

Exogenous Anandamide Protects Rat Brain against Acute Neuronal Injury *In Vivo*

M. van der Stelt,¹ W. B. Veldhuis,^{2,3} G. W. van Haaften,¹ F. Fezza,⁴ T. Bisogno,⁴ P. R. Bär,³ G. A. Veldink,¹ J. F. G. Vliegthart,¹ V. Di Marzo,⁴ and K. Nicolay²

¹Department of Bio-organic Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, 3584 CH Utrecht, The Netherlands, ²Department of Experimental *In Vivo* Nuclear Magnetic Resonance, Image Sciences Institute, Utrecht University Medical Center, 3584 CJ Utrecht, The Netherlands, ³Department of Experimental Neurology, Utrecht University Medical Center, 3584 CX Utrecht, The Netherlands, and ⁴Endocannabinoid Research Group, Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, 80078 Pozzuoli, Naples, Italy

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 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) administered 30 min before ouabain injection reduced the volume of cytotoxic edema by 43 ± 15% in a manner insensitive to the cannabinoid CB₁ receptor antagonist SR141716A. At 7 d 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 neuroprotec-

tive actions of AEA at this end point. In addition, (1) no increase in AEA and 2-arachidonoylglycerol levels was detected at 2, 8, or 24 hr after ouabain injection; (2) application of SR141716A alone did not increase the lesion volume at days 0 and 7; and (3) the AEA-uptake inhibitor, VDM11, did not affect the lesion volume. These data indicate that there was no endogenous endocannabinoid tone controlling the 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.

Key words: anandamide; 2-AG; astrogliosis; cannabinoid; excitotoxicity; ischemia; magnetic resonance imaging; neonatal rat; neuronal injury; neuroprotection; ouabain

The CNS 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 (Dirnagl et al., 1999; Doble, 1999).

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) (Reiter, 1998; Hurn and Macrae, 2000; Picano and Abbracchio, 2000). Several reports have also revealed a connection between the endogenous lipid anandamide

[*N*-arachidonylethanolamine (AEA)] and neurodegenerative diseases (H. S. Hansen et al., 1998, 2000; Di Marzo et al., 2000b; Baker et al., 2001).

AEA mimics in part the actions of Δ⁹-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 CB₁ and CB₂ 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) (Pertwee, 1997; Di Marzo et al., 1998).

Several lines of evidence indicate that AEA can serve to protect the brain against neuronal injury (H. S. Hansen et al., 1998, 2000): (1) 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 (Schmid et al., 1990; Kempe et al. 1996; Hansen et al., 1999; H. H. Hansen et al., 2000, 2001; Sugiura et al., 2000); (2) exogenous AEA protects cerebral neurons from *in vitro* ischemia (Sinor et al., 2000); (3) CB₁-mediated closing of N- and P/Q-type calcium channels protects neurons against *in vitro* secondary excitotoxicity (Shen et al., 1996; Shen and Thayer, 1998); (4) we have demonstrated recently that THC can reduce neuronal damage via the CB₁ receptor in an *in vivo* model of excitotoxicity (Van der Stelt et al., 2001); (5) WIN55.212, a synthetic cannabinoid, protected rat brains against focal and global ischemia (Nagayama et al., 1999); and (6) CB₁

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M.v.d.S. and W.B.V. contributed equally to the work.

Correspondence should be addressed to G. A. Veldink, Department of Bio-organic Chemistry, Bijvoet Center for Biomolecular Research, Padualaan 8, Utrecht University, 3584 CH Utrecht, The Netherlands. E-mail: veldink@accu.uu.nl; or V. Di Marzo, Endocannabinoid Research Group, Istituto per la Chimica di Molecole di Interesse Biologico, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, Ex Comprensorio Olivetti, Fabbricato 70, 80078 Pozzuoli, Napoli, Italy. E-mail: vdimarzo@icmib.na.cnr.it.

