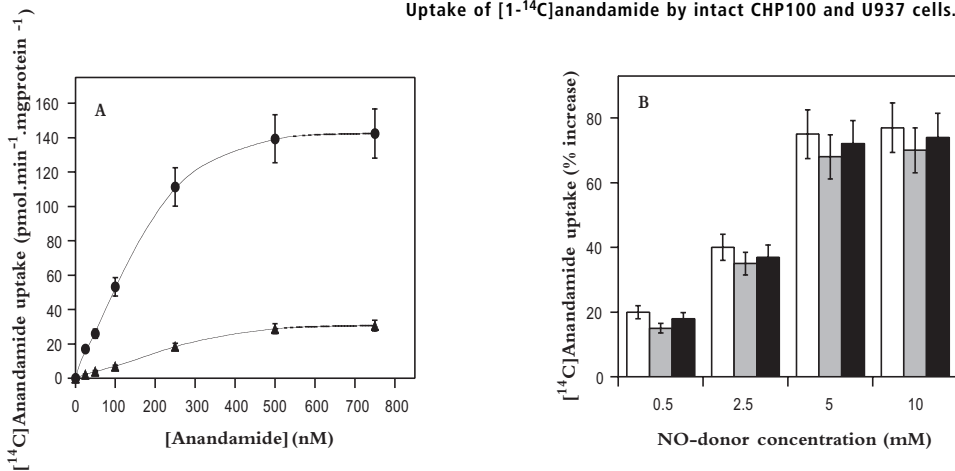
A) dependence of [1-^{14}C]AEA uptake (15 min, 37°C) on AEA concentration in human U937 (●) and CHP100 (▲) cells. B) Effect of NO donors SNP (white bars), SNAP (grey bars) and SPER/NO (black) on the uptake of 100 nM [1-^{14}C]AEA by U937 cells (15 min, 37°C). Uptake increase was expressed as percentage over the untreated control (100% = 53.0 ± 5.5 pmol.min^{-1}.mg protein^{-1})

Table 3   **Kinetic parameters of anandamide uptake in human CHP100 and U937 cells**

Uptake of [1-^{14}C]AEA was investigated in cell suspensions (2×10^6 cells/test), either untreated or treated with NO-donor SNP or alkylating agent PMSF. Apparent K_m and V_{max} values are expressed as μM and pmol.min^{-1}.mg protein^{-1}, respectively.

Human cell line	K_m	V_{max}	V_{max}/K_m
Neuroblastoma CHP100 cells	0.20 ± 0.02	30 ± 3	150
+5 mM SNP	0.20 ± 0.02	50 ± 5★	250
+100 μM PMSF	0.20 ± 0.02	15 ± 2★	75
Lymphoma U937 cells	0.13 ± 0.01	140 ± 15	1077
+5 mM SNP	0.13 ± 0.01	230 ± 22★	1769
+ 100 μM PMSF	0.13 ± 0.01	75 ± 8★	577

★ $P < 0.01$ compared with the control.

(Table 2). Enhancement of [1-^{14}C]AEA uptake by 5 mM SNP was prevented by co-incubation with either 20 μM hemoglobin, a typical NO scavenger[20], or 100 μM PMSF (data not shown). SNP and PMSF affected the uptake kinetics by changing the V_{max} value but not the K_m, thus changing the catalytic efficiency (*i.e.*, the V_{max}/K_m ratio) of the transporter (Table 3). On the other hand, 100 μM arachidonic acid or ethanolamine, and 10 μM 15-HAEA, LEA or 13-HLEA, did not significantly influence AEA uptake in either cell type, nor did 1 μM leukotriene B$_4$, 10 μM prostaglandin E$_2$ or 50 μM CCCP (Table 2).

Discussion

Meningioma is a histologically benign tumor that is brain invasive only in 4% of cases [32]. Thus, perilesional white matter surrounding the meningioma can be considered an essentially healthy brain area and was chosen in this study to characterize FAAH. Human brain showed a remarkable FAAH activity, and anti-FAAH antibodies recognized a single protein of 67 kDa with an isoelectric point of 7.6, characterized here for the first time (Fig. 2). These values were in good agreement with the size of the full-length human liver FAAH cDNA[19] and the isoelectric point predicted from FAAH sequence by the Wisconsin Sequence Analysis Package. Moreover, human brain FAAH cDNA had the same amidase consensus sequence (Fig. 3C) as FAAH cloned from human, mouse and rat livers[18,19]. It is noteworthy that the activation energy of the AEA hydrolysis catalyzed by FAAH from all three sources was identical. Furthermore, the FAAH activity in human

CHP100 and U937 cells shared several other biochemical properties, such as pH and temperature dependence and inhibition profile, with the enzyme from human brain. In addition, the enzymes contained an identical amidase sequence. This might indicate that the same enzyme was present in all human samples, although the participation of other enzymes cannot be ruled out.

Human brain FAAH was further characterized with respect to its interaction with inhibitors. Here, linoleoyl analogues of AEA and hydro(pero)xides generated thereof, which might be produced *in vivo* by brain lipoxygenases[14,33], were shown to be competitive inhibitors of FAAH activity, with apparent K_i values in the low micromolar range (Table 1). Interestingly, linoleoylethanolamide is a physiological constituent of rat neurons[3] and has recently been reported to be inactive at the CB_1–receptor[22] (See also chapter 3)

It is noteworthy that the apparent V_{max} of human brain FAAH was approximately 2-fold or 25-fold higher than that of U937 or CHP100 cells, respectively. The presence of different amounts of FAAH in the cells could explain this observation. Indeed, FAAH protein was 2- to 10-fold higher in human brain than in U937 or CHP100 cells, respectively (Fig. 3A), and similar differences were observed in the level of FAAH mRNA (Fig. 3B). Therefore, it can be suggested that a different expression (both at the transcriptional and translational level) of the same enzyme might be responsible for the different apparent V_{max} values of FAAH from the different human sources. A differential expression of FAAH might also be involved in human brain pathology, as suggested by comparison of meningioma and the surrounding (healthy) white matter (Fig. 4). This seems of interest, if one recalls that a neurotrophic effect of AEA has been proposed[8] and that AEA might act as growth factor for hematopoietic cell lines[34,35]. Therefore, a lower expression of the AEA–hydrolyzing enzyme FAAH might be instrumental in prolonging AEA-associated growth stimulus, ultimately leading to cell immortalization.

To be inactivated by FAAH, AEA has to be transported into the cell. Recent experiments performed on rat neuronal cells[3,11,12], rat basophilic leukemia (RBL–2H3) cells and mouse J774 macrophages[13] clearly showed the presence of a high–affinity AEA transporter in the outer cell membranes. A similar methodology was used here to characterize, for the first time, the AEA uptake in human neuronal (CHP100) and immune (U937) cells. Both cell types rapidly took up AEA ($t_{1/2}$= 5 min), in a temperature–dependent (Q_{10}= 1.5) and saturable way (Fig. 5A and data not shown). $[1-^{14}C]$AEA was taken up by CHP100 and U937 cells with similar high affinity, but remarkably different velocity (Table 3). Interestingly, U937 cells, which possessed higher FAAH activity than CHP100 cells, showed also a more efficient AEA uptake. The affinity of the AEA transporter in human cells was comparable to that of rat astrocytes (K_m = 0.32 μM)[12], and was almost one order of magnitude higher than the affinity reported for dopamine (K_m = 1 μM) or glutamate (K_m =1-5 μM) carriers in rat brain[36,37]. Furthermore, the uptake of AEA in human cells was affected by AEA hydrolysis products,

Scheme I   Interaction between anandamide uptake and degradation.
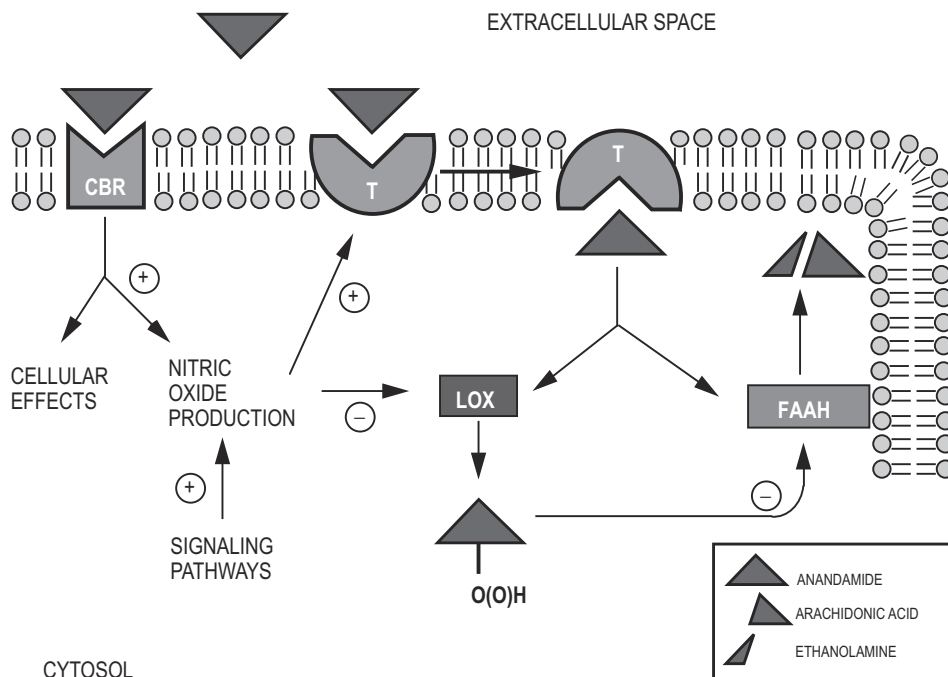
Binding of extracellular anandamide (AEA) to cannabinoid receptors (CBR) can lead to intracellular nitric oxide production, which in turn may activate transporter (T)–mediated uptake of AEA. Once uptaken, AEA can be rapidly cleaved by membrane-bound fatty acid amide hydrolase (FAAH), releasing arachidonic acid and ethanolamine. Alternatively, hydro(pero)xides of AEA can be generated by lipoxygenase (LOX) activity, leading to inhibition of FAAH. This alternate pathway is prevented by nitric oxide, short pulses of which are able to inhibit LOX activity. It should be stressed that other signaling pathways, uncoupled to AEA binding to CBR, can also enhance intracellular production of nitric oxide, thus may activate the sequestration process of this lipid mediator.

leukotriene B_4, prostaglandin E_2 and alkylating agents (Table 2) in much the same way as reported for rat neuronal and non-neuronal cells[11-13]. This suggests that AEA accumulation is selective and mediated by a transporter other than the long chain fatty acid transport protein[38] or the prostaglandin transporter[39], in keeping with recent data on the AEA carrier of rat neurons and astrocytes[12]. AEA uptake in human CHP100 and U937 cells was independent of mitochondrial energy metabolism, because the uncoupling agent CCCP[28] hardly affected AEA accumulation (Table 2). These results indicate that

AEA is accumulated by a carrier–mediated, facilitated diffusion, as recently reported for rat cells[11]. The enhancement of AEA uptake by the NO–donor SNP (Table 2) was due to increased apparent V_{max} (up to 170% of the control value), without changes in apparent K_m values. Conversely, alkylating agent PMSF reduced apparent V_{max} to 50% of the control, without changing apparent K_m (Table 3). It is tempting to suggest that the active site of the transporter may contain a cysteine residue, which could be the target of both NO–donors and alkylating agents. The effect of co–incubation with PMSF strengthens this hypothesis.

Altogether, the results reported here form the first characterization of human brain FAAH. In addition, the observations highlight the possible role of linoleoyl analogues of AEA, and hydro(pero)xides generated thereof and from AEA itself by lipoxygenase activity, as inhibitors of human brain FAAH. The AEA transporter has also been characterized for the first time in human cells, showing that it was not affected by the AEA derivatives/analogues that inhibited FAAH, but was sensitive to NO–donors.

These findings give rise to a general picture of the inactivation process of AEA in human neuronal and immune cells (Scheme I). AEA is brought into the cell by a transporter protein and is rapidly cleaved by intracellular FAAH. Lipoxygenase–generated products of AEA can competitively inhibit FAAH, which affords an elevated intracellular AEA concentration. The resulting dissipation of the AEA gradient renders the transporter inactive and leads to an enlarged extracellular AEA concentration. Enhanced CB–receptor stimulation results in prolonged pharmacological activity. On the other hand, the enhanced CB–receptor–induced NO formation potentiates the transporter protein, which clears AEA from the extracellular space. The NO–stimulated accumulation of AEA might be further enhanced by the fact that short pulses of NO are able to inhibit lipoxygenase activity[30], thus preventing inhibition of FAAH by lipoxygenase–generated hydroperoxides of AEA and congeners. Interestingly, any signaling pathway leading to NO release, either coupled or not coupled to cannabinoid receptors, might affect AEA metabolism by activating AEA (re)uptake. In this perspective, CB_1 and/or CB_2 receptors might reside on the same cell bearing the sequestration machinery or on different cells. The autacoid local inflammation antagonism[40] and glutamate excitotoxicity on neurons[41], where AEA exerts a(nta)gonistic effects on cannabinoid receptors and nitric oxide is released[10,42], might be two relevant processes in which the proposed sequestration scheme is operational. It is noteworthy that lipoxygenase activity is found in processes such as lymphocyte activation and neuronal cell death, where lipoxygenase activation[43,44] might prolong the effects of AEA[13].

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Oxygenated Metabolites of Anandamide and 2-Arachidonoylglycerol: Conformational Analysis and Interaction with Cannabinoid Receptors, Membrane Transporter and Fatty Acid Amide Hydrolase

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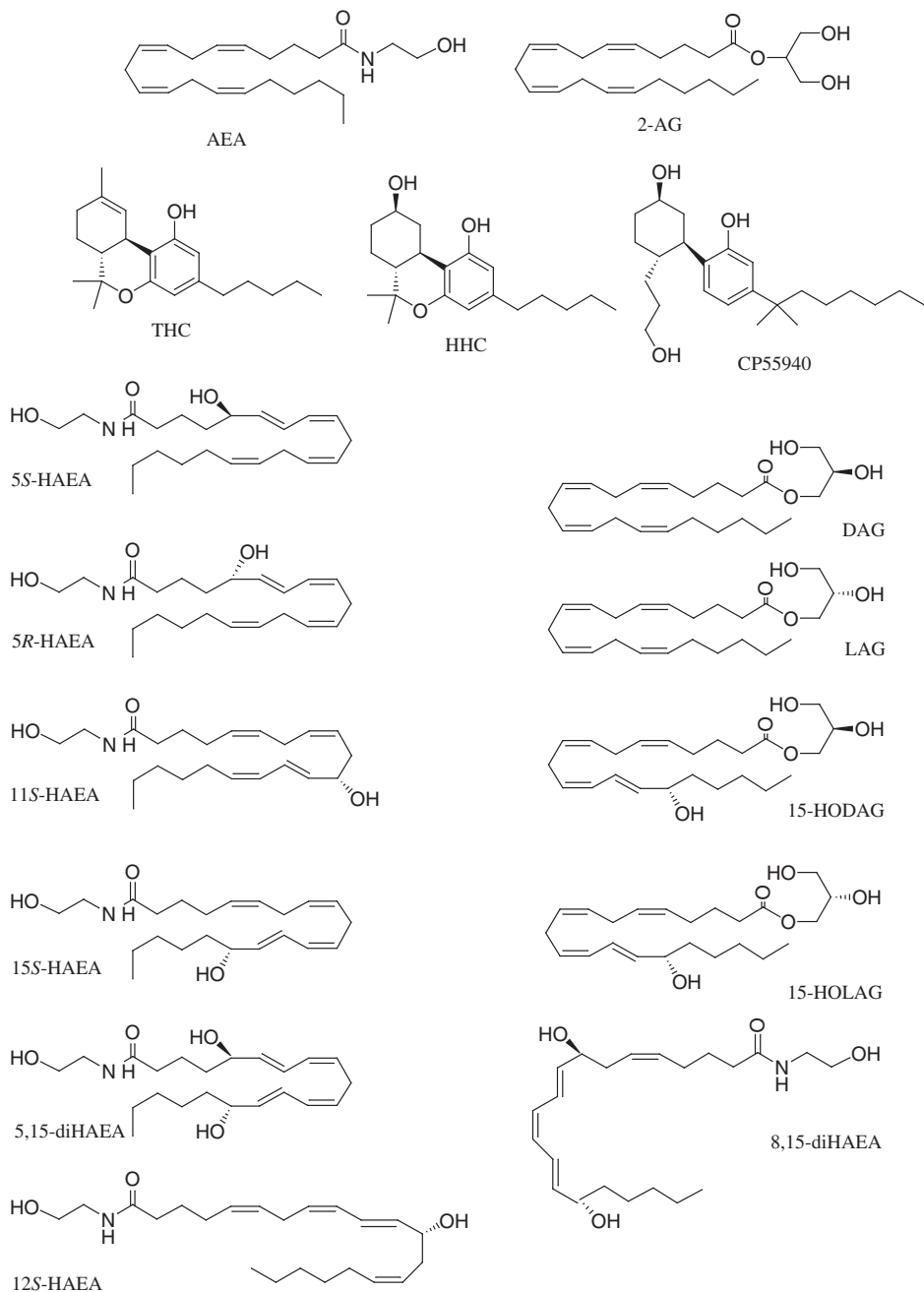
Abstract

This study was aimed at finding structural requirements for the interaction of the acyl chain of endocannabinoids with cannabinoid receptors, membrane transporter protein and fatty acid amide hydrolase (FAAH). To this end, the flexibility of the acyl chain was restricted by introduction of an 1-hydroxy-2Z,4E-pentadiene system in anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2–AG) at various positions using different lipoxygenases. This brought about selectivity and attenuated the binding potency of AEA and 2–AG. 15S-Hydroxyanandamide was found to bind selectively to the CB_1 receptor, whereas its 1-L-arachidonoylglycerol analog and 13S-hydroxylinoleoylethanolamide could selectively bind to the CB_2 receptor. 11S-Hydroxyanandamide did not bind to either receptor, whereas 12S-hydroxyanandamide did bind to both CB receptors with an affinity similar to that of AEA. All hydroxyanandamide derivatives were good inhibitors of FAAH (low μM K_i), but were ineffective on the AEA transporter.

Analysis of ^{1}H-NMR spectra revealed that chloroform did not induce notably different conformations in the acyl chain of 15S-hydroxyarachidonic acid compared with water. Molecular dynamic (MD) simulations of AEA and its analogs in the presence of explicit water molecules revealed that a tightly folded conformation of the acyl chain is not the only requirement for CB_1 binding. Structural details of the C_2-C_{15} loop, such as a sp^2-carbon at position 11, are necessary for receptor binding. The MD simulations may suggest that the average orientations of the pentyl tail of AEA and 12S-hydroxyanandamide are different from that of the low-affinity, inactive ligands.

Introduction

Anandamide (AEA; 5Z,8Z,11Z,14Z-eicosatetrateonoyl–N-(2-hydroxyethyl)–amine; arachidonoylethanolamide) is an endogenous lipid with neuro- and immunomodulatory activities. Many of the physiological activities of AEA are mediated via its interaction with cannabinoid CB_1 and CB_2 receptors, thereby mimicking some of the effects of Δ^9-tetrahydrocannabinol (THC), the psychoactive compound in marijuana (Fig.1)[1,2]. AEA and 2-arachidonoylglycerol (2–AG) are the main endocannabinoids, *i.e.* endogenous ligands of the CB receptors[3-5]. The effects of the endocannabinoids at the CB_1 and CB_2 receptors depend on the life span of these molecules in the extracellular space. AEA and 2–AG are inactivated by a rapid and selective process of transporter-mediated cellular uptake[6], followed by intracellular degradation to arachidonic acid and ethanolamine or

Chemical structures of classical, synthetic and endogenous cannabinoids and their derivatives.   Figure 1

AEA

2-AG

THC

HHC

CP55940

5S-HAEA

DAG

5R-HAEA

LAG

11S-HAEA

15-HODAG

15S-HAEA

15-HOLAG

5,15-diHAEA

8,15-diHAEA

12S-HAEA

63

glycerol by the enzyme fatty acid amide hydrolase (FAAH)[7,8]. The endocannabinoid system, which constitutes the endocannabinoids, CB receptors, transporter protein and FAAH, seems to be involved in the regulation of several physiological functions such as embryo implantation, pain, appetite and blood pressure[9-12]. Selective molecular probes targeted to one of the proteins of the endocannabinoid system are believed to yield useful therapeutics for a variety of disorders such as liver cirrhosis, multiple sclerosis and obesity, as well as for several neurodegenerative diseases including stroke[13-18]. In order to develop selective therapeutic agents, research is aimed at identifying essential structural properties of the endocannabinoids, which are needed for specific interactions with the CB receptors, AEA membrane transporter and FAAH[15,19-21].

Crystal or NMR structures of any of the proteins of the endocannabinoid system might help to elucidate the nature of the ligand–protein interaction, but they are not available yet. Therefore, a structural comparison of active and inactive classical and synthetic cannabinoids has been used to derive pharmacophore models[22-24]. These models might also be used to predict and facilitate the design of novel compounds with greater potency or selectivity at the molecular target of interest[22-24]. A prerequisite for the building of such a pharmacophore model is to characterize an ensemble of active conformations of a molecule. AEA can assume many different conformations, which originate primarily in its acyl chain. It has been suggested that a tightly folded conformation is responsible for the interaction of AEA with CB receptors[19]. However, this has not been substantiated as yet.

In order to get more insight into the structural requirements of the acyl chain of AEA to selectively interact with the proteins of the cannabinoid system and in particular the CB_1 receptor, we have combined interaction studies, ^{1}H-NMR spectroscopy and Molecular Dynamic (MD) simulations. The $1Z,4Z$-pentadiene systems of the endocannabinoids were systematically converted into 1-hydroxy-$2E,4Z$-pentadiene systems at different positions of the acyl chain by using different lipoxygenases as biocatalysts. We extended the studies of Hampson *et al.* and Edgemond *et al.* to other oxygenated metabolites to compare all derivatives in one system.[25,26] CB_1- and CB_2-binding assays were performed as well as assays in which the effects of the oxygenated metabolites on AEA–inactivation were studied. ^{1}H-NMR was used to study the solvent–effect on the conformation of the acyl chain. MD–simulations of AEA and congeners with explicit water molecules were performed to determine the influence of conjugation of the double bond system on its conformation.

Materials and Methods

5S-HAEA, 11S-HAEA, 13S-LEA 5,15-diHAEA, 8,15-diHAEA, 15S-HAEA, 15-HO-D-AG and 15-HO-L-AG were enzymatically synthesized by soybean and barley lipoxygenases as previously reported and characterized by CD-spectroscopy, ^{1}H-NMR and GC/MS[26-31]. The spectra were in accordance with their chemical structures, as previously reported[26,27,30,31]. Each lipoxygenase was incubated with LEA, AEA (Cayman Chemicals), 1-D-AG or 1-L-AG (1 U enzyme lipoxygenase per 3 μmol substrate) in 100 mM sodium borate buffer (pH 9) for soybean and 100 mM phosphate buffer (pH7) for barley grain. AEA was incubated with bovine polymorphonuclear leukocytes in phosphate-buffered saline (pH 7.4) to produce 12S-HAEA. The products of the plant lipoxygenases were reduced by 3 equivalents sodium borohydride. The HAEAs were isolated with SPE (Bakerbond, 500 mg), analyzed and purified by reversed-phase and chiral-phase HPLC as reported[27,28,31]. [^{3}H]AEA (223 Ci/mmol) and [^{3}H]CP-55,940 (126 Ci/mmol) were purchased from NEN Life Science Products, Inc.

