

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

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## Abstract

The endocannabinoid anandamide [*N*-arachidonylethanolamine; (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<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor ouabain. At 15 min after injury, AEA (10 mg/kg) administered 30 min before ouabain injection, reduced the volume of cytotoxic edema by 43 ± 15 % in a manner insensitive to the cannabinoid CB<sub>1</sub> receptor antagonist SR141716A. At seven days after ouabain treatment, 64 ± 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<sup>1,2</sup>.

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)<sup>3,4</sup>. Several reports have also revealed a connection between the endogenous lipid anandamide [*N*-arachidonylethanolamine; (AEA)] and neurodegenerative diseases<sup>5,6</sup>.

AEA mimics in part the actions of  $\Delta^9$ -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<sub>1</sub> and CB<sub>2</sub> 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)<sup>7,8</sup>.

Several lines of evidence indicate that AEA can serve to protect the brain against neuronal injury<sup>9,10</sup>: (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<sup>10-14</sup>; (ii) exogenous AEA protects cerebral neurons from *in vitro* ischemia<sup>15</sup>; (iii) CB<sub>1</sub>-mediated closing of N-, and P/Q-type calcium channels protects neurons against *in vitro* secondary excitotoxicity<sup>16,17</sup>; (iv) we have demonstrated recently that THC can reduce neuronal damage via the CB<sub>1</sub> 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<sup>18</sup>; (vi) CB<sub>1</sub> expression is enhanced in the cortical mantle zone in rats after ischemia<sup>19</sup>. 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<sup>+</sup>/K<sup>+</sup>-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<sup>52</sup>, 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<sub>2</sub> 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<sub>2</sub> (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<sub>1</sub>, FAAH) or in PBS–0.1% Tween containing 5% nonfat milk powder (CB<sub>2</sub>). 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<sub>1</sub>, Cayman Chemicals), 2 h (1: 1000, CB<sub>2</sub>, 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)<sup>53</sup>. One nmol of d<sub>8</sub>-AEA and d<sub>8</sub>-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<sup>20</sup>. 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*

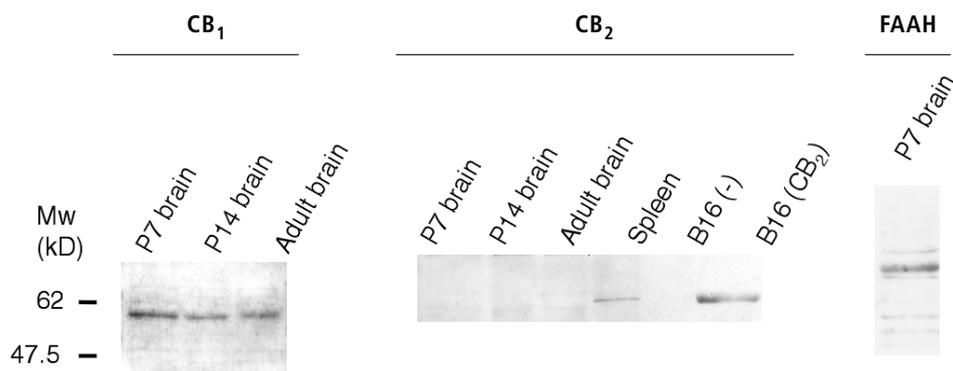
HPLC fractions were dried under a flow of nitrogen and derivatized with 15 µl *N*-methyl-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane for 2 h at room temperature,

thus yielding the trimethylsilyl derivatives of AEA and 2-AG. The two derivatized fractions were analyzed by GC/MS performed as described previously<sup>20,21</sup>. 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<sup>22</sup> were added to the amount of the 2-isomer.

## Results

The presence of CB<sub>1</sub> and CB<sub>2</sub> 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<sub>1</sub> receptor in 7- and 14-day-old rat brain, whereas the CB<sub>2</sub> receptor could not be detected (Fig. 1). FAAH was also detected in 7-day-old rats (Fig. 1). Neonatal rat brains (P7) contained  $32.5 \pm 6.5$

Western blot of CB<sub>1</sub> and CB<sub>2</sub> cannabinoid receptors and FAAH in neonatal rat (P7 and 14) and adult brain. Figure 1



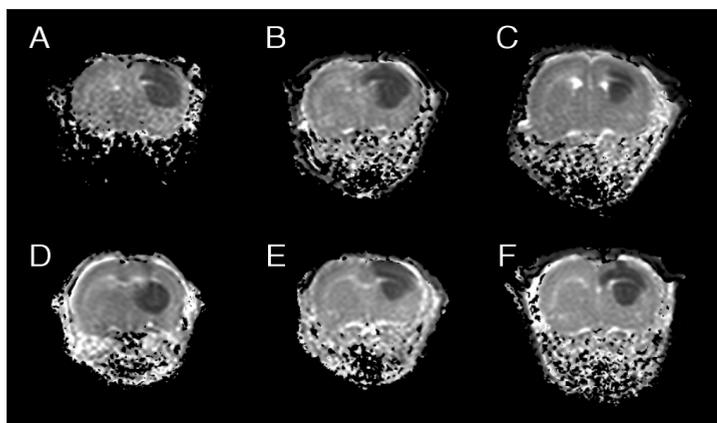
The CB<sub>2</sub> receptor was absent in rat brain, but was detected in spleen and CB<sub>2</sub>-transfected cell line.

pmol/g AEA and  $1.17 \pm 0.22$  nmol/g 2-AG, which is in the same order of magnitude as reported previously<sup>23</sup>.

