

Research Article

Endocannabinoids and β -amyloid-induced neurotoxicity *in vivo*: effect of pharmacological elevation of endocannabinoid levels

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Received 26 January 2006; received after revision 24 March 2006; accepted 12 April 2006

Online First 29 May 2006

Abstract. We investigated the involvement of endocannabinoids in the control of neuronal damage and memory retention loss in rodents treated with the β -amyloid peptide (1–42) (BAP). Twelve days after stereotaxic injection of BAP into the rat cortex, and concomitant with the appearance in the hippocampus of markers of neuronal damage, 2-arachidonoyl glycerol, but not anandamide, levels were enhanced in the hippocampus. VDM-11 (5 mg/kg, i.p.), an inhibitor of endocannabinoid cellular reuptake, significantly enhanced rat hippocampal and mouse brain

endocannabinoid levels when administered sub-chronically starting either 3 or 7 days after BAP injection and until the 12–14th day. VDM-11 concomitantly reversed hippocampal damage in rats, and loss of memory retention in the passive avoidance test in mice, but only when administered from the 3rd day after BAP injection. We suggest that early, as opposed to late, pharmacological enhancement of brain endocannabinoid levels might protect against β -amyloid neurotoxicity and its consequences.

Keywords. Anandamide, 2-arachidonoyl glycerol, cannabinoid, memory, receptor, neuroprotection, apoptosis.

Endocannabinoids are, by definition, endogenous agonists of the G-protein-coupled receptors mediating the pharmacological actions of *Cannabis* and its major component Δ^9 -tetrahydrocannabinol [1]. Of these endogenous compounds, the two best studied representatives are anandamide and 2-arachidonoyl glycerol (2-AG) [2–4]. Among several central properties of endocannabinoids [5], particular attention has been devoted recently to their possible neuroprotective actions *in vitro* [6], as well as

in animal models of acute neuronal damage, such as cerebral ischemia [7], acute brain injury [8], excitotoxicity [9–12] and epilepsy [13]. However, the neuroprotective effects of endocannabinoids, which can be due to interference with several cellular and molecular mechanisms, including apoptosis and inflammation [14, 15], are often not due to activation of the two known types of cannabinoid receptor, the CB₁ and CB₂ receptors [16]. On the other hand, another molecular target for anandamide, the transient receptor potential of vanilloid type 1 (TRPV1) receptor [17], has also been recently implicated in neuroprotection [11, 18].

Protection from chronic neuronal damage by endocannabinoids has been hypothesized during neurodegen-

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erative disorders, again through cannabinoid and non-cannabinoid receptor-mediated pathways, and via neuroadaptive, direct neuroprotective or anti-inflammatory actions [19, 20]. In particular, recent data, obtained in the postmortem human brain, have shown that the pattern of cannabinoid receptor expression is altered during Alzheimer's disease (AD) [21], possibly to play a protective action, as suggested by studies carried out *in vitro* and *in vivo* with synthetic and endogenous cannabinoids and using β -amyloid neurotoxicity and its consequences as a model [22, 23]. However, possible alterations in the brain levels of cannabinoid receptor ligands during β -amyloid neurotoxicity have not been measured to date.

Endocannabinoids are also known to affect memory as well as neurochemical substrates of memory acquisition and consolidation such as long-term potentiation [5, 24, 25]. In particular, acute activation of cannabinoid receptors can cause deficits in memory retention in passive avoidance and maze tests carried out in rodents [26–28], whereas the CB₁ receptor antagonist SR141716A improves spatial memory consolidation in rats [29, 30], thus suggesting that endocannabinoids may tonically control mnemonic functions. Interestingly, the effects of cannabinoids on memory undergo tolerance after chronic activation of CB₁ receptors [31]. Finally, a recent study suggested that endocannabinoids acting at CB₁ receptors may be in part responsible for the amnesic effects caused by β -amyloid peptide (BAP) fragments, since these effects could be attenuated by SR141716A [32].

In view of the neuroprotective and memory disruptive actions of endocannabinoids, it is important to measure the brain levels of these compounds in experimental models of β -amyloid neurotoxicity, in which rat or mouse brain is injected with BAP fragments [33, 34]. This model is characterized by progressive β -amyloid plaque deposition, extensive hippocampal neuronal damage, and subsequent loss of retention of newly acquired memory, as also occur during AD in humans [35]. More importantly, using this model, a protective effect of exogenous cannabinoids was very recently reported [23]. Interestingly, not only endocannabinoids [22], but also the non-psychotropic cannabinoid, cannabidiol, which was previously found to inhibit endocannabinoid inactivation, thus potentially leading to an enhancement of endocannabinoid levels [36], was recently shown to reduce cell toxicity induced by BAP fragments [37, 38]. Therefore, we evaluated here the effects of the pharmacological elevation of endocannabinoid levels *in vivo*, obtained by using selective inhibitors of endocannabinoid inactivation, on biochemical markers of neuronal damage and on the performance in a passive avoidance test in BAP-injected rodents.