Membrane preparation for binding assay

Male Wistar rats (250-280g) were maintained on a 12h light/dark schedule. Food and water were *ad libitum*. The experimental protocol and procedures used, met the guidelines of the Ministry of Health (G.U.No. 40, February 18, 1992) and were approved by the Animal Care Committee (University of Rome "Tor Vergata"). Rat forebrain and spleen membrane preparations were obtained using the method of Devane *et al.*,[32] and were stored at a concentration of 1 mg.ml^{-1} protein in 50 mM Tris.HCl, 2 mM Tris.EDTA, 3 mM $MgCl_2$ buffer, pH 7.4, at -80 °C for no longer than one week. The protein concentration was determined using bovine serum albumin (BSA) as a standard[33].

CB-binding assay

A rapid filtration assay was performed with [^{3}H]-labeled CP-55.940, according to Compton *et al.*[34]. Incubations were performed in a final volume of 0.5 ml 50 mM Tris.HCl, 2 mM Tris.EDTA, 3 mM $MgCl_2$, 5 mg ml^{-1} BSA buffer, pH 7.4. PMSF (final concentration 50 μM) was added freshly each time just before the incubations started. The binding was initiated by the addition of 50 μg of protein of the membrane preparation and stopped after 1h at 30°C. The washed filters were transferred to vials, which contained 0.5 ml 0.1% Triton X-100 and 3.5 ml Liquid Scintillation Cocktail (LSC) for nonaqueous solutions (Sigma Chemical Company). The vials were incubated overnight before counting. Unspecific binding was determined in the presence of 10 μM AEA. Binding data were elaborated through nonlinear regression analysis, using the Prism 3 program (GraphPAD Software for Science), and inhibition constants (K_i) were calculated.

AEA-transporter-activity

The uptake of [^{3}H]AEA by intact U937 cells was studied as described previously.[35] Incubations (15 min) were carried out with different concentrations of [^{3}H]AEA, in the range 0-1000 nM, in

order to determine the kinetic constants, *i.e.* apparent Michaelis–Menten constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i), by nonlinear regression analysis through the Prism 3 program.[35]

FAAH-activity

Fatty acid amide hydrolase activity was assayed in U937 cells by measuring the release of [^{3}H]arachidonic acid from [^{3}H]AEA using RP-HPLC as reported[29]. Kinetic studies were performed using different concentrations of [^{3}H]AEA (in the range 0-25 μM), and the kinetic constants (K_m, V_{max} and K_i) were calculated by nonlinear regression analysis through the Prism 3 program.[7]

2D-NMR

^{1}H-NMR spectra of 15-HETE were recorded at 500 MHz with a Bruker DRX–500 (Bijvoet Center for Biomolecular Research). Spectra were obtained at a probe temperatures 27°C for D_2O (0.2 mmol.l^{-1}; pH 6) and $CDCl_3$ (40 mmol.l^{-1}) solutions and 4°C for a CD_3OD (40 mmol.l^{-1}) solution. Phase sensitive (States-TPPI) 2D NOESY, 2D-off-resonance ROESY and 2D TOCSY were recorded in addition to high–resolution 1D spectra. The 2D TOCSY spectrum was obtained using an MLEV-17 isotropic mixing sequence of 15 ms at a spin lock field strength corresponding to 9.2 kHz. 2D NOESY spectra were obtained with an 800 ms mixing time. The 2D off–resonance ROESY spectrum in D_2O solution was recorded with an adiabatic spin-lock pulse of 350 ms at a field strength corresponding to 6.1 kHz. The spin-lock frequency was alternately placed 3520 Hz upfield or downfield of the centre of the spectrum, thus obtaining an average spin-lock angle <ϑ>=60°. Chemical shifts for ^{1}H are expressed in ppm relative to internal TMS (0.0 ppm) for $CDCl_3$ solutions and relative to internal acetone (2.218 ppm) for D_2O solutions.

The chemical shift and scalar coupling values were extracted through direct measurement in the high–resolution 1D NMR spectra or through simulation of the more complex spectral regions. The assignment of the ^{1}H-signals in the NMR spectra was obtained through combined analysis of the multiplet profiles in the 1D-NMR spectra, and cross peaks in the 2D-TOCSY spectrum. The NOESY and off-resonance ROESY spectrum supplied supporting information to corroborate the assignments.

Molecular dynamics calculations

Molecular dynamics simulations were performed using the GROMOS87 program package[36] on PC's running Linux. Each molecule was surrounded by SPC/E[37] water molecules in a truncated octahedron with periodic boundary conditions. All bond lengths were kept fixed using the SHAKE procedure[38]. Nonbonded interactions were calculated using the twin–range cutoff procedure with cutoff radii of 0.9 and 1.2 nm, and a time step of 2 fs was used. Simulations were performed with loose coupling to a pressure bath at 1 atm and a temperature bath at 300 K[39] with time constants of 0.5 and 0.1 ps, respectively. Atom positions were restrained by applying a harmonic oscillator force constant of 10^4 kcal mol^{-1} Å^{-2}.

Description of force field adaptions

A new atom type CS has been created to represent a bare sp^3 carbon which is in all aspects identical to CH1 and CH2, except for the van der Waals parameters that are identical to those of C or CB. To represent the double bonds, two new atom types, CEH1 and CIH1, have been created. CEH1 represents the C atoms involved in a double bond, and the external C atoms of a conjugated double bond. CIH1 represents the internal C atoms of a conjugated double bond. These two new atom types are equivalent to the CR51 and CR61 atom types except for the parameters listed in Table S1.

Supporting information:

5R/S–HAEA: Barley lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.55 (dd, J=13.6, 10.6 Hz 1H), 6.02 (t J=10.5 1H), 5.71 (dd, J=13.6 Hz, 1H), 5.39 (m, 5H), 4.16 (m, 1H), 3.72 (t, 2H), 3.42 (q. 2H) 2.97 (m, 2H); 2.81 (m, 2H), 2.22 (t, J=7.8Hz, 2H), 2.11 (m, 2H); 1.72 (q, J=6.2 Hz 2H), 1.34 (m, 6H), 0.89 (t, J=6.2 Hz, 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 515 [M$^+$], 500 [M$^+$-CH$_3$], 313 [C$_{16}$H$_{32}$OTMS$^+$], 304 [M$^+$-C$_{15}$H$_{31}$], 214 [304-TMSOH], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

11S–HAEA: Barley lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.52 (dd, J=11.0; 10.1 Hz 1H), 5.98 (t, J=11.0 Hz 1H), 5.69 (dd, J=15.1; 6.5 Hz 1H), 5.40 (m, 5H), 4.23 (q, 2H); 3.72 (t, J=4.6 Hz, 2H); 3.42 (q J=5.5 Hz; 2H), 2.81 (m, 2H), 2.32 (m, 2H), 2.22 (t, J=7.4 Hz 2H), 2.12 (m, 4H), 1.73 (q, 2H), 1.25-1.38 (m, 6H), 0.88 (t, J=6.9 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 515 [M$^+$], 500 [M$^+$-CH$_3$], 388 [M$^+$-C$_9$H$_{19}$], 229 [C$_{10}$H$_{20}$OTMS$^+$], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

12S–HAEA: Bovine leukocytes; ^{1}H NMR (CDCl$_3$) δ 6.58 (dd, J=14.2 Hz 1H), 5.99 (t, J=10.5 Hz 1H), 5.74 (dd, J=6.2 Hz 1H), 5.40 (m, 5H), 4.25 (q, 1H), 3.72 (t, J=4.6 Hz 2H), 3.42 (q, J=4.6 Hz 2H), 2.95 (m, 2H), 2.33 (m, 2H), 2.21 (t, J=7.5 Hz 2H), 2.10 (m, 4H), 1.74 (q, J=7.3 Hz, 2H), 1.28 (m, 6H), 0.89 (t, J=6.9 Hz 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 515 [M$^+$], 500 [M$^+$-CH$_3$], 402 [M$^+$-C$_8$H$_{17}$$^+$], 215 [C$_9H_{18}$OTMS$^+$], 116 [C$_2H_3$OTMS$^+$], 73 [TMS$^+$]

15S–HAEA: Soybean lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.55 (dd, J=15.4;12,2 Hz 1H), 6.00 (t, J=10.7 Hz, 1H), 5.72 (dd J=7.2 Hz 1H), 5.40 (m 5H), 4.12 (q 1H), 3.72 (t, 2H) 3.42 (q, 2H), 2.97 (m, 2H), 2,82 (m, 2H), 2.22 (t, J=7.5 Hz 2H), 2.11 (m, 2H), 1.72 (q, J=7.3 Hz, 2H), 1.56 (m, 2H), 1.31 (m, 6H), 0.89 (t, J=6.9 Hz 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 515 [M$^+$], 500 [M$^+$-CH$_3$], 444 [M$^+$-C$_5$H$_{11}$], 173 [C$_6$H$_{11}$OTMS$^+$], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

5,15–diHAEA: Soybean lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.58 (m, 2H), 6.01 (m, 2H), 5.72 (m, 2H), 5.43 (m, 2H), 4.21 (m, 2H), 3.72 (t, J=4.6 Hz 2H), 3.42 (q, J=4.6 Hz 2H), 2.97 (m, 2H), 2.28 (t, J=6.9 Hz 2H), 1.76 (m, 2H), 1.57 (m, 4H), 1.30 (m, 6H), 0.89 (t, 3H); NaBH$_4$ and H$_2$ reduced

trimethyl silylether GC/MS m/z 603 [M$^+$], 588 [M$^+$-CH$_3$], 532 [M$^+$-C$_5$H$_{11}$], 304 [M$^+$-C$_{15}$H$_{31}$], 214 [304-TMSOH], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

8,15–diHAEA: Soybean lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.70 (m, 2H), 5.97 (m, 2H) 5.74 (m, 2H), 5.46 (m,2H), 4.27 (m, 1H), 4.18 (m, 1H), 3.72 (t, J=4.6 Hz 2H), 3.42 (q, J=4.6 Hz 2H), 2.32 (m, 2H), 2.19 (t, J=6.9 Hz, 2H), 2.09 (q, 2H), 1.70 (m, 2H), 1.50 (m, 2H), 1.32 (m, 6H), 0.88 (t, 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 603 [M$^+$], 588 [M$^+$-CH$_3$], 532 [M$^+$-C$_5$H$_{11}$], 346 [M$^+$- C$_{12}$H$_{24}$OTMS], 173 [C$_6$H$_{11}$OTMS$^+$], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

13*S*-HLEA: ^{1}H NMR (CDCl$_3$) δ 6.49 (dd, J=15.0, 11 Hz, 1H), 5.98 (t, J=10.9 Hz 1H), 5.67 (dd, J=15.2, 6.8 Hz 1H), 4.16 (q, J=6.5 Hz 1H), 3.72 (t, 2H), 3.42 (q, 2H), 2.17 (m, J=7.4 4H), 1.66-1.47 (m, 6H), 1.38-1.22 (m, 10H), 0.89 (t, J=6.5 Hz 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 487 [M$^+$], 472 [M$^+$-CH$_3$], 416 [M$^+$-C$_5$H$_{11}$], 173 [C$_6$H$_{11}$OTMS$^+$], 116 [C$_2$H$_3$OTMS$^+$], 73 [TMS$^+$]

1-L-AG: ^{1}H NMR (CDCl$_3$) δ 5.40 (m, 8H), 4.19 (m, 2H), 3.93 (m, 1H), 3.65 (m, 2H), 2.84 (m, 6H), 2.37 (t, J=7.5 Hz, 2H), 2.09 (m, 4H), 1.72 (m 2H), 1.33 (m, 6H), 0.89 (t, J=6.8 Hz 3H)

1-D-AG: ^{1}H NMR (CDCl$_3$) δ 5.40 (m, 8H), 4.19 (m, 2H), 3.93 (m, 1H), 3.65 (m, 2H), 2.84 (m, 6H), 2.37 (t, J=7.5 Hz, 2H), 2.09 (m, 4H),1.72 (m, 2H), 1.33 (m, 6H), 0.89 (t, J=6.8 Hz 3H)

15-HO-L-AG: Soybean lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.53 (dd, J=15.2 Hz 1H), 6.00 (t, J=11.2 Hz 1H), 5.70 (dd, J=6.5 Hz 1H), 5.40 (m, 5H), 4.19 (m 2H), 3.92 (m 1H), 3.67 (m, 2H), 2.97 (m, 2H), 2.82 (m, 2H), 2.37 (t, J=7.4 Hz 2H), 2.12 (m, 2H), 1.72 (q, J=7.3 Hz 2H), 1.55 (m, 2H), 1.35 (m, 6H), 0.89 (t, J=7.1 Hz 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 618 [M$^+$], 603 [M$^+$-CH$_3$], 547 [M$^+$-C$_5$H$_{11}$], 173[C$_6$H$_{11}$OTMS$^+$], 103 [CH$_2$OTMS$^+$], 73 [TMS$^+$]

15-HO-D-AG: Soybean lipoxygenase; ^{1}H NMR (CDCl$_3$) δ 6.53 (dd, J=15.2 Hz 1H), 6.00 (t, J=11.1 Hz 1H), 5.70 (dd, J=6.4 Hz 1H), 5.40 (m, 5H), 4.19 (m 2H), 3.92 (m 1H), 3.65 (m, 2H), 2.97 (m, 2H), 2.82 (m, 2H), 2.37 (t, J=7.4 Hz 2H), 2.12 (m, 2H), 1.72 (q, J=7.3 Hz 2H), 1.55 (m, 2H), 1.35 (m, 6H), 0.89 (t, J=6.6 Hz 3H); NaBH$_4$ and H$_2$ reduced trimethyl silylether GC/MS m/z 618 [M$^+$], 603 [M$^+$-CH$_3$], 547 [M$^+$-C$_5$H$_{11}$], 173 [C$_6$H$_{11}$OTMS$^+$], 103 [CH$_2$OTMS$^+$], 73 [TMS$^+$]

Results and Discussion

Interaction with CB_1 and CB_2 receptors

   In accordance with previous observations, AEA inhibited the specific binding of [³H]CP-55,940 to rat brain membranes, which express CB_1 receptors, in a manner typical of competing ligands with an inhibition constant of 90 nM (Table 1)[2]. Compounds with

Displacement constants (K_i) of AEA and their oxygenated metabolites for CB_1 and CB_2 receptor binding   **Table 1**

Compound	CB$_1$ (nM)[a]	CB$_2$ (nM)[b]	Literature values CB$_1$ (nM)	Literature values CB$_2$ (nM)
AEA	90 ± 20	360 ± 50	71[c]/107[d]	94[e]
5(S)-HAEA	> 1000	> 1000	–	–
5(R)-HAEA	680 ± 140	710 ± 145	–	–
11(S)-HAEA	> 1000	> 1000	1102[c]	–
11(R/S)-HAEA	> 1000	> 1000	–	–
12(S)-HAEA	150 ± 30	500 ± 60	31[c]/207[d]	131[e]
13(S)-HLEA	> 1000	600 ± 120	–	–
15(S)-HAEA	600 ± 120	> 1000	418[c]/738[d]	1000>[e]
15(R/S)-HAEA	680 ± 140	> 1000	–	–
5,15-diHAEA	> 1000	> 1000	–	–
8,15-diHAEA	> 1000	> 1000	–	–

[a]K_i values were calculated from the displacement curves of [³H]CP-55,940 from rat forebrain membranes.

[b]K_i values were calculated from the displacement curves of [³H]CP-55,940 from rat spleen membranes.

[c]K_i values were calculated from the displacement curves of [³H]CP-55,940 from rat brain synaptosomal membranes[26]

[d]K_i values were calculated from the displacement curves of [³H]CP-55,940 from rat forebrain membranes[25]

[e]K_i values were calculated from the displacement curves of [³H]CP-55,940 from human CB$_2$-receptor expressed in CHO-cells[25]

a K_i > 1 μM were considered inactive, therefore an upper limit of 1 μM was set for binding potency[40]. Introduction of a hydroxyl-function and a conjugated diene system in AEA by various lipoxygenases reduced the capacity of AEA-derivatives to compete for CB_1-binding (Table 1). Only 12S-HAEA retained a binding affinity in the same order of magnitude as AEA. 15S-HAEA and 5R-HAEA had a ~ 7-fold higher K_i, while 11S-HAEA, 5S-HAEA, 13S-HLEA were inactive (Table 1). Introduction of a second 1-hydroxy-2E,4Z-diene system in AEA led to a complete loss of binding affinity as demonstrated by 5,15-diHAEA and 8,15-diHAEA (Table 1).

AEA also inhibited [^{3}H]CP-55,940 binding to spleen membranes, which express CB_2 receptors, with a K_i of 360 nM (Table 1). This is in agreement with previous observations[2]. The CB_2 receptor was more critical in accepting oxygenated metabolites of AEA as ligands than the CB_1 receptor. 12S-HAEA and 5R-HAEA were almost as potent as AEA (Table 1), while 5S-HAEA, 11S-HAEA, 15S-HAEA and the doubly hydroxylated AEAs (5,15-diHAEA and 8,15-HAEA) were inactive (Table 1). Interestingly, 13S-HLEA had a binding affinity towards the CB_2 receptor with a K_i of 600 nM, whereas it was inactive at the CB_1 receptor.

Interaction with AEA-transporter and FAAH

Human lymphoma U937 cells have a selective AEA-transporter with a K_m and V_{max} of 0.13 μM and 140 pmol/min per mg protein, respectively.[6] These cells also have an active FAAH with a K_m and V_{max} of 6.5 μM and 520 pmol/min per mg protein, respectively.[6] Introduction of 1-hydroxy-2E,4Z-diene into AEA at any position disrupted its ability to inhibit the transport of [^{3}H]AEA into U937 cells (Table 2). This indicates that the HAEAs have a reduced binding affinity for the AEA-transporter. It has been suggested that at least one *cis*-double bond is necessary for binding to the AEA-transporter protein, and four *cis*-double bonds for translocation into the cell[41]. Our data are in line with these observations[7,41,42].

FAAH accepted all AEA-derivatives as inhibitors (Table 2), thereby making FAAH the least selective protein of the endocannabinoid system. Introduction of the 1-hydroxy-2E,4Z-pentadiene system increased the ability of all AEA-derivatives to competitively inhibit [^{3}H]AEA-hydrolysis by human U937 cells. 13S-HLEA was the most potent inhibitor with a K_i of 0.43 μM (23-fold better than AEA), while 12S-HAEA had a 3-fold higher K_i than AEA. Introduction of a second hydroxyl group did not improve the inhibition power any further. The rank order was: 13S-HLEA > 11S-HAEA > 15S-HAEA ≈ 8,15-diHAEA > 5,15-HAEA > 5R-HAEA > 5S-HAEA > 12S-HAEA. The solubility of HAEAs in aqueous solutions was higher than that of the parent compound, due to the introduction of the hydroxyl function. This improves the feasibility of the use of HAEAs as inhibitors of FAAH, compared to saturated congeners.

Inhibition constants (K_i) of AEA and its oxygenated metabolites for FAAH activity and AEA-transporter activity Table 2

Compound	FAAH (μM)[a]	Transporter (μM)[b]
AEA	> 10	> 10
5(*S*)-HAEA	2.52 ± 0.13	> 10
5(*R*)-HAEA	1.89 ± 0.09	> 10
11(*S*)-HAEA	1.57 ± 0.03	> 10
11(*R/S*)-HAEA	1.69 ± 0.03	> 10
12(*S*)-HAEA	2.90 ± 0.15	> 10
13(*S*)-HLEA	0.43 ± 0.02	> 10
15(*S*)-HAEA	0.63 ± 0.03	> 10
15(*R/S*)-HAEA	0.63 ± 0.03	> 10
5,15-diHAEA	1.26 ± 0.06	> 10
8,15-diHAEA	0.69 ± 0.03	> 10

[a]All compounds were competitive inhibitors of FAAH activity in U937 cells. [^{3}H]AEA was used as substrate, in the 0 -25 μM concentration range.
[b]All compounds were competitive inhibitors of AEA–transporter activity in U937 cells. [^{3}H]AEA was used as substrate, in the 0–1000 nM concentration range.

Interaction of 2-AG and its congeners with the proteins of the cannabinoid system

2-AG rapidly isomerizes into 1–AG both *in vitro* and *in vivo*. The rate of this process is increased by high temperature and by acidic or basic pH. Two stereoisomers can be formed, *i.e.* 1-D-AG and 1-L-AG (Fig. 1). In endocannabinoid analysis, usually 10-40% of a racemic mixture of 1–AG is found[43]. To date, most interaction studies of 2-AG with proteins of the endocannabinoid system have not accounted for isomerisation of 2-AG into 1–AG during the incubation period. Here, it is shown that 2-AG inhibited potently the binding of [^{3}H]CP-55,940 to CB_1 and CB_2 receptors with a K_i of 100 nM (Table 3). 2-AG also inhibited [^{3}H]AEA-transport with a K_i of 3 μM, and was 2-fold more potent than AEA in inhibiting FAAH (Table 3). However, 1-D-AG and 1-L-AG did not bind to either CB receptor and did not interfere with AEA-transport (Table 3). Thus, upon isomerisation 2-AG is inactivated, thereby decreasing effective concentrations of 2-AG. This uncontrolled isomerisation may account to some extent for the large differences in

Table 3   Inhibition and displacement constants (K_i) of 2-AG and congeners for FAAH activity, AEA-transporter

activity and CB_1 and CB_2 receptor binding

Compound	CB_1 (nM)[a]	CB_2 (nM)[b]	FAAH (μM)[c]	Transporter (μM)[d]
2–AG	100 ± 20	100 ± 20	5 ± 0.25	3 ± 0.15
1–L–AG	> 1000	> 1000	3 ± 0.15	> 10
1–D–AG	> 1000	> 1000	2.5 ± 0.15	10 ± 0.50
15–HO–L–AG	> 1000	550 ± 80	6 ± 0.30	9 ± 0.45
15–HO–D–AG	> 1000	> 1000	5 ± 0.25	7 ± 0.35

[a] All compounds were competitive inhibitors of FAAH activity in U937 cells. [^{3}H]AEA was used as substrate, in the $0 - 25$ μM concentration range.

[b] All compounds were competitive inhibitors of AEA-transporter activity in U937 cells. [^{3}H]AEA was used as substrate, in the $0 - 1000$ nM concentration range.

[c] K_i values were calculated from the displacement curves of [^{3}H]CP-55,940 from rat forebrain membranes.

[d] K_i values were calculated from the displacement curves of [^{3}H]CP-55,940 from rat spleen membranes.

K_i-values reported in the literature for 2-AG binding to CB receptors, and for its inhibition of AEA–transport[6].

Interestingly, introduction of the 15-hydroxy-11Z,13E-diene system in 1-L-AG increased its CB_2-binding affinity compared to its parent compound, but this was not the case for 1-D-AG. Both 15-HO-L-AG and 15-HO-D-AG could inhibit AEA–transport at low micromolar concentrations, but were worse inhibitors of [^{3}H]AEA–hydrolysis than the parent compounds (Table 3).

It should be noted that different mammalian lipoxygenases are capable of using AEA and 2-AG as substrates in *in vitro* systems[25,26,44,45]. If this action of lipoxygenases would be functional *in vivo,* this might be instrumental to generate *in vivo* selectivity in the endocannabinoid system. In this line, it has been shown that 5-lipoxygenase inhibitors "disclose" a cryptic FAAH activity in human mast cells[46]. Furthermore, the increased capability of HAEAs to inhibit FAAH may be a way to enhance endocannabinoid signaling, *i.e* an "entourage" effect for AEA similar to that shown for 2-AG[47].

Conformation and solvation effects studied with NMR spectroscopy

In order to study the conformational effects induced by different solvents on an acyl chain, 1D and 2D-NMR spectroscopy experiments were performed with 15-hydroxyarachidonic acid (15-HETE) in chloroform and water. Double bonds and areas with conjugated double bonds induce considerable conformational restraints to

molecules. Therefore, spectral analysis was focused on regions containing signals from these restrained areas, and especially on the flexible methylene group regions that connect them.