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expression is enhanced in the cortical mantle zone in rats after ischemia (Jin et al., 2000). As yet, *in vivo* neuroprotection by AEA has never been reported.

To date no effective drugs are available to treat brain injury after 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.

MATERIALS AND METHODS

Animal model. The neonatal rat brain was used to test the neuroprotective effects of endocannabinoids because it has been widely studied in various models of cerebral ischemia and excitotoxicity (Vannucci, 1990; van Lookeren Campagne et al., 1994; Dijkhuizen et al., 1996; Renolleau et al., 1998; Hansen et al., 2001). Neonatal Wistar rats (U:Wu/Cpb; 7–8 d of age) 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 to bregma. A 1 μl syringe was lowered into the left striatum to a depth of 4.0 mm (Dijkhuizen et al., 1996). Ouabain (0.5 μl ; 1 mM, $n = 6$; Sigma Aldrich, Zwijndrecht, The Netherlands) or vehicle (0.5 μl , 40 mM Tris-HCl buffer, pH 7.4; $n = 8$) was injected at a rate of 0.125 $\mu\text{l}/\text{min}$ using a microdrive. After injection, the needle was left *in situ* for 2 min to avoid leakage of injection fluid from the needle tract. Body temperature was maintained at 37°C using a water-filled heating pad and an infrared heating lamp to compensate for AEA-induced hypothermia. Animals needed for MRI study were then positioned in the magnet and anesthesia was continued using a mixture of halothane (0.4–1%) in $\text{N}_2\text{O}/\text{O}_2$.

Pharmacological treatments. Animals used for the MRI study were treated intraperitoneally with anandamide (1 or 10 mg/kg; $n = 5$ and 6, respectively; Biomol, Heerhugowaard, The Netherlands), anandamide plus 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 (De Petrocellis et al., 2000), or vehicle alone ($n = 12$) (all drugs in 1 ml/kg body weight 18:1:1 v/v PBS/Tween 80/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. MRI was performed on a 4.7 T Varian horizontal bore spectrometer. Excitation and signal detection were accomplished by means of a Helmholtz volume coil (9 cm) and an inductively coupled surface coil (2 cm), respectively. A single-scan diffusion-trace MRI sequence [four b values = 100–1300 sec/mm^2 ; repetition time (TR) = 3 sec; echo time (TE) = 100 msec] was used to generate quantified images of the tissue water trace apparent diffusion coefficient (ADC). Diffusion-trace and T_2 -weighted imaging (TE = 18, 40, 62, and 84 msec; TR = 2 sec; number of transients = 2) were performed in all animals (2.2×2.2 cm field of view, 64×64 datamatrix), starting at $t = 15$ min after injection on day 0; both types of imaging were repeated 1 week later. As expected, no changes in T_2 -weighted MRI were detected at this early time point. Both the T_2 -weighted and the diffusion-weighted datasets consisted of seven consecutive, 1.5-mm-thick slices with a 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 neighboring 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, as described recently (de Graaf et al., 2001). The combination of gradient directions leads to cancellation of all off-diagonal tensor elements, thus 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 exper-

iments. 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 min.

Data analysis. ADC and T_2 maps were generated by monoexponential fitting using the Interactive Data Language software package (Research Systems Inc., Boulder, CO). Parametric images were analyzed in anatomic regions of interest using the same software package. Pixels in the ipsilateral hemisphere were considered pathological if their ADC value or T_2 value differed more than twice the SD of 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 (equaling the number of pathological pixels \times field-of-view in $\text{cm}^2/\text{number of points acquired per image}$) by the slice thickness. The total lesion volume was obtained by summation of the lesion volumes for all slices. The absence of a slice gap makes interpolation of lesion areas between slices unnecessary, reducing systematic errors to within-slice "averaging" of signal intensity. Statistical analysis was performed using SPSS 9.0 (SPSS Inc., Chicago, IL). 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 hr followed by 25% sucrose in PBS for 72 hr, and quickly frozen in liquid nitrogen-cooled isopentane. Coronal sections (10 μm) were cut and stained for glial fibrillary acidic protein (GFAP), for Nissl substance, or with hematoxylin-eosin using standard procedures. The position of the histological slices was matched to the position of the MRI images by known position relative to bregma, after which a gross correlation was done.