In our model, excitotoxicity was triggered by the unilateral intrastratial injection of 0.5  $\mu$ l ouabain (1 mM) in 7- to 8-day-old rats. Ouabain, a cardiac glycoside, inhibits  $\text{Na}^+/\text{K}^+$ -ATPases and induces cellular swelling, eventually leading to pancellular necrosis and infarction<sup>24-28</sup>. 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 ( $\sim 0.65 \pm 10^{-3} \text{ mm}^2\text{s}^{-1}$ ) in the ipsilateral hemisphere in all animals (Fig. 2). Normal ADC values ( $\sim 1.11 \pm 10^{-3} \text{ mm}^2\text{s}^{-1}$ ) 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  $\mu$ l 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<sup>29,30</sup>. 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

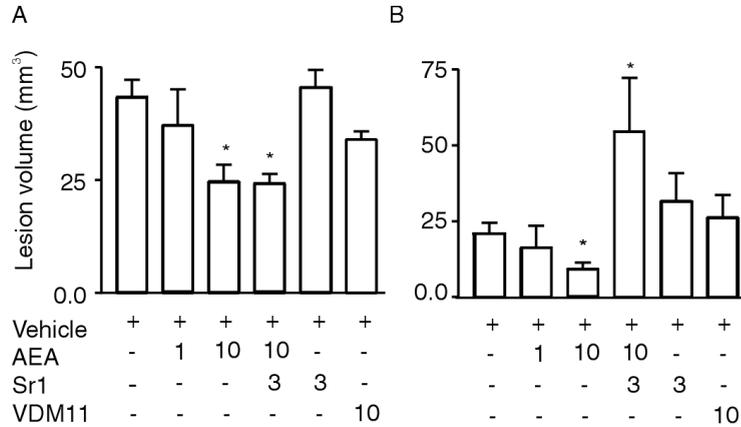
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**Figure 2** Coronal ADC maps of neonatal rat brains 15 min after ouabain injection: effect of AEA-pretreatment.



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).

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Mean lesion volumes ( $\pm$  SE) of ouabain-injected rats: effect of ligands of the cannabinoid system. Figure 3

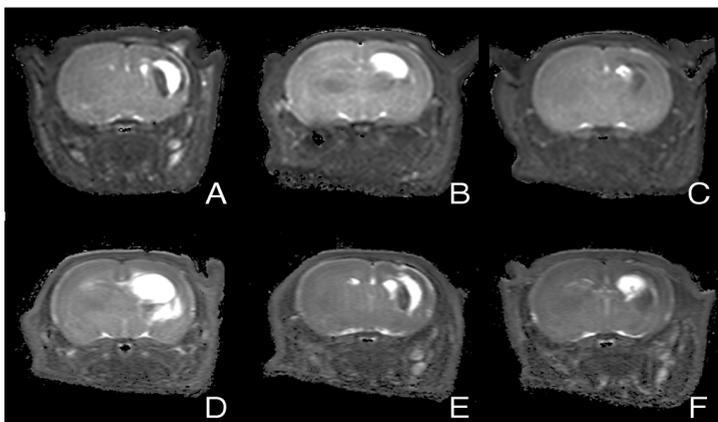
Mean lesion volumes ( $\pm$  SE) of ouabain-injected rats on day 0 (A) and 7 (B) based on ADC and  $T_2$  maps (hyperintensities), respectively. Asterisk denotes a  $p < 0.05$  compared to vehicle-treated rats. The numbers at the bottom indicate the dose of compound (mg/kg). See Materials and Methods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

cortex and striatum. Coinjection of the CB<sub>1</sub> 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)<sup>29,31</sup>. 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  $64 \pm 24\%$  ( $p < 0.05$ ) 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<sub>1</sub> antagonist ( $p < 0.05$ ) (Fig. 3B and 4). The infarct was  $\sim 2.5$  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.