The NMR-spectra of 15-HETE in D_2O and $CDCl_3$ were remarkably similar in terms of chemical shift values and coupling constants. (Fig. 2a,b and Table S2). The spectral region containing the signals for H_7 and H_{10}, which are regarded as sensitive probes for conformational differences, did not show any notable differences. In order to get more insight into the conformational properties of 15-HETE, 2D NMR spectra were recorded that reveal information about short inter-proton distances (NOESY or off-resonance ROESY). In D_2O solutions the signal-to-noise ratio was poor, despite extended measuring times, due to the low solubility of 15-HETE. Qualitative comparison of

1D-NMR-spectra of 15-HETE in water (a) and chloroform (b). Figure 2
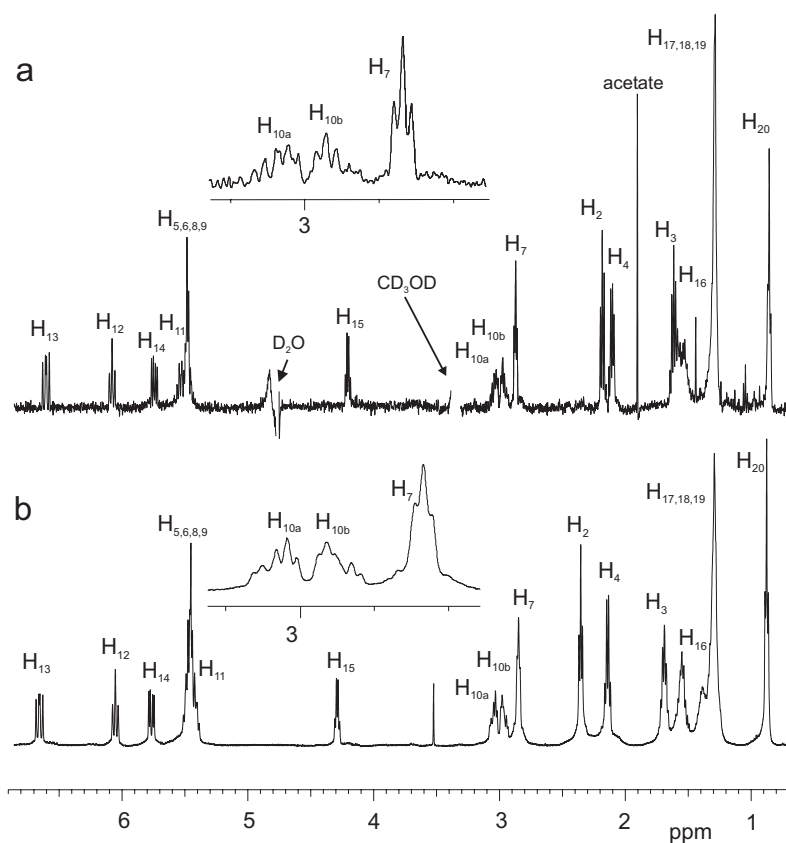
Figure 3   2D-NMR-spectrum (NOESY) of 15-HETE.
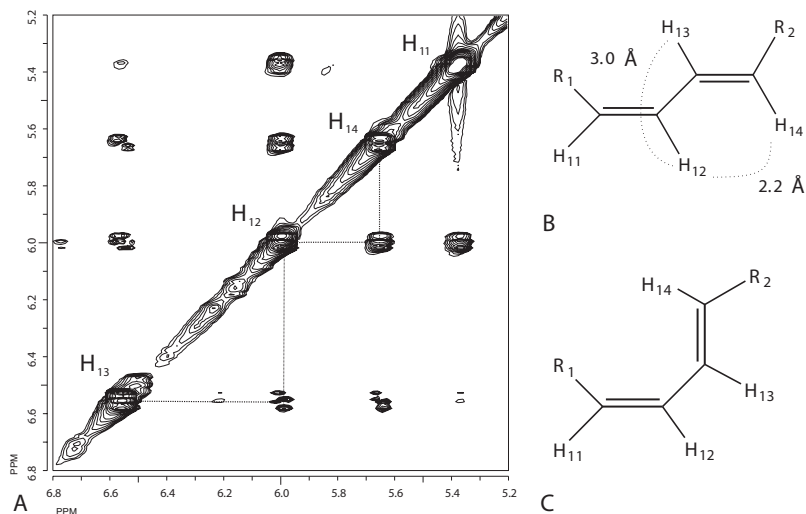
2D-NMR-spectrum (NOESY) of 15-HETE (A). The region of interest (6.8-5.2 ppm) is displayed, showing that NOE-contacts between H_{12} and H_{14} are more intense than H_{12} and H_{13}. This indicates that the conformation of the conjugated 1-hydroxy-2Z,4E-pentadiene system is represented by figure 3B than figure 3C.

NOESY spectra of 15-HETE $CDCl_3$ solution and off-resonance ROESY spectra in D_2O solution revealed that the more intense cross peaks were present in both solutions (data not shown). In order to obtain high quality 2D-NMR data of 15-HETE in a polar environment, a NOESY spectrum of 15-HETE was recorded in a CD_3OD solution. This NMR spectrum showed similar spectral properties as had been obtained for $CDCl_3$ or D_2O solutions and also the NOE cross peaks were qualitatively and quantitatively similar (data not shown). The NOESY spectra did not show any cross peaks that signify long range (primary structure) interactions. Thus, analysis of the 1D-NMR profiles, chemical shifts, scalar coupling values and the distance correlation spectra did not reveal notable differences, which could originate from different conformations of 15-HETE induced by chloroform, methanol or water.

Special attention was given to the conjugated pentadiene system. An intense cross peak in the methanol NOESY-spectrum was observed between H_{12} and H_{14}, whereas the NOE for the interresidual contact between H_{12} and H_{13} was less intense (Fig. 3a). Proton distances were estimated from cross-peak intensities and were calibrated to distances on

the basis of the distance of the vicinal protons H_{11}-H_{12} (2.20 Å). The distance between H_{12}-H_{14} was 2.24 Å, and that between H_{12}-H_{13} was 2.98 Å, which indicated that the dihedral angle C_{11}-C_{12}-C_{13}-C_{14} was almost exclusively 180° (Fig. 3b) rather than 0° (Fig. 3c). Thus, a single conformation of the conjugated pentadiene system was indeed strongly preferred.

Conformational analysis of (H)AEA

To develop an endocannabinoid pharmacophore model, an ensemble of active conformations of AEA has to be identified. The conformation of AEA is mainly determined by its acyl chain. The unsaturated acyl chain of AEA is known to adopt many conformations that are energetically almost equivalent and that are not separated by high–energy barriers. This property originates from those areas, where two double bonds are separated by one methylene group[48]. The conformational space of the two dihedral angles involved shows a large occupied region[49]. Molecular dynamics studies were performed with AEA, 5*S*-, 5*R*-, 11*S*-, 12*S*- and 15*S*-HAEA, to investigate whether the 1–hydroxy–2*E*,4*Z*-pentadiene system is responsible for inducing conformations different from AEA, which affect their ability to adopt folded conformations, and thus their bioactivities.

Previous molecular modeling studies have been performed *in vacuo*, or with solvent treated as a dielectric continuum[22-24,50]. It has been shown that the choice of the solvent influenced the shape of AEA, which was basically extended in chloroform or *in vacuo*, while it was more compact in water[50]. It has been suggested that a compact shape reduces the exposure of hydrophobic portions[19]. However, our NMR–data indicate that the choice of solvent does not notably influence the average conformation of the acyl chain of 15–HETE. We have performed MD simulations with explicit water molecules instead of chloroform to mimic physiological conditions.

Analysis of all dihedral angles of the MD trajectories of AEA and the HAEA compounds showed a number of common features. The dihedral angle of bonds between two methylene groups was predominantly 180°, but also many short visits to –60° and 60° were observed. The dihedral angle of bonds between a hydroxymethylene carbon and a sp^2 carbon of a conjugated double bond was chiefly –140°, but showed a number of transitions to 60°. The dihedral angle of bonds between a hydroxymethylene carbon and a sp^2 carbon of a (non–conjugated) double bond was mainly 170°, but visited 60° many times. The dihedral angles of bonds inside a 1Z,4Z-pentadiene system involving a methylene group and a sp^2 carbon could be any of –60°, –120°, 180°, 120°, 60° and all values in between, with some preference for –120° and 120°. The many transitions observed for all these dihedral angles indicate low energy barriers and facilitate adequate sampling of the conformational space.

Idealized conformations of AEA and HAEAs are displayed in Fig. 4, with all dihedral angles set to the most populated orientations according to the MD trajectories. It is shown that the *1Z,4Z*-pentadiene systems induce bent-shaped conformations. When both

Figure 4   Idealized conformations of oxygenated anandamides
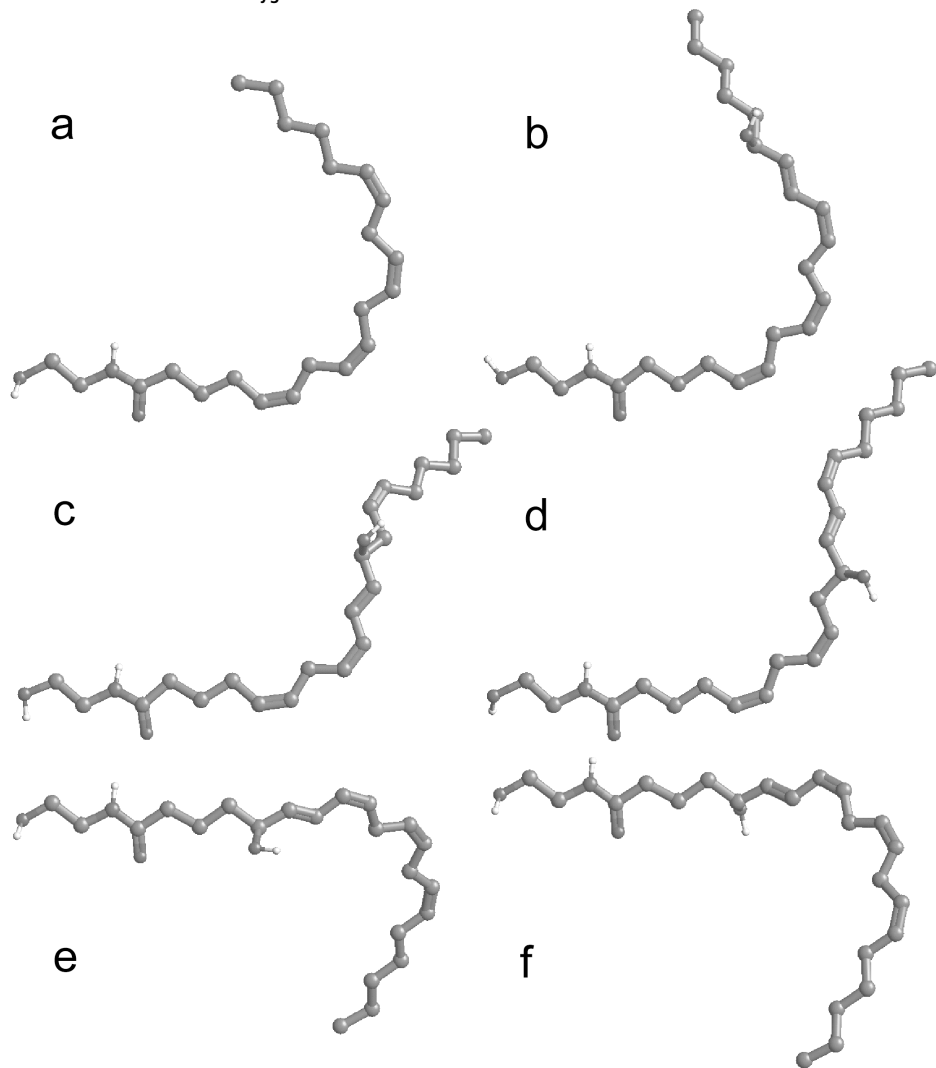
Idealized conformations of compounds (a) AEA, (b) 15S-HAEA, (c) 12S-HEAE, (d) 11S-HEAE, (e) 5R-HEAE, and (f) 5S-HEAE. The dihedral angles for the free rotational bonds in the 1Z,4Z-pentadiene systems could take any value from 60° going via 180° to –60°, and were set to 180° .

dihedral angles for the free rotational bonds in the 1Z,4Z-pentadiene systems, denoted φ_1 and φ_2, are set to 180°, a bent shape for AEA is observed. In addition, when φ_1, φ_2 have opposite signs, e.g. 120°, –120°, again a bent shape emerges. Only, when both dihedrals have the same signs, the shape of the molecule is more stretched. As opposite signs and equal signs occur with equal abundance, and dihedral angles around 180° also result in bent shapes, more than half of the low-energy conformations have shapes that are bent. This is in contrast to saturated acyl chains, where a dihedral angle of 180° is favored leading to an extended shape. This makes strongly bent shapes energetically unfavorable for saturated aliphatic chains (see also Rich[49]). Therefore, it seems that the easy adaptation of folded conformations of poly-unsaturated acyl chains originates in the inclination of 1Z,4Z-pentadiene systems to form bent shapes, and is not so much a result of the flexibility of the chains as previously suggested[19].

Introduction of a conjugated double bond in AEA, by creating a 1–hydroxy–2E,4Z-pentadiene system, transfers a non–rotatable bond with a dihedral angle of 0° to a new non–rotatable bond with a dihedral angle of 180° (1Z to 2E). Therefore, it changes the global shape of the chain. It also makes the chain rigid as it essentially fixates the middle bond in the conjugated system to 180°, which results in 6 carbon atoms lying in one plane. Conjugation of the pentadiene system (such as depicted in the specific conformation of AEA in Fig. 4a) results in more extended chains for 12S-HAEA (Fig. 4c) and 11S-HAEA (Fig. 4d), while in 5R-HAEA (Fig 4e) and 5S-HAEA (Fig. 4f) the chains are not more extended but the direction of the loops are influenced.

Structural comparison of pharmacophores of (H)AEAs with CP-55,940

To investigate whether the introduction of the 1–hydroxy–2E,4Z-pentadiene system in AEA influenced the orientation and distances of pharmacophoric groups in the HAEAs, the pharmacophores of the reference compound CP-55,940 were compared with pharmacophores in the (H)AEA series (Fig. 5). The pharmacophoric groups were identified based on the model of Tong et al [24]. This model was capable of discriminating between structurally related compounds exhibiting different pharmacological potencies for the CB$_1$ receptor, *i.e.* AEA and prostaglandinethanolamide. Furthermore, it could be used in a 3D-QSAR-study to predict the K$_i$-value of AEA. The following pharmacophoric units in CP-55,940, (*i*) the phenolic hydroxyl oxygen, (*ii*) the cyclohexyl hydroxyl oxygen, and (*iii*) the first carbon on the alkyl side chain (C'$_1$), correspond to (*i*) the oxygen of the carbonyl, (*ii*) the ethanol oxygen, and (*iii*) the first carbon of the pentyl tail (C$_{16}$) in the (H)AEA series. In the model of Tong et al. the pyran oxygen of THC (or HHC) was not included as pharmacophoric unit, as was done in the model of Thomas et al.[22], since it is not considered essential for the interaction. The model of Tong was chosen, because in our studies synthetic [³H]CP-55,940 (Fig.1), which also lacks the pyran oxygen, was used to determine displacement constants of HAEAs at both CB$_1$ and CB$_2$ receptors. Furthermore, the cyclohexyl OH in the cannabinoids and the corresponding

Figure 5   Distances and numbering of CP-55,940 and AEA.
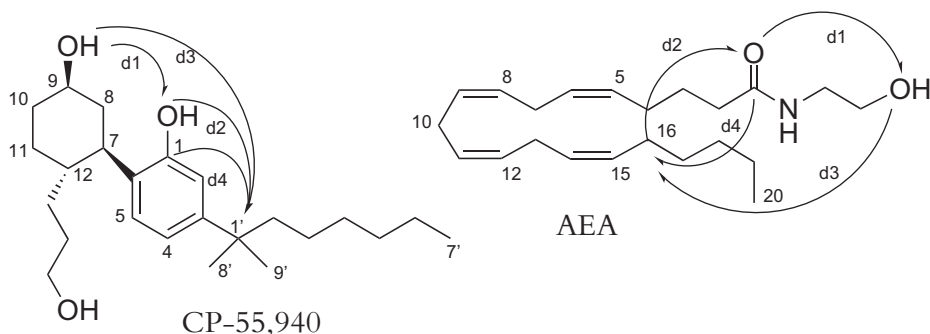
terminal OH of AEA both enhance binding, but their absence (as in THC and alkyl arachidonate derivatives) does not eliminate activity[24].

First, the conformation of CP-55,940 was analyzed to find the orientation of the hexyl ring in relation to the phenyl ring, and the orientation of the alkyl side chain. NOESY data for CP-47,497 (in chloroform) have shown that the orientation of the two rings around the C_6-C_7 bond, determined by the dihedral angle C_5-C_6-C_7-C_8 (see Fig. 5 for numbering), is –60° rather than 120°[51]. In this orientation, the OH of the cyclohexyl ring is at the same side of the phenolic ring, as is the case for HHC-DMH. This is in agreement with the proposal that the dihedral angle O_{hexyl}-C_9-C_1-O_{phenyl} (which is –90° for CP-55,940) must be negative, in order to get the most active conformation[52]. The NMR data[51] have also indicated that the 1',1'-dimethylheptyl side chain can occur in four different orientations in relation to the phenyl ring, with almost equal probability. To get the positions of the pharmacophores, a MD simulation of CP-55,940 was performed with the orientation of the rings following that of CP-47,497.

The CP-55,940 acyl chain has no single preferred orientation, and it is not yet known how it is oriented when it binds to the receptor. This hampers an easy comparison with the orientation of the tail of the acyl chain of the (H)AEA series. To facilitate the comparison of the orientation of the pentyl chain between different compounds, the orientation of the pentyl chain was expressed in polar coordinates, by using the orientation of vector $C_{1'}$-$C_{2'}$ relative to the O_{phenyl}-C_1-$C_{1'}$ plane for CP-55,940 and C_{16}-C_{17} relative to the $O_{carbonyl}$-C_1-C_{16} plane for the (H)AEA series. (For CP-55,940: φ = dihedral angle O_{phenyl}-C_1-$C_{1'}$-$C_{2'}$, and θ = angle C_1-$C_{1'}$-$C_{2'}$, and for the (H)AEA series φ = dihedral angle $O_{carbonyl}$-C_1-C_{16}-C_{17} and θ = angle C_1-C_{16}-C_{17}).

The following four atoms were used to analyze the atom positions of the pharmacophores: phenolic oxygen, cylohexyl oxygen, C_1 and $C_{1'}$ for CP-55,940, and the

carboxyl oxygen, the oxygen of the ethanol, C_1 and C_{16} for the (H)AEAs, respectively. Average distances between the pharmacophores in CP-55,940 are: phenolic and cylohexyl oxygen = 0.63 nm (**d1**), cyclohexyl oxygen and $C_{1'}$ = 0.91 nm (**d3**), phenolic oxygen and $C_{1'}$ = 0.49 nm (**d2**). The distance between the oxygen of the carboxyl and the oxygen of the ethanol in the (H)AEA series (**d2**), is somewhat smaller (0.55 nm). The distances **d2** and **d3** in the (H)AEA series demonstrate a large degree of variation, as shown in Fig. 6. In the same figure, the region of distances in CP-55,940 is indicated by an ellipse that is marked by an arrow. This CP-55,940 region falls within the much broader region of the (H)AEA series for all members, except for 5*R*-HAEA.

From the MD simulations of the (H)AEA series, all frames were selected that had appropriate $O_{hydroxyl}$-C_{16} (**d3**) and $O_{carbonyl}$-C_{16} (**d2**) and C_1-C_{16} (**d4**) distances (Fig. 7)

Scatter plots of distances d3 and d2 in HAEAs and CP.55.940 during MD simulations.   Figure 6
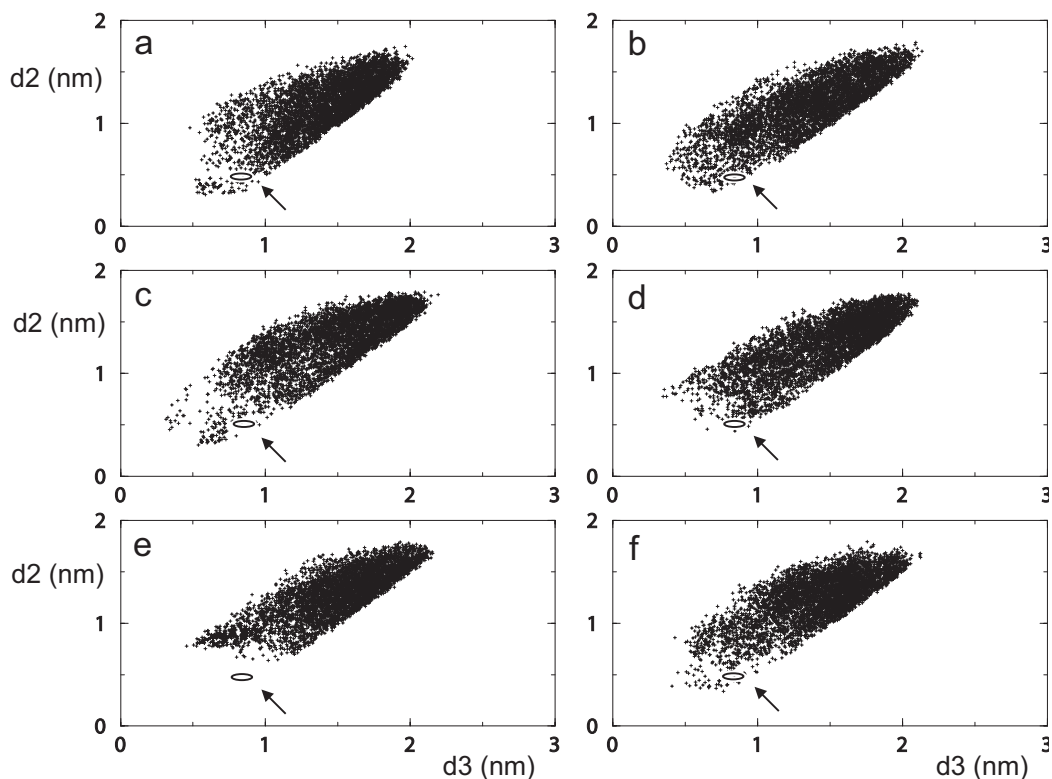
Scatter plots of distances d3 and d2 obtained during simulation of compounds (a) AEA, (b) 15*S*-HAEA, (c) 12*S*-HEAE, (d) 11*S*-HEAE, (e) 5*R*-HEAE, and (f) 5*S*-HEAE. The corresponding region of CP-55,940 is inside the ellipse and indicated by the arrow. Frames were selected with a time step of 1 ps.