Protein analysis. Mouse melanoma cells (B16-G4F) were transiently transfected with 6–10 μg of recombinant cDNA (pcDNA3 vector) encoding the CB₂ receptor (a kind gift from Dr. R. Delwel, Institute of Hematology, Erasmus University, Rotterdam, The Netherlands). Rat tissue (brain and spleen) and cells were homogenized and sonicated for 10 sec three times in buffer containing (in mM): 50 Tris, 1 EDTA, and 3 MgCl_2 , pH 7.4. Membrane fractions were prepared by centrifugation at $5000 \times g$ for 5 min. Samples were stored at -20°C until further use.

Samples containing 30 μg 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 (CB₁, FAAH) or in PBS–0.1% Tween containing 5% nonfat milk powder (CB₂). Membranes were rinsed twice and washed once for 15 min and four times for 5 min each with 20 ml of PBS–0.1% T. The membranes were incubated with polyclonal primary antibodies for 3 hr (1:1000, CB₁; Cayman Chemicals, Ann Arbor, MI), 2 hr (1:1000, CB₂; Cayman Chemicals) and 1.5 hr (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; Bio-Rad, Richmond, CA) was used as a secondary antibody (1 hr incubation). Membranes were washed and developed with Western-blotting detection reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's manual.

Lipid extraction. Neonatal rats [postnatal day 7 (P7) and P8] injected intracerebrally with ouabain or vehicle were killed by decapitation at 0, 2, 8, and 24 hr after induction of excitotoxicity ($n = 6$ for each time point). The ipsilateral and contralateral hemisphere were rapidly removed and separately homogenized in 5 ml of ice-cold Tris buffer (50 mM, pH 7.4). Lipids were extracted according to the method of Bligh and Dyer (1959). One nanomole of d_8 -AEA and 1 nmol of 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 (Bisogno et al., 1997). Mono-AGs and AEA standards were eluted after 18–23 and 27–28 min, respectively. The mono-AG 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 of N -methyl- N -trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane for 2 hr 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 (Bisogno et al., 1997, 1999). The derivatives of both deuterated and nondeuterated AEA, 2-AG, and 1-(3)AG standards were

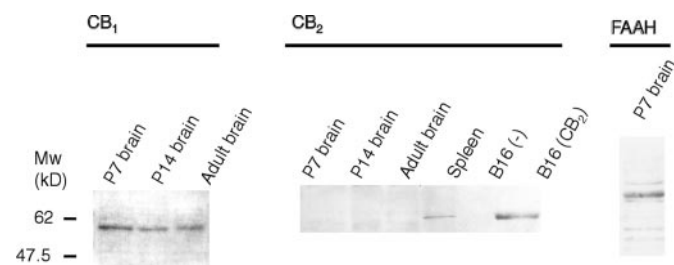


Figure 1. Western blot of CB₁ and CB₂ cannabinoid receptors and FAAH in the neonatal rat (P7 and P14) and adult brain. CB₂ receptor was absent in the rat brain but was detected in the spleen and in the CB₂-transfected cell line.

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 a mass to charge ratio (m/z) of 427 and 419 corresponding to the molecular ions for d_8 -AEA and nondeuterated AEA, and at a m/z of 412 and 404, corresponding to the loss of a methyl group in both compounds. Selected ions for 2-AG were at a m/z of 530 and 522, corresponding to the molecular ions of d_8 -2-AG and nondeuterated 2-AG, and at a m/z of 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 a m/z of 404 and 412 and at a m/z of 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 (Stella et al., 1997), was added to the amount of the 2-isomer.