Materials and methods

Animals. Adult male Wistar rats (300–350 g), aged from 15 to 18 weeks, were obtained from Harlan-Nossan (Italy). Seven-week-old male Swiss mice (40–50 g) were obtained from Charles River (Milan, Italy). After arrival in the facilities, rats were housed three per box and mice eight per box and maintained at a constant temperature on a 12-h light-dark cycle (lights on between 08:00 and 20:00) with food and water *ad libitum*. After at least 1 week of habituation in the facilities, animals were admitted to the experimental procedures. All experiments were carried out according to the European Community Council Directive 86/609/EEC and efforts were made to minimize animal suffering and to reduce the number of animals used. Three to four rats were used for each data point and for each type of experiment (i.e. histological analyses, DNA fragmentation analyses, Western blots, and measurement of endocannabinoid levels).

Drug administration to rats. β -Amyloid 1–42 fragment (BAP; Tocris, Bristol, UK) was prepared as stock solutions in sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4) and aliquoted (10 μ l per vial), frozen on dry ice, and stored at -90°C until use. The peptide was still perfectly soluble after defrosting the aliquots, and 3 μ l of freshly prepared BAP solution (10 ng/ μ l) was used for each injection. Sterile 0.1 M phosphate-buffered saline was injected into control animals. The animals were anesthetized with sodium pentobarbital (60 mg/kg) and supplemented throughout the surgery as required. They were placed in a stereotaxic frame and BAP or vehicle was injected in the deep frontal cortex (3.2 mm AP, 2 mm DV relative to bregma; depth 3 mm) according to Kowall et al. [39], who, however, used a different BAP fragment. Injections were made over 1 min using a 10- μ l Hamilton syringe fitted with a 30-gauge blunt-tipped needle, and the needle was left in place for an additional 2 min before it was slowly retracted.

In experiments with the endocannabinoid reuptake inhibitor VDM11 [synthesized in our laboratory as described in ref. 40], the compound was dissolved in DMSO/H₂O (95:100 volume ratio, 5 mg/0.1 ml) and injected intraperitoneally (5 mg/kg) every other day, starting from the 3rd or the 7th day from the operation until the 12th day when rats were sacrificed.

Histological analyses of rat brains. After killing, the brains were removed and rapidly frozen in liquid nitrogen and stored at -80°C .

Coronal sections of the brains (20 μ m thickness) were cut using a cryostat microtome (Model OTF/AS; Bright Instrument Huntingdon, UK) from the anterior to the posterior side. The sections were mounted on glass slides and stained with cresyl violet (Merck, Darmstadt, Ger-

many), according to Chan et al. [41]. Visualization was made by light microscopy using a Leica DMRB microscope. Images were acquired using the program Leica Qwin.

Western blots. Western blot analysis was performed on contralateral and ipsilateral hippocampi homogenates. Briefly, hippocampi were dissected from frozen brains, and the whole hippocampus was resuspended in 50 μ l of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl-fluoride, 1.5 μ g/ml soybean trypsin inhibitor, 7 μ g/ml pepstatin A, 5 μ g/ml leupeptin, 0.1 mM benzamidine, 0.5 mM DTT) and incubated on ice for 15 min. The tissues were lysed by rapid passage through a syringe needle five to six times, and the total protein extract was then obtained by centrifugation for 15 min at 13,000 g. Protein concentration was determined by the Bradford assay, and equivalent amounts (70 μ g) of each sample were subjected to SDS-PAGE electrophoresis. The proteins were transferred onto nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad, Hercules, Calif.). The membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in PBS and then incubated with: anti-calbindin (Sigma) (1:500 v/v), anti-caspase-3 (Sigma) (1:500 v/v), anti-S100 β (1:250 v/v) (Abcam, Cambridge, UK), anti-cyclooxygenase-2 (COX₂) (1:1000 v/v) (Transduction Laboratories, Lexington, UK), anti-inducible nitric oxide synthase (iNOS) (BD Bioscience, Milan, Italy) (1:2000 v/v), anti-CB₁ (Affinity Bioreagents, Golden Colo.) (1:250 v/v), anti-CB₂ (Cayman, Ann Arbor, Mich.) (1:250 v/v), and anti- β -actin (Sigma) (1:1000 v/v) antibodies. The membranes were washed three times with 1% Triton 100-X in PBS and then incubated with anti-mouse or anti-rabbit immunoglobulins coupled to peroxidase (1:1000 v/v) (Dako, Glostrup, Denmark). The immunocomplexes were visualised by the ECL chemiluminescence method (Amersham, Little Chalfont, UK). The relative expression of protein bands was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst; IBM, Segrate, Italy).