Figure 7   Scatter plots of the orientation of vector C_{16}-C_{17} in HAEAs during MD simulations.
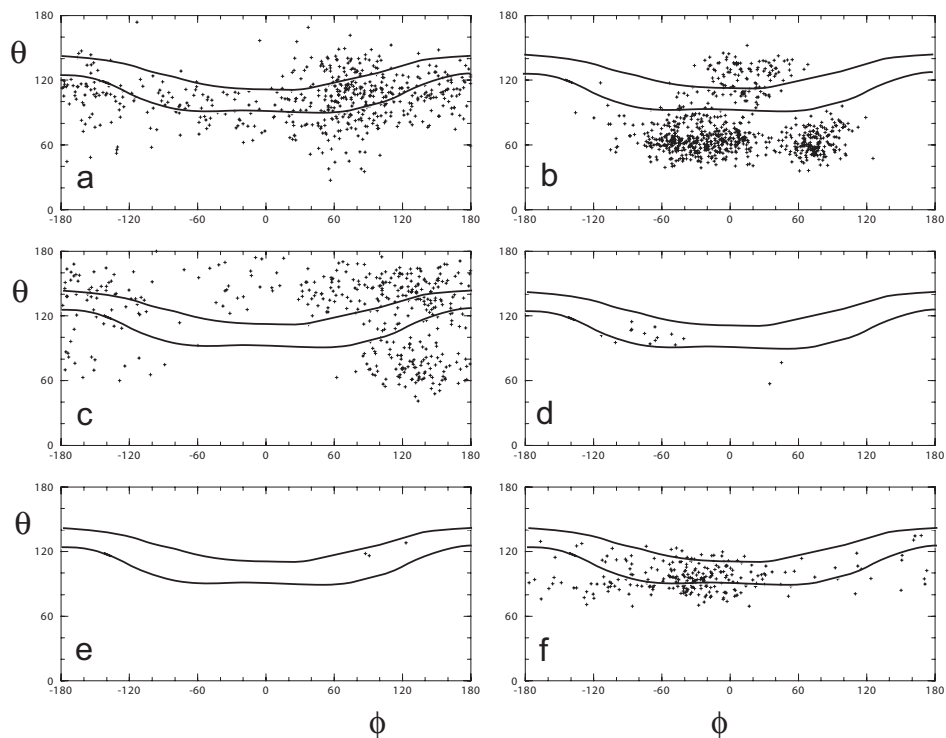
Scatter plots of the orientation of vector C_{16}-C_{17}, expressed in polar coordinates, during the 5 ns MD simulation of compounds (a) AEA, (b) 15S–HAEA, (c) 12S–HEAE, (d) 11S–HEAE, (e) 5R–HEAE, and (f) 5S–HEAE, for frames that had distances **d2** < 0.55 nm, **d3** < 0.95 nm and **d4** < 0.55 nm. Frames were selected with a time step of 0.1 ps. The corresponding results of the CP-55,940 simulation are indicated by the region between the curved lines. For CP-55,940: φ = dihedral angle O_{phenyl}-C_1-$C_{1'}$-$C_{2'}$ and θ = angle C_1-$C_{1'}$-$C_{2'}$, and for the (H)AEAs φ = dihedral angle $O_{carbonyl}$-C_1-C_{16}-C_{17} and θ = angle C_1-C_{16}-C_{17}.

and from these frames, the orientation of vector C_{16}-C_{17} was determined. The results of the CP-55,940 run are indicated in Fig. 7 by the region between the curved lines. The MD simulations of 11S–HAEA and 5R–HAEA did contain only a few frames with matching distances. As 5R–HAEA and 5S–HAEA are enantiomers, the MD runs of these compounds must furnish the same results in distances after adequate conformational sampling. This is clearly not the case after 5 ns. MD simulations show the presence of the required conformations in most of the simulations, therefore it can be concluded that the

Scatter plots of the orientation of vector C_{16}-C_{17} in HAEAs during the restrained MD simulations.   Figure 8
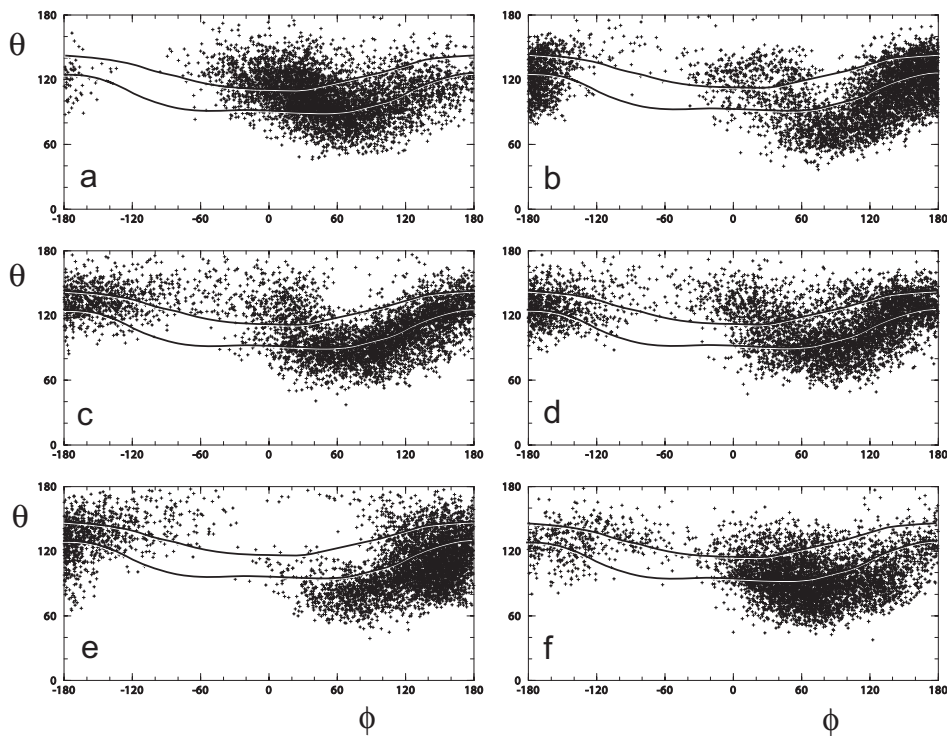
Scatter plots of the orientation of vector C_{16}-C_{17}, expressed in polar coordinates, during the 5 ns restrained MD simulation of compounds (a) AEA, (b) 15S–HAEA, (c) 12S–HEAE, (d) 11S–HEAE, (e) 5R–HEAE, and (f) 5S–HEAE. Frames were selected with a time step of 1 ps. The corresponding results of the CP-55,940 simulation are indicated by the region between the curved lines.

required conformations have sufficiently low energy to make their existence feasible. The results of the simulations of AEA and 12S–HAEA display a great similarity. For both simulations, many conformations emerge inside the CP-55,940 region enclosed by 60° > φ > 180°. This region is scarcely populated for 5R–HAEA and 5S–HAEA, and not at all for 15S–HAEA and 11S–HAEA. It is tempting to suggest a preference for analogous orientations of the pentyl tail for AEA and 12S–HAEA, which have a similar affinity for the CB_1 receptor, while the low affinity and inactive ligands do not show a preference for this pentyl tail orientation.

Structural alignment of CP-55,940 and restrained (H)AEAs

The MD simulations of the (H)AEA series do not provide enough suitable configurations for analysis, therefore, the positions of the pharmacophores in the (H)AEA series were aligned with those of the pharmacophores in CP-55,940. For that purpose, MD simulations were performed with positionally restrained atoms representing the pharmacophores according to the model of Tong et al.[24]. Stereospecificity is important, as indicated by differences in activity of the enantiomers 5*R*-HAEA and 5*S*-HAEA. To take this stereospecificity into account, a minimum of four atom positions has to be restrained. Selected atoms were the oxygen in the ethanol, the C_{16} atom of the aliphatic chain, and the carbon and oxygen in the carboxyl. Both carboxyl carbon and oxygen positions were chosen, in order to make sure that the availability of the oxygen for binding to CB_1 is not diminished by possible shielding by the position of the carbon. The results of the MD simulations for the orientation of the pentyl tail for AEA and all HAEAs are given in Fig.8. As expected, this orientation (represented by the vector C_{16}-C_{17}), in the (H)AEA series is less restricted than that of the corresponding vector $C_{1'}$-$C_{2'}$ in the acyl chain of CP-55,940. However, the overlapping regions of CP-55,940 and the (H)AEA series do not reveal great differences between the various (H)AEAs investigated. The differences in binding can not be explained by these results.

The most populated overlapping region of CP-55,940 and AEA in Fig. 8 was with $\varphi \approx 50°$ and $\theta \approx 100°$. An illustrative configuration is selected from that region and is presented in Fig. 9. It demonstrates the flexible hairpin-shaped loop C_2 to C_{15} of AEA, that does not correspond to any part of CP-55,940, and that accommodates the differences in the various (H)AEA compounds. Configurations from the restrained MD simulations of the (H)AEA compounds with a C_{16}-C_{17} vector orientation of $\varphi, \theta = 50°$, $100°$ were selected and studied for differences in the orientation and shape of this loop.

It turns out that restrained MD simulations yield conformations of the (H)AEAs that are very much the same, as demonstrated by 11*S*-HAEA and 12*S*-HAEA. This becomes evident from the orientation of the C_{16}-C_{17} vector in Figs 8 and 10. The shape of the C_2 to C_{15} loop differs slightly in the various HAEAs, due to the different positions of the hydroxyl and conjugated double bonds, that define a '6-atoms-in-a-plane' stiff part. Note that the loop formed by the carbons C_2 to C_{15} adopted various conformations during the simulations. Nevertheless, the conformational space of the orientation of the loop C_2 to C_{15}, relative to the orientation of the C_{16}-C_{17} tail vector, is essentially identical for both compounds during the simulations (data not shown).

Stereoview of snapshots CP-55,940 (upper panel) and AEA (lower panel) with a vector C_{16}-C_{17} orientation of φ, θ = 50°, 100°, depicted with dotted Van der Waals surfaces of the pharmacophores to highlight corresponding positions.

Conclusions

Towards the design of selective molecular probes for interaction with the proteins of the cannabinoid system

This study has shown that restricting the flexibility of AEA and 2–AG and introducing hydroxyl functions in their acyl chains brings selectivity and attenuates their binding potency towards the proteins of the endocannabinoid system. For example, 15S–HAEA selectively bound to the CB_1 receptor, whereas 13S–HLEA and 15–HO–LAG were the only eicosanoid ligands that selectively bound to the CB_2 receptor to date. 11S–HAEA did not bind to CB_1 or CB_2 receptors, but was a very good inhibitor of FAAH (K_i= 0.57 μM). By contrast, 12S–HAEA bound both CB receptors with an affinity similar to that of AEA, but it was a poor inhibitor of FAAH. Nevertheless, all HAEAs displayed an increased

Figure 10 Stereoview of snapshots of 12*S*-HAEA and 11*S*-HAEA.
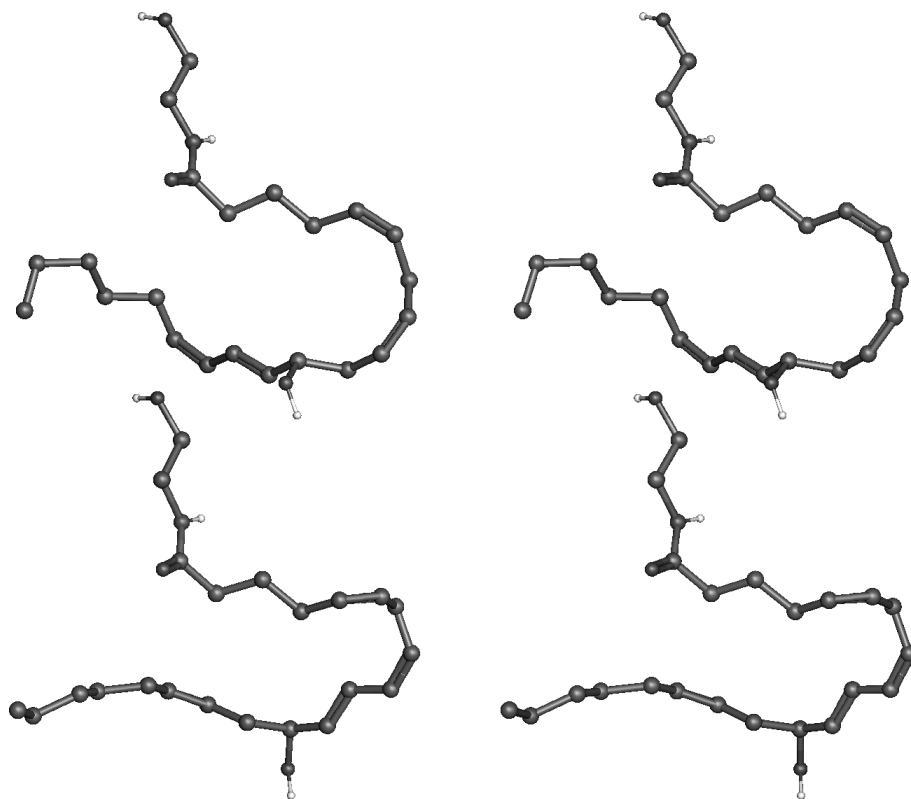
Stereoview of snapshots of 12*S*-HAEA (upper panel) and 11*S*-HAEA (lower panel) with vector C_{16}–C_{17} orientation of $\varphi, \theta = 56°, 105°$.

potency to inhibit FAAH, whereas their ability to interact with the AEA-transporter was disrupted ($K_i > 10$ μM). This makes it possible to discern the physiological contribution of FAAH and AEA-transporter in the clearance of endocannabinoids. Thus, the HAEAs can serve as structural templates to generate selectivity in the endocannabinoid system and may possibly be used as targets for drug development.

The NMR experiments indicated that chloroform and water did not induce different conformations in the acyl chain of 15-HETE. Consequently, there were no indications that 15-HETE would adopt a more compact overall conformation in aqueous solutions. For the first time, MD simulations of AEA and derivatives were performed that included explicit water molecules. Conformation analysis revealed that poly-unsaturated fatty acids

such as AEA assume more folded conformations than saturated fatty acids, because of an inclination of the 1Z,4Z-pentadiene subsystems to form bent shapes. Furthermore, our data indicated that the ability to adopt a tightly folded conformation is not the only essential feature of the acyl chain, which enables AEA to bind to the CB_1 receptor. In the free MD simulations all HAEA derivatives, both active and inactive at the CB_1 and CB_2 receptors, could adopt a folded conformation in which the pharmacophores in AEA matched those of CP–55,940. Close inspection of the free and restrained MD simulations of the HAEA series indicated that the differences must be located in the conformational details of the loop (C_2 to C_{15}), *e.g.* the position and orientation of the hydroxyl group. Furthermore, evaluation of the binding data indicated that a sp^2 carbon is required at position C_{11} in AEA-derivatives for binding to the CB_1 receptor. The free MD-simulations may suggest that the pentyl tail orientations of the high–affinity ligands AEA and 12S-HAEA are different from that of the low–affinity and inactive ligands. Distinct differences in terms of tail and loop orientations between high–affinity CB_1 receptor ligands and inactive compounds could not be detected in the restrained MD simulations. Our data also indicated that the binding of HAEAs to the CB_2 receptor is more sensitive towards steric hinder along the acyl chain. However, more conclusive statements about the nature of the interactions of the CB receptors with their ligands await purification and crystallization of the proteins with their ligands.

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500 MHz ^{1}H chemical shifts and coupling constants of 15-HETE in D_2O and $CDCl_3$. Table S1.

Proton	D_2O	$CDCl_3$	^3J	D_2O	$CDCl_3$
H-2$_{a,b}$	2.182	2.347	2,3	7.8	7.0
H-3$_{a,b}$	1.613	1.691	3,4	6.6	6.8
H-4$_{a,b}$	2.106	2.135	4,5	7.8	7.8
H-5	5.48	5.41	5,6	n.d.	n.d.
H-6	5.48	5.41	6,7	6.3	6.0
H-7$_{a,b}$	2.869	2.834	7,8	6.3	6.0
H-8	5.48	5.41	8,9	n.d.	n.d.
H-9	5.48	5.41	9,10$_a$	6.3	6.0
			9,10$_b$	7.4	7.5
H-10$_a$	2.959	2.950	10$_a$,10$_b$	–15.7	–16.5
H-10$_b$	3.037	3.030	10$_a$,11	7.9	7.8
H-11	5.54	5.41	10$_b$,11	8.0	8.0
H-12	6.074	6.000	11,12	10.6	10.5
H-13	6.600	6.595	12,13	11.1	11.1
H-14	5.739	5.717	13,14	15.3	15.3
H-15	4.202	4.258	14,15	7.2	6.0
H-16$_{a,b}$	1.548	1.553	15,16	6.8	6.3
H-17$_{a,b}$	1.283	1.393	16,17	n.d.	n.d.
H-18$_{a,b}$	1.283	1.298	17,18	n.d.	n.d.
H-19$_{a,b}$	1.283	1.298	18,19	n.d.	n.d.
H-20$_{a,b,c}$	0.854	0.887	19,20	6.3	6.7

Chemical shifts are relative to acetone = 2.218 ppm in D_2O and to TMS = 0.000 ppm in $CDCl_3$.

Table S2. Detailed force field parameter modifications.

Bond stretching term	Force constants (kcal mol^{-1} Å^{-2})	Equilibrium values (Å)	Identical to
CEH1-C$_{E/I}$H1[a]	1000.0	1.33	[b]
CEH1-CHn[c]	850.0	1.50	[b]
CIH1-CIH1	850.0	1.45	[b]

Bond angle term	Force constants (kcal mol^{-1} rad^{-2})	Equilibrium values (°)	Identical to
C$_{E/I}$H1[a]-CIH1-CIH1	100.0 §	125.0	[b]
CIH1-CEH1-CHn[c]	100.0	125.0	[b]
CEH1-CH2-CEH1	110.0	111.0	CB-CH2-CH2
CEH1-CHn[c]-CH2	110.0	111.0	CB-CH2-CH2
CEH1-CH1-OA	110.0	109.5	CH1-CH1-OA

Dihedral term	Force constants (kcal mol^{-1})	Phase angle (°)	Multi-plicity	Identical to
X[d]-CEH1-C$_{E/I}$H1[a]-X	10.0	180.0	2	X-CR61-CB-X
X-CIH1-CIH1-X	3.0	180.0	2	[e]
X-CEH1-CHn[c]-X	0.1	0.0	6	X-C-CHn[c]-X

[a]C$_{E/I}$H1 = CEH1 as well as CIH1 [b]The average value obtained from the diene systems of 35 linear structures selected from the Crystallographic Structure Database. [c]CHn = CH1 as well as CH2.
[d]X = any atom type [e]Dr. L.M.J. Kroon-Batenburg, personal communication.

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

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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 Δ^9-tetrahydrocannabinol (Δ^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 Δ^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 CB_1 cannabinoid receptor antagonist SR141716 prevented the neuroprotective actions of Δ^9-THC, indicating that Δ^9-THC afforded protection to neurons via the CB_1-receptor. In Δ^9-THC-treated rats the volume of astrogliotic tissue was 36% smaller. The CB_1 receptor antagonist did not block this effect. These results provide evidence that the cannabinoid system can serve to protect the brain against neuro-degeneration.

Introduction

The endogenous cannabinoid system comprises two cannabinoid receptors, designated CB_1 and CB_2, which have been cloned and characterized[1]. Two main endogenous ligands based on fatty acids, *i.e.* anandamide and 2-arachidonoylglycerol (2-AG) have been identified[1]. The CB_1 receptor is mainly found in the central nervous system, whereas the CB_2 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 sclerosis[3-6].

(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 diseases[7,8]. *N*-acylethanolamines, including anandamide, and their precursors and 2-AG accumulate, when tissues and cells are subjected to excitotoxic stress[3-6,9-12]. Whether this increase in endocannabinoids is neuroprotective and if so via which mechanism is still under debate[13-19].

The therapeutic effects of cannabinoids in *in vivo* models of cerebral ischemia are also not consistent. Chronic Δ^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[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, WIN 55.212-2 afforded protection to hippocampal and cortical neurons in CB_1-dependent manner in rats with a permanent middle cerebral artery occlusion or global ischemia[3]. The reason for this discrepancy is not known at the moment, but (endo)cannabinoid-induced vasorelaxation[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 *in vitro* models of excitotoxicity and *in vivo* models of cerebral ischemia, we investigated the neuroprotective properties of Δ^9-THC in an *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 Δ^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 stereotaxic frame. A small burr hole was drilled in the cranium over the left hemisphere, 2.5 mm lateral of bregma. A 1 μl syringe was lowered into the left striatum to a depth of 4.0 mm [21]. Ouabain (0.5 μl 1mM; n=30, Zwijndrecht, The Netherlands) or vehicle (0.5 μl 40 mM Tris-HCl buffer, pH 7.4; n=2) was injected at a rate of 0.125 μl/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_2O/O_2. Body temperature was maintained at 37°C using a water-filled heating pad and an infrared heating lamp. Animals were treated with Δ^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 *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[22]. 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 (= number of pathological pixels × field-of-view in cm^2 / 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 Δ^9–THC (1 mg/kg) and five animals received both Δ^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 ($\sim 0.67 \times 10^{-3}$ mm^2 s^{-1}) in the ipsilateral hemisphere in all animals (Fig. 1). Normal ADC values ($\sim 1.11 \times 10^{-3}$ 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 Δ^9–THC-group ($p<0.05$) (Figs. 1 and 2). Coinjection of the CB_1 receptor antagonist SR141716 completely abolished the Δ^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 Δ^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 Δ^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,

Figure 1 Three adjacent coronal ADC maps of neonatal rat brain 15 min after ouabain injection: effect of THC-pretreatment
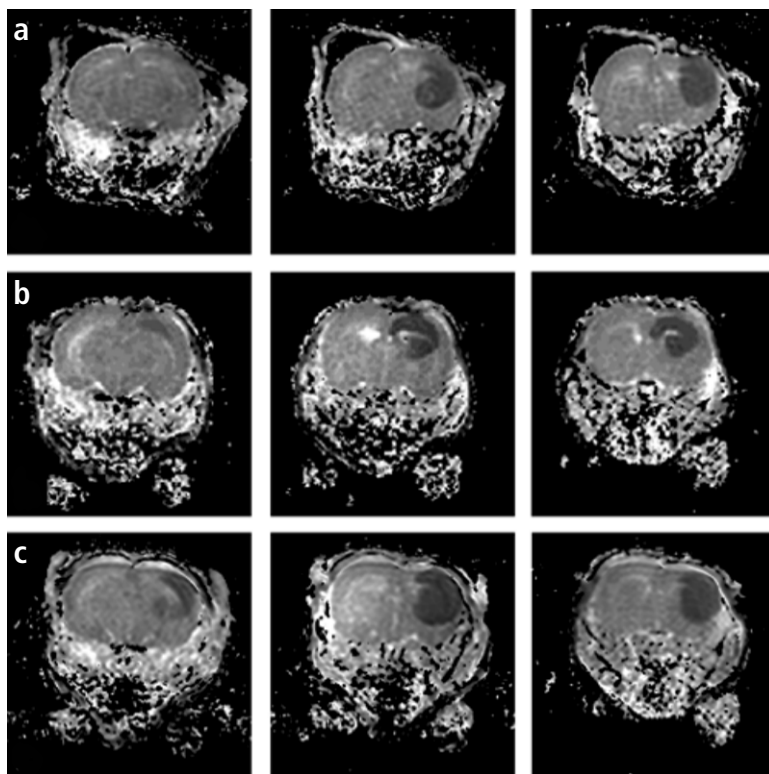
a) no treatment, b) THC-treatment, c) THC + SR141716 treatment. Hypointensities correlate to cytotoxic edema.

whereas hypointensities correlate to astrogliosis, *i.e.* phenotypic changes (hypertrophy) and proliferation of astroglial cells in response to neuronal injury (Fig. 4)[27,29]. Lesion volumes, based on the combination of hyper- and hypointense abnormalities on T_2 maps, were reduced by 36% ($p<0.005$) in Δ^9-THC-treated rats compared with the control group (Figs. 2 and 3). Infarct size based on T_2-hyperintense abnormalities was reduced by 35% in the Δ^9-THC-treated group ($p<0.05$) compared to the control animals (Figs. 2 and 3). This effect could be blocked by the CB_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_2 map analysis (data not shown).