RESULTS

The presence of CB₁ and CB₂ receptors and FAAH in the 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 CB₁ receptor in 7- and 14-d-old rat brains, whereas the CB₂ receptor could not be detected (Fig. 1). FAAH was also detected in 7-d-old rats (Fig. 1). Neonatal rat brains (P7) contained 32.5 ± 6.5 pmol/gm AEA and 1.17 ± 0.22 nmol/gm 2-AG, which is in the same order of magnitude as reported previously (Sugiura et al., 1995; Kondo et al., 1998; Berrendero et al., 1999; Bisogno et al., 1999).

In our model, excitotoxicity was triggered by the unilateral intrastriatal injection of 0.5 μ l of ouabain (1 mM) in 7- to 8-d-old rats. Ouabain, a cardiac glycoside, inhibits Na^+/K^+ -ATPases and induces cellular swelling, eventually leading to pancellular necrosis and infarction (Lees et al., 1990; Lees, 1991; Lees and Leong, 1994, 1995; Stelmashook et al., 1999). The acute cellular swelling is conveniently monitored by diffusion-weighted MRI. ADC maps of brain tissue water, calculated from diffusion-weighted MRIs acquired 15 min after ouabain injection, showed hypointensity regions with reduced ADC values ($\sim 0.65 \times 10^{-3}$ mm²/sec⁻¹) in the ipsilateral hemisphere in all animals (Fig. 2). Normal ADC values ($\sim 1.11 \times 10^{-3}$ mm²/sec⁻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 μ l of Tris-HCl, 40 mM, pH 7.4). The reduction in ADC values in the ipsilateral hemisphere after ouabain injection is thought to reflect neuronal swelling (i.e., cytotoxic edema) because of a relocation of part of the extracellular water into depolarized cells (van Lookeren Campagne et al., 1994; Dijkhuizen et al., 1996). The same brain regions, including the caudate putamen, cortex, and hippocampus, were affected in all animals (Fig. 2). In this

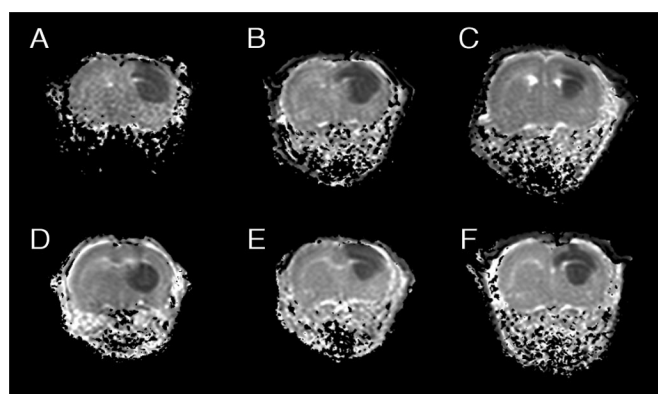


Figure 2. Coronal ADC maps of neonatal rat brains 15 min after ouabain injection. Hypointensities correlate to cytotoxic edema. Treatments: *A*, Vehicle; *B*, AEA (1 mg/kg); *C*, AEA (10 mg/kg); *D*, AEA plus SR141716A (10 and 3 mg/kg, respectively); *E*, SR141716A (3 mg/kg); *F*, VDM11 (10 mg/kg).

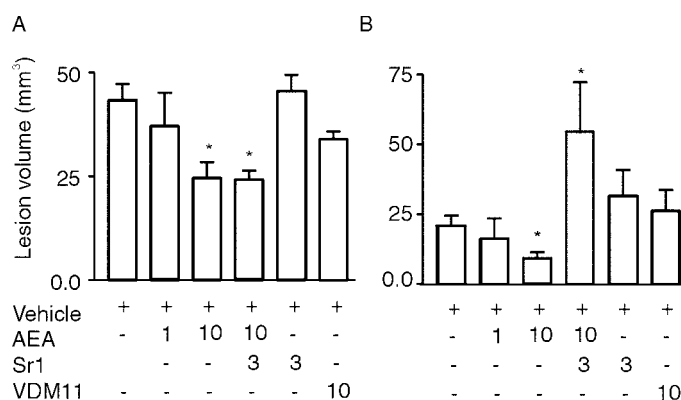


Figure 3. Mean lesion volumes (\pm SE) of ouabain-injected rats on day 0 (*A*) and day 7 (*B*) based on ADC and T₂ maps (hyperintensities), respectively. An asterisk denotes a p value of <0.05 compared with vehicle-treated rats. The numbers at the bottom indicate the dose of compound in milligrams per kilogram. See Materials and Methods for lesion volume calculation.