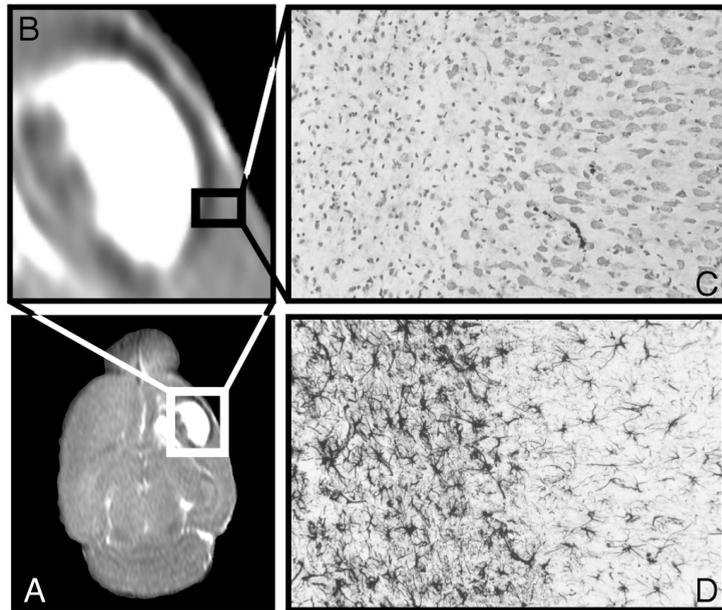
Hyperintensities correlate to vasogenic edema, tissue loss, and ventricle dilatation. Treatment: A) vehicle, B) AEA (1 mg/kg), C) AEA (10 mg/kg), D) AEA + SR141716A (10 and 3 mg/kg, respectively), E) SR141716A (3 mg/kg) and F) VDM11 (10 mg/kg).

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<sup>32</sup>.

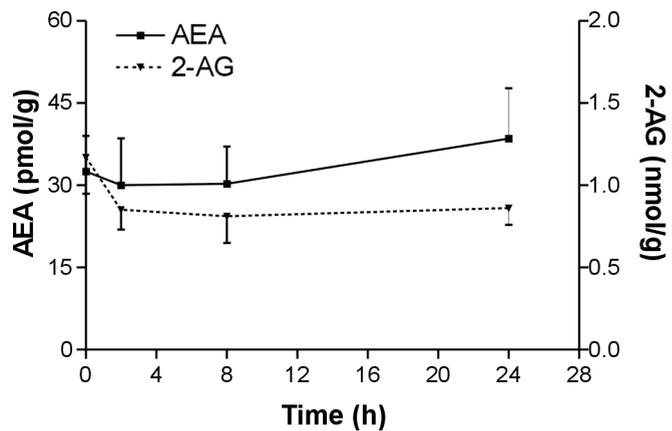
Finally, rat brain endocannabinoid levels were measured 2, 8 and 24 h after ouabain or vehicle injection. No rises in concentrations of AEA and 2-AG were observed after inducing acute neuronal damage in the ipsilateral hemisphere (Fig. 6). There were no significant differences in endocannabinoid levels 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



## Discussion and conclusions

The (patho)physiological role of the endogenous cannabinoid system is beginning to be unraveled<sup>33,34</sup>. It has been postulated that the endocannabinoid system may serve to establish a defense system for the brain during neurotoxicity and ischemia<sup>10,19</sup>; 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<sup>18,35</sup> (C.J. Hillard, personal communication). Because the cannabinoid system has complex (cerebro)vascular effects<sup>36,37</sup>, 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<sup>+</sup>/K<sup>+</sup>-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<sup>24–28</sup>.

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<sub>1</sub>-mediated manner in the same model (see chapter 4). The AEA-induced reduction in cellular swelling was not attenuated by the CB<sub>1</sub>-antagonist SR141716A. Because there are no CB<sub>2</sub> receptors detected in the brain, the early *in vivo* neuroprotective action of AEA does not seem to be mediated via the CB<sub>1</sub> or CB<sub>2</sub> 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<sub>1</sub>-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<sup>38</sup>. Moreover, some of the behavioral actions of AEA in mouse were still observed in the CB<sub>1</sub> knock-out mice<sup>38,39</sup>. Others<sup>15</sup> have shown that neuroprotection of AEA in *in vitro* experiments was also independent of CB<sub>1</sub> and CB<sub>2</sub> 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<sup>+</sup>-channels, TASK-1 channels, a non-CB<sub>1</sub> G-protein coupled AEA receptor in astrocytes and a non-CB<sub>1</sub> non-CB<sub>2</sub> G-protein coupled receptor for AEA and WIN55,212-2 in mouse brain<sup>40,41</sup>, 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<sub>1</sub> G-protein-coupled AEA-receptor<sup>42</sup> or the inhibition of L-type calcium channels<sup>43</sup> 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<sub>2</sub>-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<sub>1</sub> 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<sup>2,44</sup>. We have suggested previously that THC protected rat brains in the late phase via the CB<sub>1</sub>-mediated closing of N-, and P/Q-type calcium channels and inhibition of glutamatergic transmission in the same model<sup>(45; see chapter 4)</sup>. It is reasonable to assume that this CB<sub>1</sub>-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.<sup>46</sup> This enhancement could only be observed when CB<sub>1</sub> 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<sub>1</sub> receptors by constitutive levels of AEA, 2-AG or any other novel CB<sub>1</sub> ligand, such as noladin ether<sup>47</sup>, 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<sub>1</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sup>14,48</sup>.

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.<sup>14</sup> 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.<sup>49</sup> 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<sup>49-51</sup>.

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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