Mean lesion volumes (± SE) of ouabain-injected rats on day 0 and day 7, based on ADC and T2 map analysis.   **Figure 2**
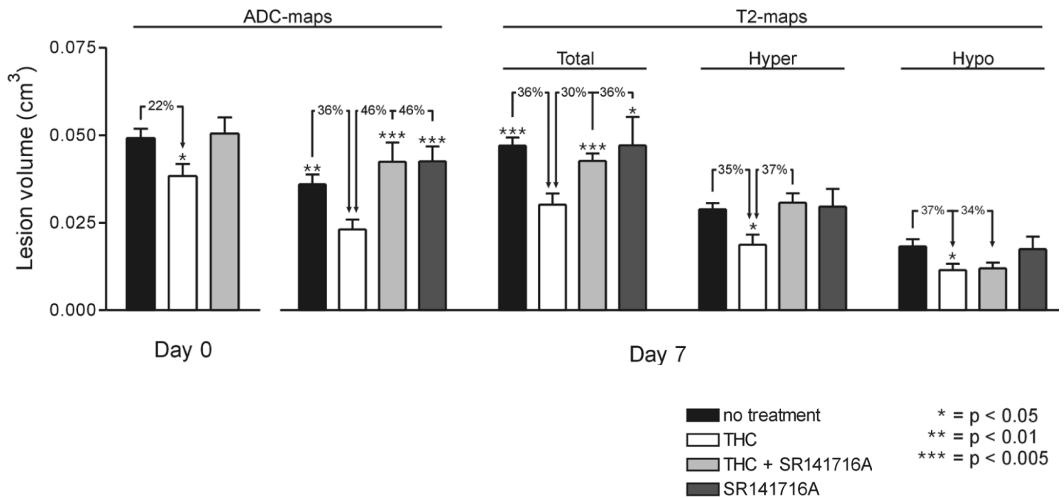
The hypointense regions on the T_2 maps corresponded to regions exhibiting increased staining for GFAP staining, which is typical of astrogliosis, on brain sections of ouabain-treated rats (Fig. 4). No indications were found for hemorrhage. Astrogliotic tissue constituted 40% of the lesion on T_2-maps and usually surrounded the edematous tissue and the dilatated ventricles (Fig. 3). The volume of astrogliotic tissue in Δ^9-THC-treated rats was reduced by 37% compared with nontreated rats (p<0.05). Importantly, this effect was not blocked by the CB_1 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

Figure 3   Three adjacent coronal T$_2$-maps of neonatal rat brain 7 days after ouabain injection: effect of THC-pretreatment.
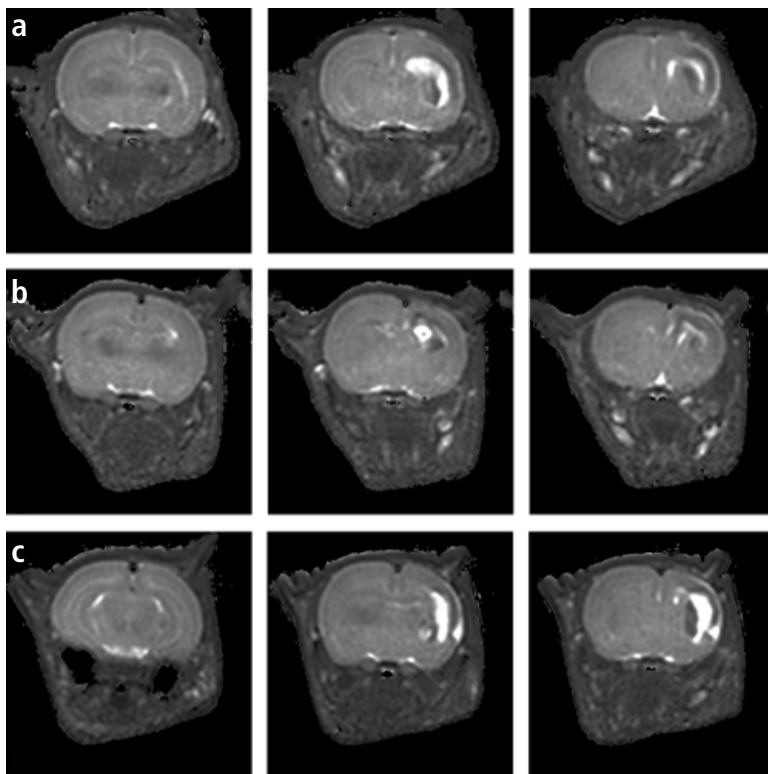
a) no treatment, b) THC treatment, c) THC + SR141716 treatment. Hyperintensities correlate to ventricle dilatation, vasogenic edema and tissue loss, whereas hypo-intensities correlate to astrogliosis.

pathology[23-26,30]. 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[23-26,30].

The diffusion-weighted MRI data acquired 15 min after the injection of ouabain showed that activation of the CB$_1$ receptor by Δ^9-THC attenuates *in vivo* cell swelling in an early phase after the induction of excitotoxicity. Activation of the CB$_1$ receptor on presynaptic-neuron terminals can lead to inhibition of the Ca^{2+} influx via N-, and P/Q-type voltage-sensitive calcium channels, thereby preventing the release of glutamate and subsequent depolarization of other neurons[17,31]. Furthermore, cannabinoids can induce hyperpolarization via the CB$_1$-mediated activation of inward rectifying and A-type K$^+$-channels[32]. Hyperpolarization raises the threshold to depolarization, which therefore may

contribute to the observed reduction in the development of cytotoxic edema.

ADC and T_2 data acquired after seven days, demonstrated that Δ^9-THC or its CB_1-active metabolite 11-HO-Δ^9-THC reduced neuronal damage by 36%. Various mechanisms could underlie the observed effects:

(*i*) Δ^9-THC-induced hypothermia[2]. 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 Δ^9-THC[16]. Anti-oxidant activity most likely does not play a major role in our model, because a) the neuroprotective effects were blocked by the CB_1 antagonist and b) the dose of Δ^9-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[33]

(*iii*) Down-regulation of brain-resident mast cells by activation of a CB_2-like receptor [14,15]. This process is also unlikely to be effective in our model, because a) the neuroprotective effects were blocked by SR141716, b) Δ^9-THC has been shown to have a low efficacy on the stimulation of the CB_2 receptor[2] and c) a CB_2 like receptor could not be detected.

(*iv*) Closing of N- and P/Q-type calcium channels via a CB_1-receptor-mediated mechanism[17,34]. Reduced influx of calcium decreases directly the activation of destructive pathways, *e.g.* it prevents the activation of neuronal NO-synthase[35] and it reduces glutamatergic transmission, *i.e* induction of excitotoxicity[17,36,37]. This CB_1-mediated mechanism is likely to dominate the observed neuroprotective effects in the late phase in our model.

GFAP-staining of a brain section of a ouabain-injected rat.   Figure 4
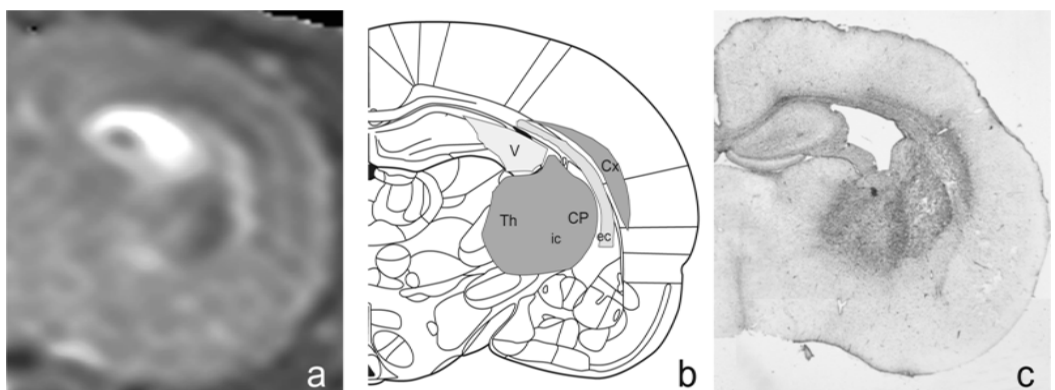
Markedly increased staining was observed in the thalamus, external capsule and cortex of the injected hemisphere, whereas normal staining was seen in the contralateral hemisphere.

Neuroprotection by Δ^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. Δ^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, dexanabinol, a non–psychotropic cannabinoid, inhibits tumor necrosis factor-α (TNF-α) release from astrocytes[39]. It is thought that TNF-α sets the stage for inflammatory reactions including glial cell activation and proliferation[29]. Δ^9-THC is known to inhibit the release of TNF-α from immune cells[40]. Thus, Δ^9-THC or one of its metabolites might also inhibit the release of TNF-α 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 Δ^9-THC reduce astrogliosis via this mechanism. Further research is required to investigate the mechanism of Δ^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 CB_1-receptor, which strongly corroborates our findings (R. Mechoulam, personal communication).

In summary, we have shown that in an *in vivo* model of neurodegeneration Δ^9-THC reduces neuronal damage via a CB_1 receptor–mediated mechanism. This holds in both the acute and late phase after induction of excitotoxicity. Δ^9-THC inhibits astrogliosis via a non-CB_1-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.

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Exogenous anandamide protects rat brain against acute neuronal injury *in vivo*

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Abstract

The endocannabinoid anandamide [*N*-arachidonoylethanolamine; (AEA)] is thought to function as an endogenous protective factor of the brain against acute neuronal damage. However, this has never been tested in an in vivo model of acute brain injury. Here, we show in a longitudinal pharmacological magnetic resonance imaging (MRI) study that exogenously administered AEA dose-dependently reduced neuronal damage in neonatal rats injected intracerebrally with the Na^+/K^+-ATPase inhibitor ouabain. At 15 min after injury, AEA (10 mg/kg) administrated 30 min before ouabain injection, reduced the volume of cytotoxic edema by 43 ± 15 % in a manner insensitive to the cannabinoid CB_1 receptor antagonist SR141716A. At seven days after ouabain treatment, $64 \pm 24\%$ less neuronal damage was observed in AEA-treated (10 mg/kg) rats compared with control animals. Coadministration of SR141716A prevented the neuroprotective actions of AEA at this end point. In addition, (i) no increase in AEA and 2-arachidonoylglycerol levels was detected at 2, 8 or 24 h after ouabain injection; (ii) application of SR141716A alone did not increase the lesion volume at days 0 and 7; and (iii) the AEA-uptake inhibitor, VDM11, did not affect the lesion volume. These data indicate that there was no endogenous endocannabinoid tone controlling acute neuronal damage induced by ouabain. Although our data seem to question a possible role of the endogenous cannabinoid system in establishing a brain defense system in our model, AEA may be used as a structural template to develop neuroprotective agents.

Introduction

The central nervous system is highly vulnerable to ischemia. Neuronal death caused by ischemia is executed via a complex array of processes in which excitotoxicity plays a major role. In excitotoxicity, cell death is triggered by the overstimulation of excitatory amino acid receptors. This leads to cytotoxic levels of calcium and to subsequent activation of destructive pathways, involving among others caspases, calpains, and the generation of reactive oxygen species[1,2].

Compounds that interfere with excitotoxicity may be used as neuroprotective therapeutic agents. Interestingly, the brain has various endogenous protection factors at its disposal, (*e.g.* adenosine, melatonin, and estrogens)[3,4]. Several reports have also revealed a connection between the endogenous lipid anandamide [*N*-arachidonoylethanolamine; (AEA)] and neurodegenerative diseases[5,6].

AEA mimics in part the actions of Δ^9-tetrahydrocannabinol (THC), the psychoactive compound in marijuana. Together with 2-arachidonoylglycerol (2-AG), AEA represents a class of lipids, termed endocannabinoids, because of their ability to activate the CB_1 and CB_2 cannabinoid receptors. AEA is rapidly translocated into the cell via a transporter protein, and is then immediately inactivated by a fatty acid amide hydrolase (FAAH)[7,8].

Several lines of evidence indicate that AEA can serve to protect the brain against neuronal injury[9,10]: (*i*) AEA and its precursor *N*-arachidonoylphosphatidylethanolamine are normally found in low concentrations in the brain, but their levels increase in a calcium–dependent manner *postmortem* and with severe neuronal injury[10-14]; (*ii*) exogenous AEA protects cerebral neurons from *in vitro* ischemia[15]; (*iii*) CB_1–mediated closing of N–, and P/Q–type calcium channels protects neurons against *in vitro* secondary excitotoxicity[16,17]; (*iv*) we have demonstrated recently that THC can reduce neuronal damage via the CB_1 receptor in an *in vivo* model of excitotoxicity (See Chapter 4); (*v*) WIN55.212, a synthetic cannabinoid, protected rat brain against focal and global ischemia[18]; (*vi*) CB_1 expression is enhanced in the cortical mantle zone in rats after ischemia[19]. As yet, *in vivo* neuroprotection by AEA has never been reported.

To date no effective drugs are available to treat brain injury following transient (global) or permanent focal cerebral ischemia. Insights into how the brain defends itself may lead to novel strategies to develop new therapeutic agents. Therefore, it was our goal to test whether the endogenous cannabinoid system affords the brain protection in an *in vivo* model of neuronal injury. In this study we combined longitudinal pharmacological magnetic resonance imaging (MRI) and isotope dilution gas chromatography / mass spectrometry (GC/MS) techniques. Our data indicate that there is no endogenous endocannabinoid tone controlling the acute neuronal damage induced by ouabain, a Na^+/K^+–ATPase–inhibitor, although exogenous AEA can effectively reduce toxin–induced injury in the neonatal rat brain.

Animals, Materials and Methods

Animal model

See chapter 4.

Pharmacological Treatments

Animals used for the MRI study were treated *i.p.* with AEA (1 or 10 mg/kg, n= 5 and 6, respectively, Biomol, Heerhugowaard, The Netherlands), AEA + SR141716A (10 and 3 mg/kg, n=4, Sanofi Recherche, Montpellier, France), SR141716A alone (3 mg/kg, n=5), the selective AEA membrane transporter inhibitor, VDM11 (10 mg/kg, n=5), synthesized as described previously[52], or vehicle alone (n=12) (all drugs in 1 ml/kg body weight 18:1:1 v/v phosphate buffered saline (PBS)/Tween80/Ethanol) 30 min before toxin injection. There was no difference in body weight

and growth rate between any of the groups. Utrecht University's Animal Experimentation Committee approved all protocols.

MRI-experiments, data analysis and histology
  See Chapter 4.

Protein analysis
  Mouse melanoma cells (B16–G4F) were transiently transfected with 6-10 μg recombinant cDNA (pcDNA3-vector) encoding the CB_2 receptor (kind gift of Dr. R. Delwel, Institute of Hematology, Erasmus University, Rotterdam, the Netherlands). Rat tissue (brain and spleen) and cells were homogenized and sonicated (3 × 10 s) in buffer containing 50 mM Tris-HCl, 1 mM EDTA and 3 mM $MgCl_2$ (pH 7.4). Membrane fractions were prepared by centrifugation at 5000 ? *g* for 5 min. Samples were stored at −20 °C until further use.

  Samples containing 30 mg of protein were separated by 10% SDS–PAGE (reducing) and transferred to a nitrocellulose membrane. Membranes were blocked overnight in 1% gelatin in PBS-0.1% Tween (CB_1, FAAH) or in PBS-0.1% Tween containing 5% nonfat milk powder (CB_2). Membranes were rinsed twice and washed once for 15 min and four times 5 min with 20 ml PBS-0.1% Tween. The membranes were incubated with polyclonal primary antibodies for 3 h (1: 1000, CB_1, Cayman Chemicals), 2 h (1: 1000, CB_2, Cayman Chemicals) and 1.5 h (1:5000, FAAH, a kind gift from Dr. M. Maccarrone, University of Rome "Tor Vergata", Rome, Italy) and washed. A donkey anti-rabbit antibody conjugated to horseradish peroxidase (1:5000, Biorad) was used as a secondary antibody (1h incubation). Membranes were washed and developed with Western-blotting detection reagents (ECL, Amersham Pharmacia Biotech) according to the manufacturer's manual.

Lipid extraction
  Neonatal rats (postnatal day 7 (P7) and P8) *i.c.* injected with ouabain or vehicle were killed by decapitation at 0, 2, 8 and 24 h after induction of excitotoxicity (n= 6 for each time point). Ipsilateral and contralateral hemisphere were rapidly removed and separately homogenized in 5 ml ice-cold Tris buffer (50 mM, pH 7.4). Lipids were extracted according to the method of Bligh and Dyer (1957)[53]. One nmol of d_8–AEA and d_8–2-AG (Cayman Chemicals) were added as internal standards. The organic phases were dried under nitrogen, and purified by normal phase-HPLC performed as described previously[20]. Mono–AGs and AEA standards were eluted after 18-23 min and 27-28 min, respectively. The mono–AGs fraction contained the 1- (3)- and 2-stereoisomers.

  To limit *postmortem* accumulation of endocannabinoids, the time between decapitation and homogenization in cold organic solvents was kept as short and constant as possible (< 5 min) and the tissues were kept on ice.

GC/MS analysis
  HPLC fractions were dried under a flow of nitrogen and derivatized with 15 μl *N*-methyl-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane for 2 h at room temperature,

thus yielding the trimethylsilyl derivatives of AEA and 2-AG. The two derivatized fractions were analyzed by GC/MS performed as described previously[20,21]. The derivatives of both deuterated and non-deuterated AEA, 2-AG and 1-(3)AG standards were eluted after 18, 19 and 19.5 min, respectively. MS-detection was run in the selected ion-monitoring mode to improve sensitivity. Selected ions for AEA were at m/z 427 and 419 corresponding to the molecular ions for d_8-AEA and nondeuterated AEA, and m/z 412 and 404, corresponding to the loss of a methyl group in both compounds. Selected ions for 2-AG were at m/z 530 and 522, corresponding to the molecular ions of d_8-2-AG and nondeuterated 2-AG and m/z 515 and 507, corresponding to the loss of a methyl group in both compounds. The endocannabinoids were identified on the basis of the same retention time as the deuterated internal standards of the corresponding MS signals with the appropriate relative abundance. The amounts of AEA and 2-AG were calculated from the peak area ratios between the signals at m/z 404 and 412, and m/z 507 and 515, respectively. A linear correlation between these area ratios and the amounts of standards was observed in separate studies. In the case of 2-AG, the amount of the 1-(3) isomer, which is almost exclusively formed during tissue workup and lipid purification[22] were added to the amount of the 2-isomer.

Results

The presence of CB_1 and CB_2 receptors and FAAH in neonatal rat brain was verified by Western blotting; endocannabinoid levels were determined by isotope dilution GC/MS. Western blot analysis demonstrated the presence of the CB_1 receptor in 7- and 14-day-old rat brain, whereas the CB_2 receptor could not be detected (Fig. 1). FAAH was also detected in 7-day-old rats (Fig. 1). Neonatal rat brains (P7) contained 32.5 ± 6.5

Western blot of CB$_1$ and CB$_2$ cannabinoid receptors and FAAH in neonatal rat (P7 and 14) and adult brain. **Figure 1**
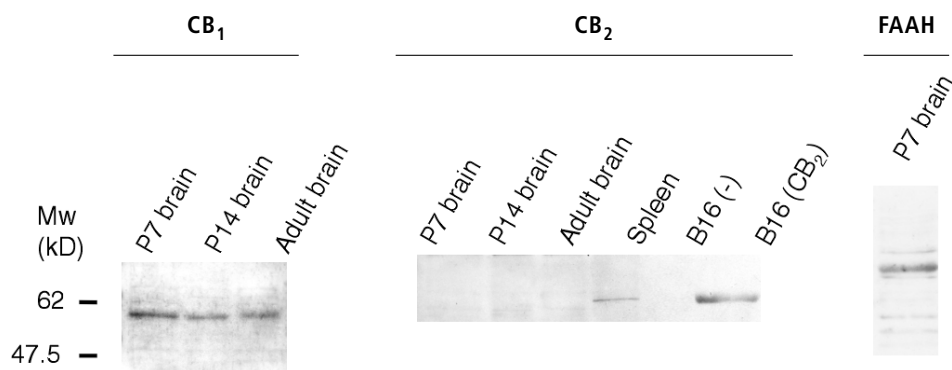
The CB_2 receptor was absent in rat brain, but was detected in spleen and CB_2-transfected cell line.

pmol/g AEA and 1.17 ± 0.22 nmol/g 2–AG, which is in the same order of magnitude as reported previously[23].

In our model, excitotoxicity was triggered by the unilateral intrastriatal injection of 0.5 µl ouabain (1 mM) in 7- to 8-day-old rats. Ouabain, a cardiac glycoside, inhibits Na^+/K^+-ATPases and induces cellular swelling, eventually leading to pancellular necrosis and infarction[24-28]. The acute cellular swelling is conveniently monitored by diffusion-weighted MRI. ADC-maps of brain tissue water, calculated from diffusion–weighted MR images acquired 15 min after ouabain-injection, showed hypointense regions with reduced ADC values ($\sim 0.65 \pm 10^{-3}$ $mm^2 s^{-1}$) in the ipsilateral hemisphere in all animals (Fig. 2). Normal ADC values ($\sim 1.11 \pm 10^{-3}$ $mm^2 s^{-1}$) were measured in the contralateral hemisphere of the ouabain-injected rats (Fig. 2) and in the brains 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 hemisphere 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[29,30]. The same brain regions, including the caudate putamen, cortex and hippocampus, were affected in all animals (Fig. 2). In this acute phase, AEA reduced the volume of brain tissue with cytotoxic edema dose-dependently. The volume of tissue at risk to go into infarction was reduced by $43 \pm 15\%$ ($p<0.05$) at 10 mg/kg AEA (Fig. 2 and 3A). This effect was observed at the borders of the affected tissue, namely

Figure 2   Coronal ADC maps of neonatal rat brains 15 min after ouabain injection: effect of AEA-pretreatment.
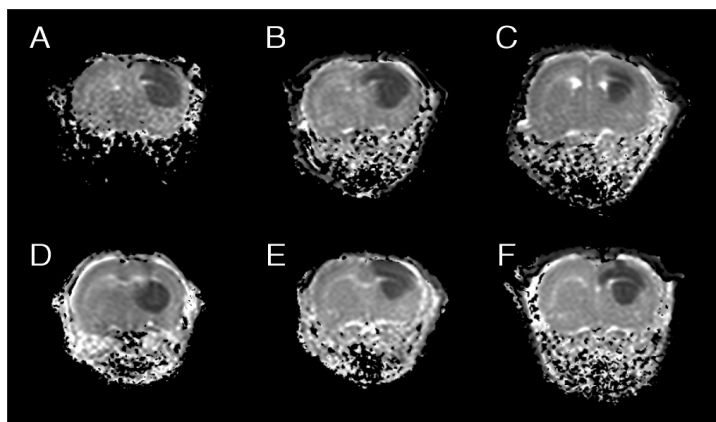
Hypointensities correlate to cytotoxic edema. Treatments: A) vehicle, B) AEA (1 mg/kg), C) AEA (10 mg/kg), D) AEA + SR141716A (10 and 3 mg/kg, respectively), E) SR141716A (3 mg/kg) and F) VDM11 (10 mg/kg).

Mean lesion volumes (± SE) of ouabain-injected rats: effect of ligands of the cannabinoid system.   **Figure 3**
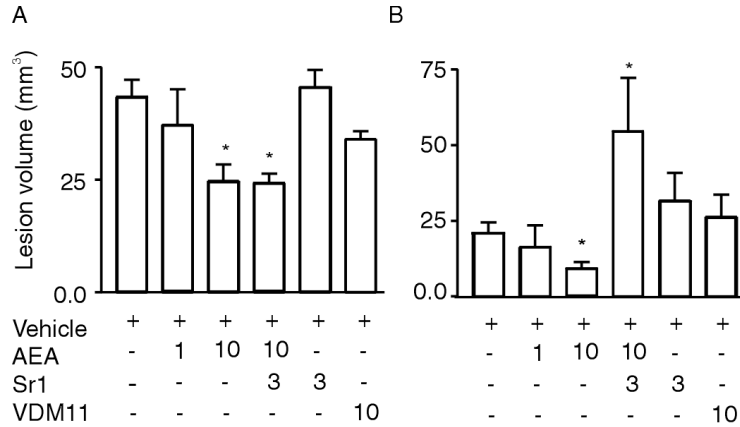
Mean lesion volumes (± SE) of ouabain-injected rats on day 0 (A) and 7 (B) based on ADC and T_2 maps (hyperintensities), respectively. Asterisk denotes a $p<0.05$ compared to vehicle-treated rats. The numbers at the bottom indicate the dose of compound (mg/kg). See Materials and Methods for lesion volume calculation.

cortex and striatum. Coinjection of the CB_1 receptor antagonist SR141716A with AEA did not reverse AEA action (Fig. 2 and 3A). Application of SR141716A or VDM11 (an endocannabinoid uptake inhibitor), alone did not change lesion volume at day 0, compared with vehicle-treated animals (Fig. 2 and 3A).