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 (Figs. 2 and 3*A*). This effect was observed at the borders of the affected tissue, namely the cortex and striatum. Coinjection of the CB₁ receptor antagonist SR141716A with AEA did not reverse AEA action (Figs. 2 and 3*A*). Application of SR141716A or VDM11 (an endocannabinoid uptake inhibitor) alone did not change lesion volume at day 0 compared with vehicle-treated animals (Figs. 2 and 3*A*).

After 7 d, the effect of AEA treatment on neuronal damage was assessed using T₂-weighted imaging and verified by standard histology. Normal T₂ values ($T_2 = 73 \pm 1$ msec) were observed in contralateral hemispheres and in the brains of control animals (Fig. 4). The T₂ maps of ouabain-injected animals demonstrated both hyperintensities and hypointensities (Fig. 4). Both types of T₂ 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) (Feuerstein et al., 1994; van Lookeren Campagne et al., 1994). Infarct size based on

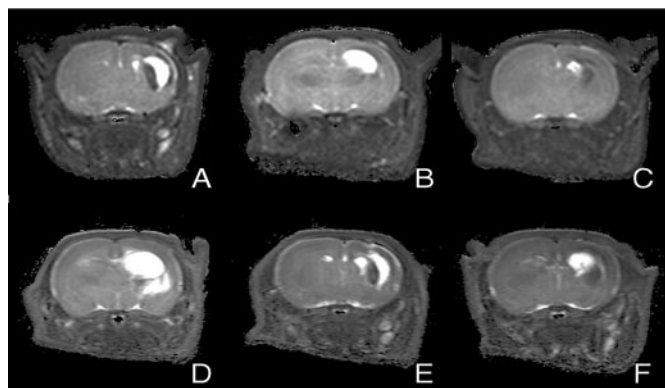


Figure 4. Coronal T_2 maps of neonatal rat brains 7 d after ouabain injection. Hyperintensities correlate to vasogenic edema, tissue loss, and ventricle dilatation. Treatment: *A*, Vehicle; *B*, AEA (1 mg/kg); *C*, AEA (10 mg/kg); *D*, AEA plus SR141716A (10 and 3 mg/kg, respectively); *E*, SR141716A (3 mg/kg); *F*, VDM11 (10 mg/kg).

T_2 hyperintense abnormalities were dose-dependently reduced in the AEA-treated group compared with the control animals (Figs. 3*B* and 4). The infarct volume was $64 \pm 24\%$ ($p < 0.05$) at 10 mg/kg AEA, smaller 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 ($p < 0.05$) (Figs. 3*B* and 4). The infarct was ~ 2.5 -fold larger than that in vehicle-treated animals ($p < 0.05$) and primarily involved the hippocampus 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. 5*A–D*) (Van der Stelt et al., 2001). No indications for hemorrhage were found. Astrogliotic tissue constituted $\sim 44\%$ of the lesion on T_2 maps of nontreated animals and usually surrounded the edematous tissue and the dilated ventricles (Fig. 5*B*). The volume of astrogliotic tissue in AEA-treated rats was not affected compared with 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 (Van der Stelt et al., 2001) (our unpublished results). This reinforces the notion that classical cannabinoids also have other modes of action in addition to their interaction with the CB_1 and CB_2 receptors (Burstein, 1999).

Finally, rat brain endocannabinoid levels were measured 2, 8, and 24 hr 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).

DISCUSSION

The (patho)physiological role of the endogenous cannabinoid system is beginning to be unraveled (Giuffrida et al., 1999; Galve-Roperh et al., 2000; Di Marzo et al., 2001). It has been postulated that the endocannabinoid system may serve to establish a defense system for the brain during neurotoxicity and

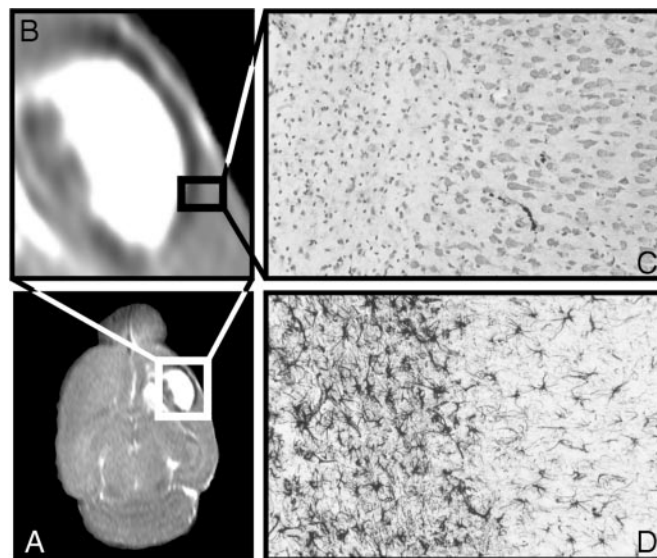


Figure 5. Transversal T_2 map (*A*) of an ouabain-injected animal showing that vasogenic edema is surrounded by astrogliosis (hypointense area) (*B*). Nissl-staining (*C*) and GFAP-staining (*D*) of a brain section of an ouabain-injected rat demonstrate a sharp line between affected and healthy tissue.

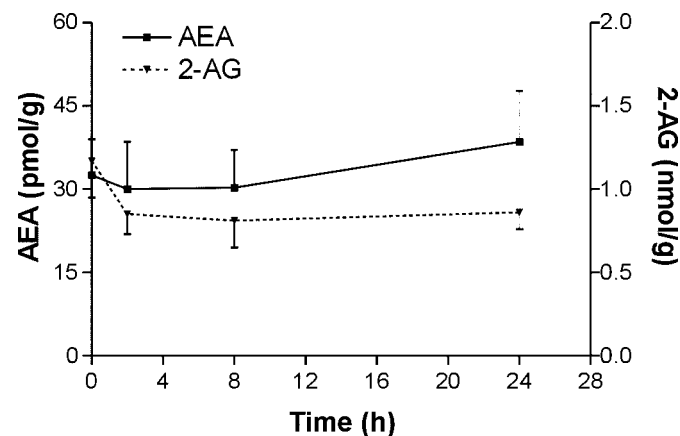


Figure 6. Endocannabinoid levels in a rat brain 2, 8, and 24 hr after ouabain injection.

ischemia (H. S. Hansen et al., 2000; Jin et al., 2000; Van der Stelt et al., 2001). 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 (Nagayama et al., 1999; Louw et al., 2000; C. J. Hillard, personal communication). Because the cannabinoid system has complex (cerebro)vascular effects (Randall and Kendall, 1998; Kunos et al., 2000), 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 and induces cellular swelling and glutamate-dependent damage of cells, which can be prevented in part by blockade of the NMDA receptor (Lees et al.,

1990; Cousin et al., 1995; Greene and Greenamyre, 1996; Basar-sky et al., 1999).

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 CB₁-mediated manner in the same model (Van der Stelt et al., 2001). The reduction in cellular swelling was not attenuated by the CB₁ antagonist SR141716A. Because there are no CB₂ receptors detected in the brain, the early *in vivo* neuroprotective action of AEA does not seem to be mediated via the CB₁ or CB₂ receptors.