After seven days the effect of AEA treatment on neuronal damage was assessed using T_2-weighted imaging and verified by standard histology. Normal T_2 values ($T_2= 73 \pm 1$ ms) were observed in contralateral hemispheres and in the brains of control animals (Fig. 4). The T_2 maps of ouabain-injected animals demonstrated both hyperintensities and hypointensities (Fig. 4).

Both types of T_2 abnormalities indicate pathological changes. Hyperintense areas correspond to vasogenic edema, tissue loss, and ventricle dilation, whereas hypointensities can correlate to astrogliosis, *i.e.* phenotypic changes (hypertrophy) and proliferation of astroglial cells in response to neuronal injury (Fig. 4)[29,31]. Infarct size based on T_2 hyperintense abnormalities was dose-dependently reduced in the AEA-treated group compared with the control animals (Fig. 3B and 4). The infarct volume was 64±24% ($p<0.05$) smaller at 10 mg/kg AEA, than in the vehicle-treated animals. Protection was primarily observed in the caudate putamen, cortex and hippocampus. This effect was blocked by the CB_1 antagonist ($p<0.05$) (Fig. 3B and 4). The infarct was ~2.5 fold larger than that in vehicle-treated animals ($p<0.05$), and primarily involved the hippocampus

Figure 4   Coronal T$_2$ maps of neonatal rat brains seven days after ouabain injection: effect of AEA-pretreatment.
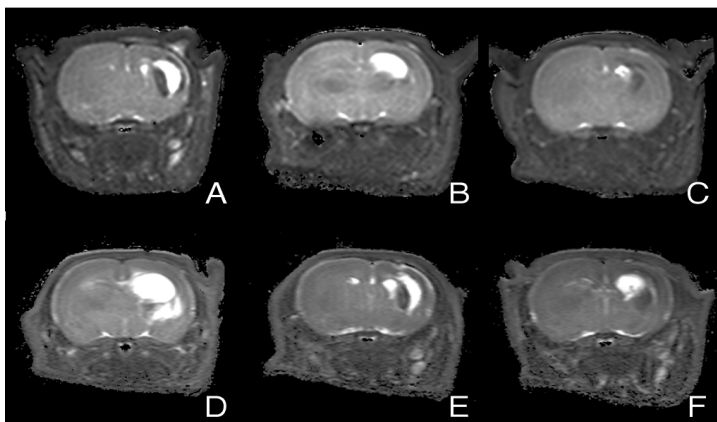
Hyperintensities correlate to vasogenenic edema, tissue loss, and ventricle dilatation. Treatment: A) vehicle, B) AEA (1 mg/kg), C) AEA (10 mg/kg), D) AEA + SR141716A (10 and 3 mg/kg, respectively), E) SR141716A (3 mg/kg) and F) VDM11 (10 mg/kg).

and caudate putamen. Application of SR141716A and VDM11 alone did not affect lesion size. Conventional histology (Nissl- and hematoxylin/eosin-staining) showed a similar lesion pattern on brain sections and confirmed the assessment made by T$_2$ map analysis (data not shown).

The hypointense regions on the T$_2$ maps corresponded to regions exhibiting increased staining for GFAP on brain sections of ouabain-treated rats, which is typical of astrogliosis (Fig. 5B-D; See also chapter 4). No indications for hemorrhage were found. Astrogliotic tissue constituted ~44% of the lesion on T$_2$ maps of nontreated animals and usually surrounded the edematous tissue and the dilated ventricles (Fig. 5B). The volume of astrogliotic tissue in AEA-treated rats was not affected compared to nontreated rats ($p>0.05$), which is in accordance with our previous observation that THC reduces astrogliosis via a CB$_1$ and CB$_2$ independent mode of action in our model (chapter 4; our unpublished results). This reinforces the notion that also classical cannabinoids have other modes of action, in addition to their interaction with the CB$_1$ and CB$_2$ receptors[32].

Finally, rat brain endocannabinoid levels were measured 2, 8 and 24 h after ouabain or vehicle injection. No rises in concentrations of AEA and 2-AG were observed after inducing acute neuronal damage in the ipsilateral hemisphere (Fig. 6). There were no significant differences in endocannabinoid levels between ipsilateral and contralateral hemispheres and between vehicle and ouabain-injected animals (data not shown).

Astrogliosis in neonatal rat brain 7 days after ouabain injection.   **Figure 5**
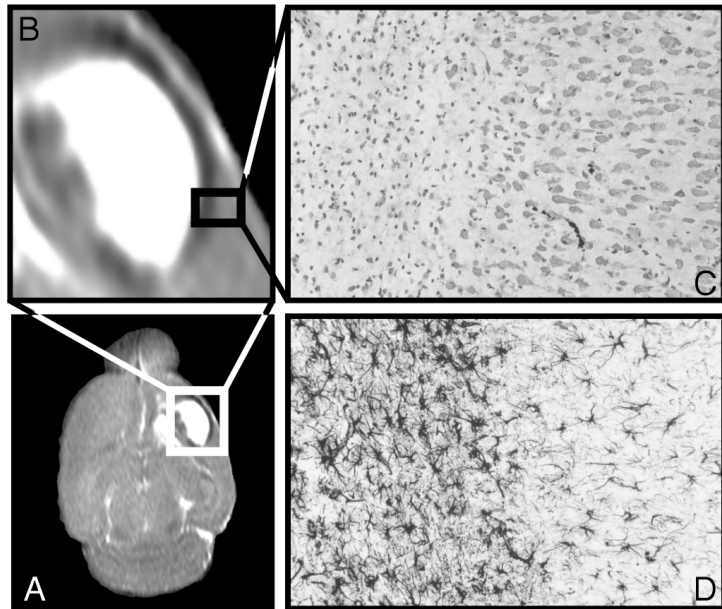
Transversal T_2 map (A) of an ouabain–injected animal showing that vasogenic edema is surrounded by astrogliosis (hypointense area) (B). Nissl–staining (C) and GFAP(D)–staining of a brain section of an ouabain–injected rat demonstrate a sharp line between affected and healthy tissue.

Endocannabinoid levels in rat brain 2, 8 and 24 h after ouabain injection.   **Figure 6**
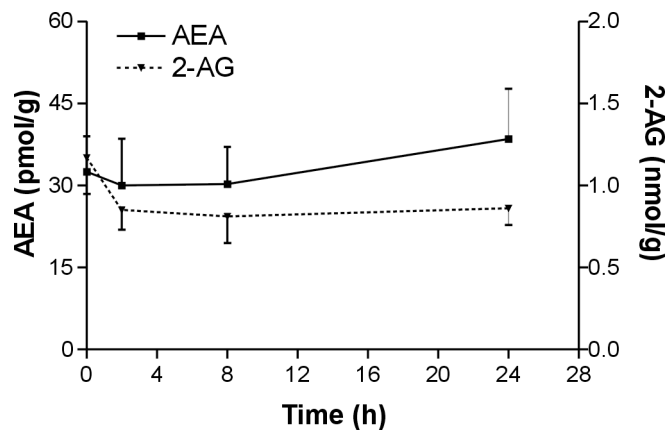
Discusson and conclusions

The (patho)physiological role of the endogenous cannabinoid system is beginning to be unraveled[33,34]. It has been postulated that the endocannabinoid system may serve to establish a defense system for the brain during neurotoxicity and ischemia[10,19]; see also chapter 4). Yet the therapeutic effects of classical and synthetic cannabinoids were contradictory in models in which transient (20-120 min) or permanent cerebral ischemia was induced[18,35] (C.J. Hillard, personal communication). Because the cannabinoid system has complex (cerebro)vascular effects[36,37], this might explain the difference in therapeutic outcome in the various models of stroke.

We investigated the presumed neuroprotective properties of the most studied endocannabinoid, AEA, in an *in vivo* model of secondary excitotoxicity, in which neuronal injury was induced by unilateral intrastriatal injection of the Na^+/K^+-ATPase-inhibitor ouabain without direct cerebrovascular intervention. Ouabain rapidly perturbs ion homeostasis, induces cellular swelling and glutamate-dependent damage of cells, which can be prevented in part by blockade of the NMDA-receptor[24-28].

Diffusion-weighted MRI data acquired 15 min after the injection of ouabain showed that exogenous AEA dose-dependently reduces *in vivo* cellular swelling in the early phase after the induction of excitotoxicity. We have shown previously that THC was able to reduce *in vivo* cellular swelling in a CB_1-mediated manner in the same model (see chapter 4). The AEA-induced reduction in cellular swelling was not attenuated by the CB_1-antagonist SR141716A. Because there are no CB_2 receptors detected in the brain, the early *in vivo* neuroprotective action of AEA does not seem to be mediated via the CB_1 or CB_2 receptors.

The failure of SR141716A to block the reduction in cytotoxic edema by AEA in the early phase does not seem to be a matter of dose and pharmacokinetics. The antagonist (3 mg/kg) did block the late effects (after 7 days) of AEA (10 mg/kg). Furthermore, a lower dose of SR141716A (1 mg/kg) was more than sufficient to block the neuroprotective actions of THC (1 mg/kg) at days 0 and 7 (see chapter 4). It is noteworthy that THC is a more potent agonist of the CB_1-receptor than AEA. In addition, SR141716A is effective in blocking the behavioral effects of THC in the mouse "tetrad" with AD50s of ~0.1 mg/kg, whereas some of the actions of AEA were insensitive to SR141716A[38]. Moreover, some of the behavioral actions of AEA in mouse were still observed in the CB_1 knock-out mice[38,39]. Others[15] have shown that neuroprotection of AEA in *in vitro* experiments was also independent of CB_1 and CB_2 receptors.

Recent data demonstrate that AEA is capable of interacting with other molecular targets, such as 5-hydroxytryptamine receptors, *N*-methyl-*D*-aspartate receptors, vanilloid receptors, L-type calcium channels, Shaker related K^+-channels, TASK-1 channels, a non-CB_1 G-protein coupled AEA receptor in astrocytes and a non-CB_1 non-CB_2 G-protein coupled receptor for AEA and WIN55,212-2 in mouse brain[40,41], some of which may

contribute to a reduction in cellular swelling. For example, the inhibition of gap junctions and intracellular calcium signaling in striatal astrocytes by the non–CB_1 G-protein-coupled AEA-receptor[42] or the inhibition of L-type calcium channels[43] may prevent glutamate exocytosis and the spreading of excitotoxicity. We cannot rule out the possibility that metabolites of AEA may account for some of the observed effects. Additional studies are necessary to understand the molecular mechanism of AEA-induced reduction of cytotoxic edema.

T_2-weighted MRI data recorded one week after ouabain injection showed that exogenous AEA reduced neuronal damage by $64 \pm 24\%$ ($p<0.05$). Compared with the early phase, AEA-induced neuroprotection was blocked by the CB_1 receptor antagonist after 7 days. This can be explained by the different stages in the cascade of events induced by excitotoxicity. Calcium entry is held responsible for delayed neurodegenerative events, which can occur even if the initial cellular swelling is reversed or prevented [2,44]. We have suggested previously that THC protected rat brains in the late phase via the CB_1–mediated closing of N–, and P/Q–type calcium channels and inhibition of glutamatergic transmission in the same model ([45]; see chapter 4). It is reasonable to assume that this CB_1-mediated process of closing voltage-sensitive calcium channels also contributes to the observed neuroprotection of AEA after seven days. As noted before for THC, AEA-induced neuroprotection was observed in brain regions such as cortex, striatum and hippocampus (See chapter 4).

Strikingly, the combination of AEA and the antagonist produced an infarct that was 2.5 times larger than seen for the control group. This observation is not likely to be explained by (*i*) blockade of the effect of endogenously released cannabinoids; (*ii*) inverse agonism of SR141716A or (*iii*) toxic effects of SR141716A. In fact, treatment of rats with SR141716A at 1 and 3 mg/kg or with SR141716A+THC (1 mg/kg) did not increase infarct size significantly (See chapter 4). In addition, endocannabinoid levels do not appear to increase after ouabain injection.

Hampson et al. have shown that AEA can enhance calcium influx presumably via direct activation of NMDA-receptors.[46] This enhancement could only be observed when CB_1 receptors were blocked by SR141716A. This might explain the extra deleterious effect of the combination AEA and SR141716A. Interestingly, the site and size of the infarcted region in these animals was similar to those observed in animals that received a unilateral intrastriatal injection with NMDA (unpublished results).

Several lines of evidence gained in this study indicate that there is no endogenous cannabinoid tone controlling the acute neuronal damage induced by ouabain: (*i*) No increases in AEA and 2-AG levels were detected at 2, 8 or 24 h after ouabain injection; (*ii*) Application of SR141716A alone (3 mg/kg) did not increase the lesion volume at day 0 nor at day 7. This implies that activation of CB_1 receptors by constitutive levels of AEA, 2-AG or any other novel CB_1 ligand, such as noladin ether[47], does not tonically protect the brain; (*iii*) The AEA uptake inhibitor, VDM11, did not reduce neuronal swelling at day

0, nor did it reduce the infarct volume after seven days. This also argues against a CB_1 receptor–independent tonic protective role of endogenous AEA and 2-AG. Thus, in our *in vivo* model of acute neuronal damage the data do not support the previously proposed role of the endogenous cannabinoid system in neuroprotection.

However, it cannot be excluded that technical issues have prevented the detection of a tonic protection by endocannabinoids. The lesion was considered pathological when ADC or T_2 values differed more than twice the SD of the mean value in the contra-lateral hemisphere. Thus, the periphery of the infarct with smaller changes in ADC or T_2 was not taken into account, but may have benefited from a possible endogenous release of cannabinoids. In addition, endocannabinoid levels were measured in total hemispheres, and a possible local up–regulation of AEA and 2-AG could have been missed. However, this last possibility appears unlikely if one considers the massive accumulation of endocannabinoids observed in other models of neuronal injury[14,48].

In addition, Hansen et al. (2001) have demonstrated that the increase in *N*-acyl–phosphatidylethanolamines varies in different *in vivo* models of neuronal damage and is dependent on the type of cell death.[14] High levels of these lipids, which act as biosynthetic precursors for AEA and its congeners, were found in NMDA–injected neonatal rats. However, only moderate and low increases were observed in a closed head injury model and in an apoptotic model, respectively. Although our model does not represent a model of apoptotic cell death, ouabain–induced injury was not severe enough to elicit endocannabinoid formation.

In this respect it is interesting to note that Wilson and Nicoll, 2001 have suggested that release of relevant levels of endocannabinoids probably only occurs in response to particularly intense stimuli.[49] Thus, endogenous AEA may only be released after the intense stimulus, and, hence, too late to exert a protective action, whereas exogenous AEA may inhibit the ouabain–induced glutamatergic transmission, thereby preventing the spreading and reducing the effect of the toxic stimulus. This explanation is consistent with recent studies in which endogenous cannabinoids were shown to mediate retrograde signaling from postsynaptic neurons to presynaptic terminals in hippocampal and cerebellar synapses[49-51].

In summary, we were able to accumulate data that strongly suggest that there is no endogenous endocannabinoid tone controlling the acute neuronal damage induced by ouabain. In contrast, our data, together with previous reports, indicate that exogenous AEA can protect the neonatal rat brain via a variety of mechanisms. Although our findings do question the role of the endogenous cannabinoid system in establishing a tonic brain defense system in our model, AEA may be used as a structural template to develop new neuroprotective agents.

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Acute neuronal injury: excitotoxicity, neuroprotection and cannabinoids

High and Healthy: neuroprotection by cannabinoids

The endocannabinoid system is a valuable target for drug discovery. The endocannabinoid system seems to be involved in the regulation of several physiological functions such as embryo implantation and development, motor coordination, learning and memory, pain, appetite and blood pressure[1-4]. Selective molecular probes targeted to one of the proteins of the endocannabinoid system may yield useful therapeutics for a variety of disorders such as liver cirrhosis, multiple sclerosis and obesity[5-8]. Furthermore, (endo)cannabinoid-based drugs may potentially be useful to reduce the effects of neurodegeneration.

The central nervous system is highly vulnerable to ischemia induced by a stroke or traumatic brain injury. In western societies stroke is the third cause of mortality and the first in disability[9]. At the moment no effective drugs are available to improve outcome after neuronal injury. Neuronal death caused by ischemia is executed via a complex array of processes in which excitotoxicity plays a major role. In excitotoxicity, cell death is triggered by the overstimulation of excitatory amino acid receptors, such as NMDA and AMPA receptors, by high concentrations of glutamate. This overstimulation leads to cytotoxic levels of calcium and subsequent activation of destructive enzymatic pathways and the generation of reactive oxygen species.[9,10] In conjunction with providing a theoretical concept underlying acute neuronal damage, the excitotoxicity hypothesis is also used to explain the biochemical basis of many chronic and slowly neurodegenerative disorders such as amyotrophic lateral sclerosis, Parkinson's, Huntington's, and Alzheimer's diseases[9,10].

In vitro neuroprotection by cannabinoids

CB-dependent protection

Various *in vitro* and *in vivo* models are used to test neuroprotective agents and to study the molecular mechanisms of neuronal cell death[10,11]. Depending on the model, classical and synthetic cannabinoids have been shown to exert neuroprotection via different mechanisms. For example, (*i*) cerebellar granule neurons were rescued from glutamate toxicity by administration of WIN55.212-2, Δ^8-THC, 11-OH-THC and palmitoylethanolamide 15 min after glutamate exposure (Table 1; no 1)[12]. The mechanism of protection is unknown, but it was suggested that palmitoylethanolamide interfered with downstream consequences of an excitotoxic stimulus upon binding to CB_2-like receptors. However, the involvement of such a CB_2-like receptor was not demonstrated. (*ii*) In another study it was shown that WIN55.212-2 and CP55.940 protected hippocampal neurons *in vitro* via the CB_1 receptor against secondary excitotoxicity, which was induced by reduction of Mg^{2+}-levels (Table 1, no 2)[13]. Presynaptic CB_1-mediated inhibition of Ca^{2+}-spiking and glutamate release were assumed to be responsible for the neuroprotective effects[13]. This is in line with the observation that the cannabinoids could

not rescue the cells from direct glutamate exposure (Table 1, no 2).[13] (*iii*) In contrast, THC could protect cultured mouse spinal neurons against direct kainate toxicity in a CB_1-dependent manner (Table 1, no 3)[14]. It should be noted that in studies [13] and [14] the distribution of the CB_1 receptor was different. In spinal neurons the CB_1 receptor was located on cell bodies[14], whereas a CB_1-like receptor was thought be located at presynaptic sites in hippocampal neurons[13,15]. It is noteworthy that AEA could reduce NMDA-induced calcium influx via CB_1-mediated closing of voltage sensitive calcium channels in rat brain slices[16]. A CB_1-mediated closing of voltage sensitive calcium channels may also be responsible for the observed neuroprotection in spinal neurons. (*iv*) In addition, it was shown that CP-55,940 could protect cortical neurons against glutamate exposure at low concentrations in a CB_1-dependent manner via closing of N- and P/Q-type calcium channels (Table 1, no 6)[17]. It was shown that the protective effects of CP-55,940 *in vitro* could only be observed when the cAMP levels were elevated.

CB-independent neuroprotection

In other studies cannabinoids were shown to protect neurons independently of CB receptors. For example, (*v*) WIN55212-2 protected cerebral cortical neurons from *in vitro* hypoxia and glucose deprivation in a CB_1- and CB_2-independent manner (Table 1, no 4)[18]. (*vi*) Exogenous AEA and 2-AG protected cultured cerebral neurons in the same model also in a CB-independent manner (Table 1, no 7)[19]. The mechanism of neuroprotection of the synthetic and endocannabinoids was not investigated in these studies. (*vii*) In another study cannabidiol and THC protected via their anti-oxidative properties rat cortical neurons exposed for a short time to glutamate, AMPA and kainate receptor ligands (Table 1, no 5)[20]. The neuroprotection was unaffected by CB receptor antagonists. THC and cannabidiol were anti-oxidants as was demonstrated by cyclic voltammetry. Furthermore, THC and cannabidiol prevented H_2O_2-induced damage in neuronal cultures[20]. Interestingly, it has been reported that 2-AG surpressed the formation of reactive oxygen species and tumor necrosis factor-α (TNF-α), a neurotoxic inflammatory mediator, by murine macrophages *in vitro* following stimulation with lipopolysaccharide (LPS)[21].

Thus, the mechanism of neuroprotection by cannabinoids differs in the various *in vitro* models and could involve a) activation of CB_2-like receptors, b) CB_1-mediated inhibition of glutamatergic transmission via closing of N-, and P/Q-type calcium channels, c) reduction of calcium influx and subsequent inhibition of deleterious cascades, d) anti-oxidant activity, and e) inhibition of TNF-α formation.

In vivo neuroprotection of classical and synthetic cannabinoids

Stroke models

To date, the neuroprotective effects of cannabinoids have only been addressed in a few *in vivo* models of neuronal injury. However, the results show a large degree of variation. (*i*) Chronic Δ^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 (Table 1, no 12)[22]. THC at was injected *i.p.* either a low (0.1 mg/kg) or high (10 mg/kg) dose every 12 h for 7 days prior to ischemia. Three weeks after neuronal injury rat brains were analyzed via a histological procedure. No protection was observed in the hippocampus at either dose, whereas animals treated with the high dose showed significantly less neocortical injury. The striatum was protected in all THC-treated animals. The involvement of the CB_1 receptor was not studied. (*ii*) In contrast, no protective effect could be found for the CB receptor agonist WIN55.212-2 in rats when the middle cerebral artery was occluded for 2 h. Surprisingly, CB_1 receptor antagonists were protective, which may suggest that endogenously released endocannabinoid are toxic.[a] The CB_1 antagonists SR141716A and LY320135 reduced the size of the infarct and improved neurological outcome. (*iii*) Yet, in another study WIN55.212-2 afforded dose–dependent protection to hippocampal and cortical neurons in a CB_1-dependent manner in rats with a permanent middle cerebral artery occlusion or global ischemia (Table 1, no 10 and 11)[18]. WIN55.212–2 (1 mg/kg, *i.p.*) could reduce the infarct volume in rat brains when administered up to 30 min after permanent focal ischemia (Table 1, no 11)[18]. (*iv*) Finally, in gerbils subjected to transient global ischemia CP-55,940 (4 mg/kg *i.p.*) reduced the ischemia–induced hyperlocomotion and improved electroencephalographic (EEG) spectral power after 24h (Table 1, no 14)[23]. These CP-55,940-induced effects were still observed after 7 days. Coadministration of SR141716A completely blocked the effect of CP-55,940, which indicated that the CB_1 receptor was involved.

It should be noted that in each of these stroke models different perturbations in cerebral blood pressure and flow were induced, which may explain the varying results. It has recently been shown that endocannabinoids generated in monocytes and platelets contributed to hypotension in acute myocardial infarction[24]. It might be possible that (endo)cannabinoid–induced vasorelaxation is affecting the extent and pathway of neuronal demise in a different manner in each of the stroke models. Therefore, we have tested the neuroprotective effects of THC in a model of acute neuronal damage without directly affecting blood pressure and flow to the brain (Chapter 4).

Excitotoxicity model

In our *in vivo* model acute neuronal damage was elicited in neonatal rats by inhibiting the Na^+/K^+-ATPase, thereby producing secondary excitotoxicity (Table 1, no 13; Chapter

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[a] Muthian, S. and Hillard, C.J. Symposium on the Cannabinoids, Burlington, Vermont, ICRS 2000, p. 107

4). THC (1 mg/kg, *i.p.*) could reduce the volume of cytotoxic edema in a CB_1-dependent manner already 15 min after blockade of the Na^+/K^+-ATPase. After seven days the infarct volume in THC-treated rats was ~40% smaller compared with control animals. The effect was abolished by coadministration of SR14716A. We have suggested that a CB_1-mediated reduction in calcium influx and a reduced glutamate release were responsible for the neuroprotection in the hippocampus, striatum and cortex. This suggestion was based on several studies, which had shown that both synthetic and classic cannabinoids as well as AEA could inhibit presynaptic glutamatergic transmission via CB_1-mediated closing of voltage sensitive calcium channels in different areas of rat brain such as striatum, hippocampus[25-27], substantia nigra pars reticulata[28], as well as in prefrontal cortex pyrimidal neurons[29] (see also Chapter 1).

Endogenously released endocannabinoids: to be protective?

It has been postulated that the release of endocannabinoids during neuronal injury might be an endogenous protective response. If this is the case, inhibitors of endocannabinoid clearance may provide useful therapeutics for neurodegenerative diseases. The lines of evidence are the following:

(1) 2-AG, AEA and its precursor *N*-arachidonoylphosphatidylethanolamine are normally found in low concentrations in the brain, but their levels increase in a calcium-dependent manner *postmortem* and upon (severe) neuronal injury[30-34]. AEA levels were increased in cultured primary neocortical neurons treated with NMDA or glutamate[35]. High levels of NAPEs were found in neonatal rat brains after receiving a striatal injection of NMDA (25 nmol)[36]. In the last model, total NAE levels were increased 46-fold in cerebral cortex after 24 h and AEA concentrations were elevated by a factor of 13.[34] In addition, a large increase in 2-AG was found in a closed head injury model in mice[37] and in rats treated with picrotoxinin[38].

(2) CB_1 expression is enhanced in the cortical mantle zone in rats after 20 min occlusion of the middle cerebral arteries[39]. Western blotting and immunohistochemistry showed that the increased CB_1 expression began by 2 h and persisted for 72 h or more after ischemia. CB_1 transcripts and [^{3}H]CP55.940 binding capacity were also elevated in the border regions of the cortex in mice subjected to mild concussive head trauma[34].

(3) The neuroprotective effects of classical, synthetic and endogenous cannabinoids have been reported (see above). However, only one *in vitro* study described a neuroprotective effect of endocannabinoids (Table 1, no 7)[19] and *in vivo* studies were still lacking. Recently, it has been shown that 2-AG may have a protective role at least in part via the CB_1 receptor after closed head injury to mouse brain (Table 1, no 15).[37] Synthetical 2-AG administered to mice after closed head injury reduced brain edema, improved clinical recovery, reduced infarct volume and reduced hippocampal cell death compared to controls. The reduction in brain edema by 2-AG was dose-dependently attenuated by SR141716A.

Table 1: Neuroprotection by (endo)cannabinoids in different i*n vitro* and *in vivo* models of neuronal injury.

No	Compound	Dose	Time of of application relative to injury	Type	Model and toxin used
1	WIN55.212-2 Δ^8-THC 11-OH-THC PEA	24.5 µM[1] 2.8 µM[1] 0.88 µM[1] 54.6 µM[1]	15 min post toxin	*In vitro*	glutamate
2	WIN55.212-2 CP55940 WIN55.212-2	100 nM[2] 100 nM[2] 100 nM[2]	coapplication	*In vitro*	reduced $[Mg^{2+}]$ glutamate
3	Δ^9-THC	0.5 µM[2]	coapplication	*In vitro*	kainate
4	WIN55.212-2 THC	30 nM[2] 10 µM[2]	coapplication	*In vitro*	hypoxia and glucose deprivation
5	Δ^9-THC CBD	3.7 µM[1] 30 µM[2]	coapplication	*In vitro*	glutamate AMPA-ligand kainate-ligand
6	CP55940	10 nM[2]	coapplication	*In vitro*	glutamate
7	AEA 2-AG	100 nM[2] 1000 nM[2]	coapplication	*In vitro* *In vitro*	hypoxia and glucose deprivation
8	AEA	100 µM[1]	15 min post toxin		glutamate
9	AEA PEA	10 µM[2] 10 µM[2]	pre and post toxin	*In vitro*	glutamate
10	WIN55.212-2	0.1-1 mg/kg[2], *i.p.*	40 min pre injury	*In vivo*	global ischemia; four vessel occlusion
11	WIN55.212-2	1mg/kg[2], *i.p.*	30 min post injury	*In vivo*	focal ischemia, MCA-occlussion
12	Δ^9-THC	0.1-10 mg/kg[2], *i.p*	7-day pre injury	*In vivo*	ischemic reperfusion reduced blood pressure bicarotid occlusion
13	Δ^9-THC	1 mg/kg[2], *i.p.*	30 min pre injury	*In vivo*	ouabain-induced excitotoxicity
14	CP55940	4 mg/kg[2], *i.p.*,	5 min post injury	*In vivo*	global ischemia; bicarotid occlusion
15	2-AG	0.1-10 mg/kg[2], *i.v,*	15 min post injury	*In vivo*	traumatic brain injury
16	AEA	1-10 mg/kg[2], *i.p.*	30 min pre injury	*In vivo*	ouabain-induced excitotoxicity

1) ED_{50} values. 2) highest (effective) concentration tested.

Dose of toxin and duration of toxin application	Species	Neuron type	Protection?	Time of assessment after injury	CB dependent?	Ref.
500 μM, 5 min	rat	cerebellar granule	yes	24 h	CB_2-like	12
18-24 h	rat	hippocampal	yes	0 h	CB_1	13
30 μM, 18-24 h			no			
100 μM, 24 h	mouse	spinal	yes	24 h	CB_1	14
8 h,	rat	cortical	yes / no	16 h	CB_1 and CB_2 independent	18
250 μM, 10 min 1.5 μM, 20 h 10 μM, 20 h	rat	cortical	yes	18-20 h	Anti-oxidant	20
200 μM, 15 min	rat	cortical	yes	18-20h	CB_1	17
8 h	rat	cortical	yes	16 h	CB_1 and CB_2 independent	19
500 μM, 5 min	rat	cerebellar granule	no	24 h		12
100 μM, 1 or 24 h	chicken	telencephalon	no	24 h		40
15 min	rat	hippocampal	yes	3 d	CB_1	18
permanent	rat	cortical	reduction in infarct volume	24 h	CB_1	18
12 min	rat	striatal, cortical	yes	3 wk	N.D.	22
0.5 nmol intrastriatal	rat	hippocampal, striatal, cortical	reduction in infarct volume	15 min and 7 d	CB_1	Ch4
10 min	gerbil	–	improvement of locomotion and EEG	1, 3 and 7 d	CB_1	23
	mouse	hippocampal	reduction in infarct volume	1 and 7 d	In part CB_1	37
0.5 nmol intrastriatal	rat	hippocampal, striatal, cortical	reduction in infarct volume	15 min and 7 d	In part CB_1	Ch5

At the same time, we have shown in a longitudinal MRI-study that exogenously applied AEA reduced dose–dependently neuronal damage in neonatal rats injected *i.c.* with the Na^+/K^+–ATPase inhibitor ouabain (Table 1, no 16; Chapter 5). Fifteen minutes after injury, AEA (10 mg/kg)-administration, 30 min before ouabain injection, reduced the volume of cytoxic edema in a manner insensitive to the CB_1 receptor antagonist. Seven days after ouabain treatment, the lesion volume was 64% smaller in AEA-treated rats. After this period of time, neuroprotective actions of AEA were abolished by SR141716A.

Endogenously released endocannabinoids: or not to be protective?

However, several other observations question the role of the endocannabinoid system as a general endogenous protection system.

(1) It has been demonstrated that the increase in NAPE and AEA varies in different *in vivo* models of neuronal damage and is dependent on the type of cell death.[36] As stated above, high levels of these lipids were found in NMDA-injected neonatal rats, but only moderate and low increases were observed in a closed head injury model and in an apoptotic model, respectively. No increase in 2-AG levels was found after traumatic brain injury or NMDA-injection in rats.[34] In our secondary excitotoxicity model in which neurodegeneration was elicited by ouabain, AEA and 2-AG concentrations were not elevated (Chapter 5).

(2) Some *in vitro* studies do not support a neuroprotective action of endocannabinoids. (*i*) AEA did not protect cerebellar granule neurons in a post–glutamate paradigm, whereas THC and palmitoylethanolamide protected these neurons (Table 1, no 8)[12]. (*ii*) AEA was also ineffective against prolonged glutamate exposure of chicken telencephalon neurons (Table 1, no 9)[40]. (*iii*) Hippocampal neurons exposed to THC died in a CB_1-dependent manner, probably due to an activated arachidonic acid pathway[41].

(3) Observations in four different *in vivo* models indicate that endogenously released or constitutive endocannabinoids do not protect the brain against acute neuronal injury. (*i*) In a mice traumatic brain injury model application of SR141716A (20 mg/kg) did not increase the volume of edematous tissue and the application of entourage compounds did not reduce the volume of edematous tissue.[37] (*ii*) No increase in infarct volume upon application of SR141716 was found in global and (*iii*) focal ischemia models (MCA-occlusion)[18]. (*iv*) In our secondary excitotoxicity model compared to control animals, application of AEA-uptake inhibitor, VDM11, or SR141716A alone did not affect lesion volumes at day 0 nor at day 7. (Chapters 4 and 5).

(4) In an ischemic reperfusion model, application of SR141716A was neuroprotective[a]. In this study neuronal damage was assessed after 24 h, whereas in most of the other *in vivo* studies neuronal injury was quantified after 3 days or more (Table 1, no 10-16).

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[a] Muthian, S. and Hillard, C.J. Symposium on the Cannabinoids, Burlington, Vermont, ICRS 2000, p. 107

Some possible explanations:

Why can exogenously applied (endo)cannabinoids prevent neuronal loss, while endogenously released endocannabinoids do not seem to be able to reduce neuronal damage? Several explanations can be put forward, which can act independently or in concert with each other.

(1) As described above, the neurodegenerative insult may not always lead to an upregulation of endocannabinoid biosynthesis. It seems to be dependent on the species and on the type of injury. For example, traumatic brain injury in mice resulted in a substantial increase in 2-AG levels[37], whereas in neonatal rats no increase was found[36]. Intrastriatal injection of NMDA in neonatal rats led to an increase in AEA[36], but not with an ouabain injection (Chapter 5). Recently, it has been shown that the simultaneous application of glutamate and carbachol (an acetylcholine receptor agonist), but not of either agent alone, caused a marked increase in AEA biosynthesis in cortical neurons[42]. Thus, membrane depolarization was necessary for the biosynthesis, but was insufficient per se to initiate AEA biosynthesis. At the moment the biosynthetic pathways of endocannabinoids have been characterized, but their regulation is largely unknown. Isolation or cloning of the proteins responsible for endocannabinoid formation may help to understand the regulation of endocannabinoid biosynthesis in response to neuronal injury.

(2) The distribution and localization of the CB_1 receptor is also of importance. The CB_1 receptor is highly expressed in several areas of the central nervous system at presynaptic and postsynaptic sites as well as on cell bodies. As noted before in *in vitro* models, presynaptic CB_1 receptors can block secondary excitotoxicity by inhibiting glutamate release. However, these presynaptic CB_1 receptors are ineffective against a direct glutamate-induced elevation of postsynaptic calcium concentrations[25,26], such as occurring in glutamate toxicity models.[43]

It is noteworthy that endocannabinoids have been shown to act as retrograde messengers in the hippocampus and cerebellum.[44-47] They are released from the postsynaptic membrane and have to diffuse back to the presynaptic CB_1 receptors. Upon activation of these receptors the release of GABA or glutamate is inhibited. If during the *in vivo* toxic stimulus glutamate has been released in large quantities (or exogenously applied), then endogenously released endocannabinoids might be too late to exert a protective action. The damage has already been inflicted. In this way the endocannabinoid system may not be able to function as an endogenous protection system. Conversely, pretreatment with exogenous (endo)cannabinoids may inhibit the toxic stimulus-induced glutamate exocytosis in advance, thereby preventing the spreading and reducing the effect of the toxic stimulus.

(3) The ability of endocannabinoids to influence downstream effects of increased calcium concentrations are also dependent on the cell type, strength, duration, and stimulus type. In most studies cannabinoids have been shown to reduce intracellular

calcium concentrations via CB_1-mediated closing of voltage sensitive calcium channels, but several studies have indicated that cannabinoids may also increase calcium concentrations.[48] For example, CB_1 and CB_2 receptor activation by AEA and 2-AG in N18TG2 neuroblastoma and NG108-15 neuroblastoma-glioma hybrid cells has been shown to induce a rapid transient increase in intracellular free calcium via its release from IP_3-sensitive calcium stores.[49,50] It has been shown that synthetic cannabinoids can enhance peak amplitude of NMDA-elicited signals via calcium release of intracellular stores in cerebellar granule neurons by a CB_1-dependent mechanism[51]. Moreover, AEA was shown to directly modulate NMDA receptors in the presence of SR141716, thereby potentiating calcium currents[16].

(4) Another possibility is that the inflicted damage in the *in vivo* models of acute neuronal injury is too severe. It has been shown that severe excitotoxic injury leads to a loss of CB_1 receptor expression[36]. If so, endogenously released endocannabinoids may only be effective in the border zone of the damaged brain area or in mild to moderate brain injury in which the expression of the CB_1 receptor is not lost[18,36,39] (Chapter 4 and 5). Thus, the extent of the neurodegenerative process may be a major determinant of the ability of endocannabinoids to exert neuroprotection.

Conversely, it is interesting to speculate that if the endocannabinoid system is dysfunctioning, (*e.g.,* endocannabinoid biosynthesis is inhibited, CB_1 receptor is inactive or its expression is lost), then glutamate homeostasis is lost and excitotoxicity may be initiated. For example, in a genetic model of Huntington's disease CB_1 mRNA was decreased prior to the development of either Huntington's disease phenotype or neurodegeneration[52]. Also a loss of CB receptor binding capacity was evident before degeneration of the nerve terminals was seen. It is tempting to suggest that the early downregulation of CB receptors induces excitotoxicity and subsequent neurodegeneration. Furthermore, it has been speculated that the CB_1 knock-out mice, which die suddenly without any obvious sign of disease, might succumb to neurological problems such as seizures[53].

(5) The lack of CB_1 receptor antagonists to demonstrate endogenous neuroprotective actions of endocannaboids, may result from neuroprotective actions of AEA and 2-AG via other molecular targets than CB_1 receptors. For example, palmitoylethanolamide reduced glutamate toxicity via a CB_1-independent manner.[12] This observation has not been extended yet to an *in vivo* model, but palmitoylethanolamide was a potent anticonvulsant in electroshock and chemically-induced seizures in mice[54]. Furthermore, AEA and 2-AG have been shown to reduce neuronal damage both *in vitro* and *in vivo,* partially via a CB-independent pathway. It is unclear which molecular targets may be responsible for these effects. Recent data demonstrate that AEA is capable of interacting with many other proteins, such as 5-hydroxytryptamine receptors, vanilloid receptors, L-type calcium channels, Shaker related K^+-channels, TASK-1 channels, a non-CB_1 G-protein coupled

AEA-receptor in astrocytes and a non-CB_1 non-CB_2 G-protein coupled receptor for AEA and WIN55,212-2 in mouse brain.[44] The inhibition of gap junctions and intracellular calcium signaling in striatal astrocytes by the non-CB_1 G-protein-coupled AEA-receptor or the inhibition of L-type calcium channels might help to prevent glutamate exocytosis and the spreading of excitotoxicity.[55,56] Recently, a new CB-type receptor was suggested to be responsible for WIN55,212-2-induced reduction in glutamatergic transmission in the hippocampus of CB_1 knock-out mice[15]. Thus, endocannabinoids may provide neuroprotection through other molecular targets. However, it should be noted that the AEA-uptake inhibitor VDM11 (Chapter 5) and entourage compounds of 2-AG did not affect lesion volumes[33], which argues against an endogenous endocannabinoid tone controlling acute neuronal damage via any extracellular molecular target.

Perspectives

Recent developments, such as the generation of CB_1 and CB_2 knock-out mice and synthesis of selective antagonists of the CB_1 and CB_2 receptors, have provided insight into the (patho)physiological roles of the endocannabinoid system. However, some important fundamental aspects of the endogenous cannabinoind system remain to be discovered. For example, the proteins responsible for the biosynthesis of endocannabinoids and their transport into cells have not been identified yet. The regulation of the biosynthetic and inactivation pathways of AEA and 2-AG is also largely unknown. It is likely that novel CB receptor subtypes as well as novel endogenous ligands will be found. The understanding of the complex interplay of the endocannabinoid system with other neurotransmitters in the CNS and their function as retrograde messengers will greatly enhance our knowledge about the physiological roles of the endocannabinoid system. This may provide useful information to exploit the cannabinoid system for therapeutic intervention in various diseases, ranging from liver cirrhosis, multiple sclerosis, pain, obesity to schizophrenia. Crystal or NMR structures of the proteins of the cannabinoid system may help to elucidate the nature of the ligand-protein interaction, thereby facilitating the design of selective and potent molecular probes for each of these proteins.

The studies reported in this thesis indicate that agonists of CB_1 receptors might also be useful to enhance the neurological outcome after acute brain damage. It is interesting to note that THC and CP-55,940 are partial agonists which can reduce excitotoxicity without completely blocking glutamatergic transmission. Complete inhibition of glutamatergic neurotransmission may lead to serious complications such as hallucinations[57]. Nevertheless, many questions have to be answered, before a CB_1 agonist can be used as a neuroprotective agent, such as a) which type of human brain injury can be treated? b) to what extent do cannabinoids improve functional and neurological outcome after brain damage? c) What is the therapeutic time-window? d) Does the cannabinoid-induced vasorelaxation and psychotropic side effects pose a problem? At the

moment it is not clear whether CB_2 agonists, which do not cause psychotropic effects, are also able to reduce neuronal damage *in vivo*, but other anti-inflammatory drugs have been proven to reduce neuronal injury and improve functional recovery. It is noteworthy that dexanabinol (HU-211), a non-psychotropic cannabinoid which do not bind to CB receptors, has been shown to be a powerful neuroprotective agent. Its neuroprotection is thought to be mediated via direct antagonism of the NMDA receptor, anti-oxidative properties and blockade of TNF-α production[58]. The fact that dexanabinol has recently entered phase III clinical trials against brain trauma, substantiates the hope that cannabinoid-based drugs will be useful as therapeutic agents for acute brain damage. If the extent of neuronal damage is not too large, inhibitors of endocannabinoid clearance may prove to be suitable leads for drug development, because they have probably limited side effects compared with CB_1 agonists and NMDA receptor antagonists.

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Endocannabinoids: the Body's own Marijuana

Marijuana and its constituents: the classical cannabinoids

Marijuana is the most widely used illegal drug throughout the world and has aroused great controversies. In the Netherlands, public debate centres upon the possible legalization of marijuana for recreational and therapeutic uses. Nowadays, marijuana is used by multiple sclerosis patients to alleviate tremors and by aids-patients as well as cancer-patients with chemotherapy to stimulate appetite and to relieve nausea. However, in 1996, the health council of the Netherlands came to the conclusion, based on a literature survey, that the scientific evidence to justify the medical use of marijuana was insufficient. The council believes that physicians cannot accept responsibility for a product of unknown composition that has not been subjected to a quality control. The council was unable to comment on the use of any other preparation of the hemp plant, and its active ingredient Δ^9-tetrahydrocannabinol (THC), or other components, since there were no published (clinical) reports. The recent and rapidly evolving developments in the field of cannabinoid research may give rise to a new assessment of these issues.

Marijuana (cannabis, bangh, hashish) is an extract from the plant *cannabis sativa* and contains at least 400 chemical components of which 60 belong to the cannabinoid-class. Marijuana and its main psychoactive compound THC have been used for centuries. It took until 1964 before THC was isolated and characterized. The structure elucidation of THC led to the design of a series of analogs. By 1986, over 300 cannabinoid derivatives were synthesized and used to determine the structural prerequisites, necessary for cannabinoids to exert their typical psychotropic properties.

Proteins are responsible for the action of cannabinoids

The mechanism of action of THC has also been subject of discussion. At first it

was thought that cannabinoids perturbed the membrane due to their lipophilic nature, thereby producing cellular effects. However, the requirement of one enantiomer and stringent structural features of THC to produce pharmacological responses raised the question whether cannabinoids could act via membrane proteins. The development of the potent bicyclic cannabinoid CP-55.940, which could be radio-labelled, allowed Devane and coworkers to demonstrate selective and specific binding sites in the brain in 1988. The identification and cloning of a protein responsible for this binding followed within two years. This protein was termed cannabinoid (CB_1) receptor. The existence of another subtype cannabinoid receptor (CB_2) was demonstrated in 1993. CB_1 receptors have been detected both in the central nervous system and in certain peripheral tissues. The central distribution pattern of CB_1 receptor is heterogeneous and was found in high levels in several brain regions. The activation of CB_1 receptors in these brain areas is thought to mediate typical cannabinoid effects, such as impairment of motor behaviour (cerebellum, basal ganglia), memory (hippocampus) and cognition (cerebral cortex). The CB_2 receptor is found in cells of the immune system.

The cannabinoid receptors have the structural characteristics of G-protein coupled receptors. Both CB_1 and CB_2 receptors inhibit cAMP formation via G_i-proteins and activate mitogen activated protein kinases. In addition, CB_1 receptors activate ion channels such as A-type and inwardly rectifying potassium channels and inhibit voltage sensitive N-type and P/Q-type calcium channels.

The body's own marijuana: endocannabinoids

The presence of cannabinoid receptors in mammalians suggested the existence of endogenous compounds in brain, which could bind and activate these proteins. In 1992, Mechoulam *et al* isolated the first endogenous compound for these receptors from porcine brain. This component proved to be an amide of ethanolamine and arachidonate. The compound was named anandamide. Ananda is Sanskrit for internal bliss. Anandamide [5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl-*N*-(2-hydroxyethyl)-amine] was able to mimic many biochemical, pharmacological and behavioural properties of the classical cannabinoids. In contrast to most other neurotransmitters, anandamide is not stored in vesicles, but is released from cells upon demand by stimulus-dependent cleavage of membrane phospholipid precursors. Already in the early 80's Schmid *et al* had characterized a biosynthetic pathway for *N*-acylethanolamines. According to this scheme *N*-acylethanolamines, including anandamide, are formed through sequential *N*-acylation and phosphodiesterase activities. A membrane-bound calcium-dependent transacylase catalyzes the transfer of fatty acids from the first position of various lipids to the ethanolamine of phosphatidylethanolamine, which yields the precursor NAPE. The formation of NAPE seems to be rate limiting.

Enzymatic hydrolysis of NAPE by a phosphodiesterase of the phospholipase D-type, which seems to be an unregulated enzyme, generates the *N*-acylethanolamines. So far, no

fatty acid selectivity for either the biosynthesis or hydrolysis of NAPE could be demonstrated. Any selectivity in anandamide generation must therefore rely on other mechanisms. Another problem with this scheme concerns the very low amounts of arachidonic acid that are found at the first position of phospholipids. It is almost exclusively in the second position of brain phospholipids. It is noteworthy that the *N*-acyltransferase and the phospolipase D have not been purified or cloned yet.

Three years after the discovery of anandamide, 2-arachidonoylglycerol (2-AG) was found as a second endogenous ligand for the cannabinoid receptors. Anandamide and 2-AG are termed endocannabinoids. Very recently a third endogenous compound, 2-arachidonoyl glyceryl ether, was isolated from porcine brain, which could activate CB_1 receptors.

How the body terminates the action of endocannabinoids

In order to function as a neurotransmitter the concentrations of the endocannabinoids have to be regulated in a strict manner. We have shown that after release, anandamide can be eliminated by a two-step mechanism in human brain and immune cells (Chapter 2). It consists of anandamide transport into cells followed by enzymatic hydrolysis. Both human neuronal and immune cells rapidly take up extracellular anandamide through a mechanism that meets four key criteria of carrier-mediated transport: fast rate, temperature dependent, saturability, and substrate selectivity. Importantly and in contrast with transport systems for classical neurotransmitters, anandamide uptake is neither dependent on external Na^+ nor affected by metabolic inhibitors. This suggests that the uptake is a process of carrier-facilitated diffusion. Anandamide-uptake was not affected by fatty acids, neutral lipids, neurotransmitters, biogenic amines, prostaglandins and leukotrienes. To date, the protein responsible for anandamide-uptake has not been purified or cloned, but its activity has been demonstrated in several human cells and in rat brain slices.