The failure of SR141716A to block a 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 d) of AEA (10 mg/kg). In addition, 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 (Van der Stelt et al., 2001). It is noteworthy that THC is a more potent agonist of the CB₁ 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 (Adams et al., 1998). Moreover, some of the behavioral actions of AEA in mice were still observed in the CB₁ knock-out mice (Di Marzo et al., 2000a). Others (Sinor et al., 2000) have shown that neuroprotection of AEA in *in vitro* experiments was also independent of CB₁ and CB₂ receptors.

Recent data demonstrate that AEA is capable of interacting with other molecular targets, such as 5-hydroxytryptamine receptors, NMDA receptors, vanilloid receptors, L-type calcium channels, Shaker-related K⁺ channels, TASK-1 channels, a non-CB₁ G-protein-coupled AEA receptor in astrocytes, and a non-CB₁, non-CB₂ G-protein-coupled receptor for AEA and WIN55.212-2 in the mouse brain (Howlett and Mukhopadhyay, 2000; Breivogel et al., 2001), 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-CB₁ G-protein-coupled AEA receptor (Venance et al., 1995) or the inhibition of L-type calcium channels (Johnson et al., 1993) 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.

T₂-weighted MRI data recorded 1 week after ouabain injection showed that exogenous AEA reduced neuronal damage by 64 ± 24% ($p < 0.05$). Compared with the early phase, AEA-induced neuroprotection was blocked by the CB₁ receptor antagonist after 7 d. 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 (Choi, 1987; Doble, 1999). We have suggested previously that THC protected rat brains in the late phase via the CB₁-mediated closing of N- and P/Q-type calcium channels and inhibition of glutamatergic transmission in the same model (Huang et al., 2001; Van der Stelt et al., 2001). It is reasonable to assume that this CB₁-mediated process of closing voltage-sensitive calcium channels also contributes to the observed neuroprotection of AEA after 7 d. As noted before for THC, AEA-induced neuroprotection was observed in

brain regions such as the cortex, striatum, and hippocampus (Van der Stelt et al., 2001).

Strikingly, the combination of AEA and the antagonist produced an infarct that was 2.5 times larger than that seen for the control group. This observation is not likely to be explained by (1) blockade of the effect of endogenously released cannabinoids, (2) inverse agonism of SR141716A, or (3) toxic effects of SR141716A. In fact, treatment of rats with SR141716A at 1 and 3 mg/kg or with SR141716A plus THC (1 mg/kg) did not increase infarct size significantly (Van der Stelt et al., 2001). In addition, endocannabinoid levels do not appear to increase after ouabain injection.

Hampson et al. (1998) have shown that AEA can enhance calcium influx presumably via direct activation of NMDA receptors. This enhancement could only be observed when CB₁ 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 were similar to those observed in animals that received a unilateral intrastriatal injection with NMDA (our 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: (1) No increases in AEA and 2-AG levels were detected at 2, 8, or 24 hr after ouabain injection. (2) Application of SR141716A alone (3 mg/kg) did not increase the lesion volume at day 0 or at day 7. This implies that activation of CB₁ receptors by constitutive levels of AEA, 2-AG, or any other novel CB₁ ligand, such as noladin ether (Hanus et al., 2001), does not tonically protect the brain. (3) The AEA uptake inhibitor VDM11 did not reduce neuronal swelling at day 0, nor did it reduce the infarct volume after 7 d. This also argues against a CB₁ 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, we cannot exclude the possibility that technical issues have prevented the detection of a tonic protection by endocannabinoids. The lesion was considered pathological when ADC or T₂ values differed more than twice the SD of the mean value in the contralateral hemisphere. Thus, the periphery of the infarct with smaller changes in ADC or T₂ 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 upregulation 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 (Sugiura et al., 2000; Hansen et al., 2001).

In addition, Hansen et al. (2001) have demonstrated that the increase in *N*-acylphosphatidylethanolamines varies in different *in vivo* models of neuronal damage and is dependent on the type of cell death. 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. 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 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 (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001).

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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