Once inside the cell, anandamide is inactivated by hydrolysis of its amide bond to arachidonic acid and ethanolamine. A similar activity was already identified by Schmid and co-workers in rat liver in 1984. After the discovery of anandamide, biochemical evidence suggested that the same enzyme activity could degrade anandamide. In 1996, the enzyme responsible for the degradation of anandamide was purified and cloned from rat liver and termed fatty acid amide hydrolase (FAAH). FAAH is an intracellular membrane-bound 64 kDA protein with a conserved amidase sequence. We have shown that FAAH is also present and active in human brain (Chapter 2). Interestingly, FAAH-activity and expression were downregulated in meningioma tumors. FAAH has been shown to degrade a broad spectrum of fatty acid amides and esters. Among them were oleamide (a novel sleep inducing factor), palmitoylethanolamide and 2-arachidonoylglycerol. The CB receptors, endocannabinoids and the proteins of the inactivation process constitute the endogenous cannabinoid system.

Anandamide is not only inactivated via FAAH, but it is also converted by other enzymes such as lipoxygenases. Various oxidative metabolites of anandamide were synthesized via a lipoxygenase reaction and their cannabinoid properties were characterized (Chapter 3). The oxidative metabolites were shown to competively inhibit FAAH. Furthermore, depending on the position of the hydroxyl group in the metabolites, they were able to selectively bind to the CB_1 or CB_2 receptor. The metabolites did not interfere with anandamide uptake into the cell. These results indicate that small changes in the chemical structure of anandamide can lead to the generation of novel compounds which can specifically interact with each of the proteins of the endogenous cannabinoid system (Chapter 3). With computer simulations we have tried to identify the essential structural elements of anandamide, which are necessary for the interaction with the CB_1 receptor (Chapter 3).

How endocannabinoids influence the communication between nerve cells

The feeling of getting 'high' is experienced when CB_1 receptors are activated in various brain areas. When these receptors are activated, they alter the communication between the nerve cells in the brain. Glutamate is the main excitatory neurotransmitter in the brain which carries the signal from one nerve cell to the other. THC and anandamide reduce the release of glutamate from the nerve ending of the cell and thereby prevent the activation of the second nerve cell and slow down communication. The relation of the cannabinoid system to various neurotransmitter systems is of importance to understand its action on the central and peripheral nervous system. The cannabinoid system is involved via presynaptic and postsynaptic CB_1 receptors in regulation of the release, uptake and actions of various other neurotransmitters such as GABA and dopamine.

The relation of endocannabinoid to brain diseases

Glutamate is not only important for normal communication between nerve cells. It also plays a role in several brain diseases such as Parkinson's disease, amyotrophic lateral sclerosis and in acute brain damage. When concentrations of glutamate are rising above a certain level, then nerve cells cannot cope with the sustained activation anymore. The nerve cells will eventually die. Since cannabinoids can reduce glutamate release from nerve ending, we speculated that cannabinoids could protect nerve cells against acute brain injury. We tested this hypothesis in the following way (Chapters 4, 5 and 6).

Seven days old rats were directly injected in the brain with a toxin which causes nerve cells to die. This process of dying was followed by magnetic resonance imaging. We followed this process for one week. One group of rats received a solution with THC or anandamide 30 min prior to the toxin. Another group received THC or anandamide plus a compound which blocked the action of the cannabinoids at the CB_1 receptor. One group of rats was used as a control.

We observed that the animals which had received THC or anandamide had an infarct which was ~ 40 or 64% smaller than the non-treated animals after seven days, respectively (Chapters 4 and 5). Thus, both classical and endogenous cannabinoids can protect rat brain against acute brain damage. The animals which received cannabinoids plus a blocker had an infarct which was as large as in the control animals. This indicated that the protective effects of the cannabinoids were mediated via the CB_1 receptor. We could not observe a protection by endogenous endocannabinoids.

Future 'high' lights

The discovery of the endocannabinoids and the way they work in the brain have opened a new road to study the effects of marijuana. In addition, it has become possible to investigate its participation in normal body function and in disease. It is thought that the cannabinoid system is involved in the regulation of a variety of physiological processes such as pain, appetite, memory, blood pressure and motor coordination. In several diseases such as liver cirrhosis, Parkinson's disease, multiple sclerosis, schizophrenia and glaucoma components of the cannabinoid system are involved. Possibly, selective and potent compounds targeted towards proteins of the cannabinoid system can be used to design new drugs for such diseases. This will circumvent the need of a marijuana extract as a medicine, thereby avoiding marijuana's complex, unknown and varying composition and possibly its psychotropic side effects. For example, the development of compounds, which selectively activate the CB_2 receptor, which is not present in brain, may lead to therapeutic agents which prevent the patient from becoming "high". Our results indicate that compounds, which activate the CB_1 receptor might be used to reduce acute neuronal damage. However, much research effort is necessary before we will witness the introduction of the first cannabinoid-based drug.

Marihuana, endocannabinoïden en acute hersenschade

Marihuana en haar ingrediënten: de cannabinoïden

Marihuana is het meest gebruikte illegale genotmiddel ter wereld en heeft aanleiding gegeven tot vele controverses. In Nederland richt de discussie zich op het legaliseren van het gebruik voor zowel medische als recreatieve toepassingen. Tegenwoordig wordt marihuana gebruikt door multiple sclerose patiënten om ongecontroleerde trillingen te onderdrukken en door aids-patiënten alsmede door mensen die een chemotherapie ondergaan om de eetlust op te wekken. Wetenschappelijke onderzoek om de claims te ondersteunen is summier. In 1996 adviseerde de Gezondheidsraad minister Borst van Volksgezondheid dan ook om marihuana niet als medicijn te registreren. De afwezigheid van een goede kwaliteitscontrole van een product met onbekende en variërende samenstelling maakt het onverantwoord voor artsen om het als medicijn voor te schrijven. Over afgeleide actieve componenten deed de gezondheidsraad geen uitspraak omdat daar te weinig over bekend was. De Nederlandse regering heeft in het najaar van 2001 dit advies naast zich neergelegd en besloten in navolging van Canada, dat marihuana gelegaliseerd wordt voor medicinale toepassingen. In dit proefschrift wordt de huidige stand van het wetenschappelijk onderzoek beschreven en wordt een aantal nieuwe aspecten van Δ^9-tetrahydrocannabinol (THC), de meest actieve stof in marihuana, en de lichaamseigen stoffen met een marihuana-achtige werking onderzocht. Er is speciale aandacht voor de mogelijk positieve effecten van cannabinoïden op acute hersenschade.

Marihuana is een product van de plant *cannabis sativa* en bevat op zijn minst 400 verschillende componenten, waarvan 60 stoffen tot de klasse van cannabinoïden behoren. Hoewel marihuana al eeuwen wordt gebruikt, werd in 1964 pas voor het eerst de actieve stof THC geïsoleerd en gekarakteriseerd. De structuuropheldering van THC leidde tot het ontwerp van een serie van verbindingen met vergelijkbare activiteit. In 1986 waren

meer dan 300 verbindingen gemaakt. Deze stoffen werden gebruikt om er achter te komen welk deel van de structuur van THC verantwoordelijk was voor het "high" worden.

Eiwitten zijn verantwoordelijk voor de werking van de cannabinoïden

Het werkingsmechanisme van THC was het middelpunt van een heftige discussie. In eerste instantie werd gedacht dat cannabinoïden via een a-specifieke interactie de vloeibaarheid van de celmembraan van hersencellen veranderden, waardoor het "high"-zijn werd veroorzaakt. Aangezien niet alle cannabinoïden dit effect vertoonden en een kleine verandering in THC de werking uitschakelde, vroeg men zich af of de effecten niet via bepaalde eiwitten tot stand werden gebracht. Dit bleek inderdaad het geval te zijn. In 1988 werd met behulp van een radioactieve cannabinoïd specifieke bindingsplaatsen in de hersenen van ratten aangetoond. Het eiwit dat verantwoordelijk was voor de specifieke binding van THC werd in 1990 gevonden. Dit eiwit werd de cannabinoïd (CB_1) receptor genoemd. Drie jaar later werd het bestaan een tweede eiwit aangetoond: de CB_2 receptor. De twee eiwitten komen op verschillende plaatsen in het lichaam voor. De CB_1 receptor wordt vooral gevonden in de hersenen en de CB_2 receptor in cellen van het immuun systeem. De CB_1 receptor komt ondermeer voor in hersengebieden die verantwoordelijk zijn voor beweging (cerebellum, basale ganglia), het geheugen (hippocampus) en cognitie (cerebrale cortex). Als THC aan de CB_1 receptoren bindt, dan wordt het eiwit geactiveerd en de signaal overdracht in de cel gedempt. De productie van het signaalmolecuul cyclisch AMP wordt in de cel geremd en calcium ionen kunnen niet meer de cel instromen.

Lichaamseigen marihuana: de endocannabinoïden

De aanwezigheid van CB_1 receptoren in de hersenen van muizen, ratten en ook mensen suggereerde het bestaan van een stof, die van nature zou voorkomen in de hersenen en die de CB_1 receptor zou kunnen activeren. Deze stof werd voor het eerst gevonden in varkenshersenen in 1992. Het bleek een vetzuuramide te zijn, dat was samengesteld uit arachidonzuur en ethanolamine. Deze lichaamseigen stof wordt anandamide genoemd naar het Sanskrit voor "gelukzalig". Anandamide bootst vele biochemische, farmacologische en gedragseffecten van THC na. Moleculen die van nature in de hersenen voorkomen en de CB_1 receptor kunnen activeren, worden endocannabinoïden genoemd. Na de ontdekking van anandamide werden nog meer van deze stoffen gevonden. Anandamide is echter nog steeds één van de belangrijkste vertegenwoordigers van de klasse van endocannabinoïden. Anandamide wordt vrijgemaakt uit de celmembraan van zenuwcellen, nadat de cellen geactiveerd zijn. Al in het begin van de jaren tachtig werd ontdekt hoe dit proces voor andere vergelijkbare moleculen in zijn werk ging. De eiwitten verantwoordelijk voor de biosynthese van anandamide zijn echter nog niet gevonden.

Beëindiging van de werking van anandamide

Als anandamide zijn werk gedaan heeft op de CB_1 receptor, dan wordt anandamide afgebroken. Wij hebben laten zien dat menselijke cellen van zowel het immuun systeem als de hersenen anandamide kunnen elimineren via een proces dat uit twee stappen bestaat. In hoofdstuk twee wordt dit in detail beschreven. Anandamide werd in eerste instantie de cel in getransporteerd en daarna afgebroken door een eiwit dat vetzuuramidehydrolase wordt genoemd. Anandamide werd opgenomen door een proces dat voldoet aan de vier criteria van 'drager'-gemedieerd transport: snel, temperatuur afhankelijk, verzadigbaar and structuur afhankelijk. In tegenstelling tot transportsystemen voor klassieke signaalmoleculen in de hersenen was de opname van anandamide niet afhankelijk van een natrium ion gradiënt en het kostte geen energie. Het transport van anandamide werd niet beïnvloed door een variëteit aan andere vergelijkbare moleculen. Tot op heden is het eiwit dat verantwoordelijk lijkt te zijn voor het transport van anandamide nog niet gevonden.

Als anandamide eenmaal in een hersencel is, dan wordt de binding tussen het ethanolamine en het arachidonzuur verbroken. Een vergelijkbare activiteit was al eens aangetoond in de lever van ratten voor andere vetzuuramide moleculen in 1984. Na de vondst van anandamide in varkenshersenen werd gesuggereerd dat één eiwit verantwoordelijk was voor de afbraak van deze vetzuuramide moleculen. Dit eiwit werd in 1996 in rattenlever gevonden en vetzuuramidehydrolase genoemd. Wij hebben in hoofdstuk twee aangetoond dat dit eiwit ook voorkomt en actief is in humane hersenen. Vetzuuramidehydrolase kan een zeer uiteenlopend spectrum van vetzuuramide moleculen afbreken, waaronder ook een stofje dat slaap veroorzaakt. De CB receptoren, de endocannabinoïden en de eiwitten van het inactivatie-proces vormen met elkaar het endogene cannabis systeem.

Het blijkt echter dat anandamide niet alleen wordt aangepakt door het vetzuuramidehydrolase. Er zijn ook eiwitten die de structuur van anandamide een klein beetje kunnen veranderen. Eén van deze klasse van eiwitten zijn de lipoxygenases. Deze eiwitten bouwen een zuurstof molecuul op verschillende plaatsen in anandamide. Wij hebben in hoofdstuk drie aangetoond dat deze varianten van anandamide ook aan de CB_1 en CB_2 receptor kunnen binden, maar minder goed dan anandamide zelf. De sterkte van de binding was afhankelijk van de plaats van de nieuwe zuurstof-atoom in de structuur van anandamide. Deze nieuwe anandamide varianten waren in staat om het vetzuuramidehydrolase zeer goed te remmen in zijn taak om ananamide af te breken, maar verhinderden niet de opname van anandamide in de cel. De resultaten gaven ook aan dat kleine veranderingen in de structuur van anandamide kunnen leiden tot nieuwe stoffen met specifieke interacties met de CB_1 of met de CB_2 receptor. We hebben in hoofdstuk drie ook geprobeerd met computer simulaties te achterhalen welke structuurelementen van anandamide verantwoordelijk waren voor de specifieke interacties.

Endocannabinoïden en communicatie tussen hersencellen

Het gevoel 'high' te zijn wordt ervaren wanneer de CB_1 receptor wordt geactiveerd in verschillende hersengebieden. Wanneer deze receptoren geactiveerd zijn, veranderen zij de communicatie tussen zenuwcellen in de hersenen. Glutamaat is een van de belangrijkste neurotransmitters in de hersenen, die het signaal van de ene zenuwceluiteinde overbrengt naar het begin van een andere zenuwcel. THC en anandamide verminderen de vrijgifte van glutamaat uit de zenuwceluiteinden. Daarmee voorkomen zij dat de volgende zenuwcel wordt geactiveerd en veranderen zij de communicatie tussen de zenuwcellen.

De relatie met acute hersenziekten

Glutamaat is niet alleen belangrijk voor normale communicatie tussen zenuwcellen. Het speelt ook een rol bij verschillende hersenziekten, zoals de ziekte van Parkinson en amyotrofe lateraal sclerose (ALS) en in acute hersenschade. Als de concentraties van glutamaat boven een bepaalde waarde stijgen, dan kunnen de zenuwcellen niet meer omgaan met de langdurige activatie. De zenuwcellen gaan uiteindelijk dood. Aangezien cannabinoïden de vrijgifte van glutamaat beperken, speculeerden wij dat THC en anandamide wel eens zenuwcellen zouden kunnen beschermen tegen acute hersenschade. We hebben deze hypothese in hoofdstukken vier en vijf op de volgende manier getest.

Zeven dagen oude ratten kregen een injectie in de hersenen met een toxine, waardoor de hersencellen afstierven. Dit proces werd een week gevolgd met behulp van magnetische resonantie beeldvormende (MRI) technieken. Een groep ratten kreeg dertig minuten voor de toxine injectie een oplossing met THC of anandamide. Een andere groep ontving THC of anandamide plus een stof die de werking van de CB_1 receptor blokkeerde en een laatste groep ratten werd gebruikt als controle groep.

Na zeven dagen zagen we dat de groep ratten die THC of anandamide hadden gekregen een kleiner infarct hadden ontwikkeld dan de controle dieren. De dieren die ook behandeld waren met de blokker, vertoonden een infarct dat net zo groot (of zelfs groter) was als bij de controle dieren. Dit wijst erop dat de beschermende effecten van zowel THC als anandamide via de CB_1 receptor tot stand kwamen.

Toekomst perspectieven

De ontdekking van de endocannabinoïden en hoe zij werken in de hersenen hebben geleid tot een nieuwe manier van denken over de werking van marihuana. Het is mogelijk geworden om de medische claims die aan marihuana worden toegeschreven op een fundamenteel moleculair niveau te onderzoeken. Er wordt gedacht dat het endogene cannabis systeem een regulerende werking heeft bij verschillende fysiologische processen, zoals pijn, eetlust, geheugen, bloeddruk en beweging. Het lijkt erop dat het cannabis systeem betrokken is bij het ontstaan van verschillende ziekten, zoals lever cirrose, multiple sclerose, schizofrenie en glaucoom. Mogelijkerwijs kunnen selectieve en potente stoffen die een interactie aangaan met de eiwitten van het endogene cannabis systeem worden

gebruikt om medicijnen te ontwikkelen voor deze ziekten. Dit zal het gebruik van marihuana als medicijn overbodig maken. Hiermee worden de nadelen van marihuana, zoals haar complexe en voor een groot deel onbekende samenstelling en psychotrope bijwerking omzeild. Het ontwikkelen van stoffen die alleen de CB_2 receptor kunnen activeren, zal bijvoorbeeld kunnen leiden tot medicijnen die de patiënt niet 'high' maken, omdat de CB_2 receptor niet voorkomt in de hersenen. Onze resultaten geven aan dat moleculen die de CB_1 receptor activeren mogelijkerwijs gebruikt kunnen worden om acute hersenschade te verminderen. Stoffen die de afbraak van anandamide verhinderen spelen in dit opzicht ook een belangrijke rol, omdat zij op een indirecte wijze de CB_1 receptor kunnen activeren. Dit zal waarschijnlijk niet leiden tot een algehele activatie van het cannabis systeem en dus een verminderde beleving van het 'high' zijn.

Marihuana gedogen als medicijn?

Mario van der Stelt en Wouter Veldhuis

-Opinie artikel in het Utrechts Nieuwsblad, 20 juli 2001 –

Marihuana is het meest gebruikte illegale genotmiddel ter wereld en het middelpunt van een verhit debat tussen voor- en tegenstanders van legalisering van deze softdrug. Met enige regelmaat verschijnt in de kolommen van de krant weer een artikel over de vermeende positieve of slechte eigenschappen. Marihuana zou schadelijk zijn voor de hersenen, omdat het een delicaat evenwicht zou verstoren. Marihuana zou jongeren aanzetten tot het overstappen naar harddrugs en Nederwiet zou zelf een harddrug zijn vanwege het hoge percentage THC, de belangrijkste psychoactieve stof in marihuana. Deze claims zijn echter niet gebaseerd op wetenschappelijk onderzoek en nooit bewezen. Ze missen derhalve elke basis om als argument te dienen in de politieke besluitvorming omtrent legalisering van marihuana als softdrug.

Aan de andere kant heb je de fervente voorstanders die elke strohalm aangrijpen om hun pleidooi kracht bij te zetten voor legalisering van drugs in zijn algemeenheid en marihuana in het bijzonder. De medicinale aspecten van marihuana dienen als kruiwagen om de drug zelf te legaliseren. Het is de vraag echter of marihuana geschikt is als medicijn voor verschillende kwalen. Marihuana bestaat uit meer dan 60 verschillende THC-achtige stoffen waarvan de werking voor het merendeel onbekend is. Hoewel marihuana tegenwoordig wordt gebruikt door multiple sclerose patiënten om ongecontroleerde trillingen te onderdrukken en door aids-patiënten alsmede door mensen die een chemotherapie ondergaan om de eetlust op te wekken, is de wetenschappelijke onderbouwing om de claims te ondersteunen summier. Wetenschappelijk onderzoek met goede controles ontbreekt nog. In 1996 adviseerde de Gezondheidsraad minister Borst

van Volksgezondheid dan ook om marihuana niet als medicijn te registreren. De afwezigheid van een goede kwaliteitscontrole van een product met onbekende en variërende samenstelling maakt het onverantwoord voor artsen om het als medicijn voor te schrijven. Over afgeleide actieve componenten deed de gezondheidsraad geen uitspraak omdat daar te weinig over bekend was.

De laatste tien jaar is door wetenschappelijk onderzoek van marihuana en met name naar zijn actieve componenten zoals THC, de werking beter bekend geworden. Onderzoek naar zuivere componenten uit marihuana en afgeleide producten biedt nu de mogelijkheid om de medicinale claims te onderzoeken en eventueel afgeleide medicijnen te ontwikkelen. Uit onderzoek uitgevoerd met behulp van muizen in het Verenigd Koninkrijk blijkt dat er inderdaad een wetenschappelijk basis bestaat voor de positieve effecten van marihuana in ms-patiënten. Waarschijnlijk wordt het zelfs mogelijk om de psychotrope bijwerkingen te omzeilen en toch baat te hebben bij een behandeling met een afgeleid product. Dit geldt ook voor onderzoek uitgevoerd in Spanje waaruit bleek dat een bepaald type hersentumor bestreden kon worden door de ratten te behandelen met zuivere THC.

Ons onderzoek toont nu aan dat preventieve behandeling met THC de hersenschade beperkt in een proefdiermodel voor herseninfarcten. Dit is een eerste stap op weg naar een mogelijke toepassing als medicijn voor bepaalde neurodegeneratieve hersenziekten. De suggestie die gewekt is dat het roken van een joint bij mensen de gevolgen van een herseninfarct beperkt, ligt voor de hand, maar mag niet worden afgeleid uit ons onderzoek. Roken vergroot zelfs de kans op hart- en vaatziekten. Wel maakt onze benadering het mogelijk producten te ontwikkelen die niet het "high"-zijn veroorzaken, maar wel de medicinale werking hebben. De discussie over het behandelen van mensen door het roken van een joint zal dan gelukkig overbodig zijn en tot het verleden behoren. Tot die tijd is het onverstandig om marihuana te gedogen als medicijn. Patiënten die baat vinden bij het gebruik van marihuana zijn zelf verantwoordelijk voor de risico's. De discussie of marihuana gelegaliseerd moet worden als softdrug is een politiek vraagstuk en zal losgekoppeld moeten worden van de medicinale aspecten.

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Mario

Curriculum Vitae

Mario van der Stelt is geboren op 20 september 1975 te Werkendam. Na zijn VWO op het Altena College te Sleeuwijk is hij in 1993 scheikunde gaan studeren aan de Universiteit Utrecht. De propedeuse werd in 1994 (*cum laude*) behaald. Tijdens zijn doctoraal heeft hij stage gelopen bij het Rudolf Magnus Instituut voor Neurowetenschappen onder leiding van dr. R.A.H. Adan, dr. M. Verhage en prof.dr. W.H. Gispen. Een Erasmus-stage werd uitgevoerd bij dr. M. Maccarrone en prof. dr. A. Finazzi-Agrò aan de Universiteit van Rome "Tor Vergata" in Italië. Het hoofdvak werd gevolgd in de sectie Bio-organische Chemie bij prof. dr. J.F.G. Vliegenthart en prof. dr. G.A. Veldink. Het doctoraal diploma werd in 1998 (*cum laude*) behaald. Vanaf 1 juni 1998 was hij werkzaam als Assistent-in-Opleiding bij de sectie Bio-organische Chemie van het Bijvoet Centrum voor Biomoleculair onderzoek. Onder leiding van prof. dr. J.F.G. Vliegenthart en prof. dr. G.A. Veldink werd gewerkt aan een zelf geschreven onderzoeksvoorstel. Een deel van de resultaten zijn beschreven in dit proefschrift. De resultaten zijn ondermeer gepresenteerd op het congres van de "International Society for Magnetic Resonance in Medicine" in Denver (2000) en op symposia van de "International Cannabinoid Research Society" in Montpellier (1998), Baltimore (2000) en Madrid (2001). Op de ICRS-symposia in 2000 en 2001 werd de Coy W. Waller Student Merit Award toegekend voor de presentatie van zijn werk. Naast het onderzoek heeft hij onderwijstaken uitgevoerd. Tevens is hij sinds januari 2001 bestuurslid bij D66-Utrecht.