DNA fragmentation. Tissue DNA was prepared according to Iuvone et al. [37], with some modification. Briefly, hippocampi were dissected from frozen brains, and the DNA from the whole hippocampus was isolated using a DNA isolation kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. A 20- μ l aliquot of each DNA sample was analyzed on a 1.5% agarose gel containing ethidium bromide (1 μ g/ml) in TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA) and run for 90 min. After electrophoresis, the DNA was visualized under UV light and photographed.

Drug administration to mice. BAP was prepared as stock solutions in sterile 0.1 M PBs (pH 7.4) prior to freezing. Sterile 0.1 M PBs was injected into control animals. BAP (400 pmol) was administered intracerebroventricularly (i.c.v.) using a microsyringe with a 28-gauge stainless steel needle 3.0 mm long (Hamilton, Bonaduz, Switzerland). In brief, the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears, and perpendicular to the plane of the skull [42]. I.c.v. injection was used because of its simplicity with respect to stereotaxis in mice, and also to ensure diffusion of BAP in the whole brain. BAP or vehicle for peptide solution (2 μ l) was delivered gradually over 3 s. Mice exhibited normal locomotor behavior within 1 min after injection. The selective inhibitor of endocannabinoid cellular uptake, VDM-11 [5 mg/kg, intraperitoneal (i.p.)], and N-arachidonoyl-serotonin (AA-5-HT; 5 mg/kg, i.p.), a selective inhibitor of fatty acid amide hydrolase (FAAH), synthesized in our laboratory as described in Bisogno et al. [43], were diluted in DMSO/H₂O (95:100 volume ratio). These preparations were injected in a total volume of 0.1 ml/100 g body weight. The same volume of DMSO/H₂O solution was used for control mice. The compounds were administered every other day, starting from the 3rd or the 7th day from the operation until the 14th day, when mice were sacrificed.

Passive-avoidance test. The apparatus for the step-through passive-avoidance test was an automated shuttle-box (Cat. 7551 Passive Avoidance Controller and Cat. 7553 Passive Avoidance Mouse Cage; Basile, Comerio VA, Italy), divided into an illuminated compartment and a dark compartment of the same size by a wall with a guillotine door.

Adaptation, training trial, and retention test. In the experimental session, each mouse was trained to adapt to the step-through passive-avoidance apparatus [44]. The animal was put into the illuminated compartment, facing away from the dark compartment. After 10 s, the door between these two boxes was opened and the mouse was allowed to move into the dark compartment freely. The latency to leave the illuminated compartment was recorded. Two hours after the adaptation trial, the mouse was again put into the illuminated compartment. The learning trial was similar to the adaptation trial except that the door was closed automatically as soon as the mouse stepped into the dark compartment and an inescapable foot-shock (0.2 mA, 2 s) was delivered through the grid floor. The retention of the passive-avoidance response was measured 1 and 7 days after the learning trial. Each animal was again put into the illuminated compartment and the latency to re-enter the dark compartment was recorded. No foot-shock was delivered during the retention test.

The maximum cut-off time for step-through latency was 300 s [44].

Experimental procedures. After 1 week of habituation in the facilities, mice were injected i.c.v. with BAP (400 pmol); control animals were treated with the vehicle used for the peptide preparation. For the acute treatment, mice ($n = 10$ per data point) were treated with VDM-11 or AA-5-HT (both 5 mg/kg) 14 days after β -amyloid injection, 30 min prior to the test. For the chronic treatment, three days after β -amyloid injection, VDM-11 or AA-5-HT (both 5 mg/kg) were administered to animals for 11 days, every other day, while other groups of animals began the treatment with these drugs 7 days after β -amyloid injection for 1 week. Respective control groups, treated with vehicle, were subjected to the same procedures. The dose of 5 mg/kg for the two drugs was selected based on previous experiments carried out both in mice and rats, and showing a potentiation of exogenous anandamide effects on nociception and locomotion but no effect of the two inhibitors per se on these two parameters [45]. At the end of the chronic treatment with VDM-11 or AA-5-HT, 14 days after i.c.v. injection of BAP 1–42, mice were tested in the passive-avoidance task, as described above. Their memory retention was assessed 1 and 7 days after the learning trial. Three mice from each group were sacrificed 15 min after the last administration of VDM-11 or AA-5-HT, and the brains were frozen and used for biochemical analysis.

Data analysis. The results (time in seconds employed for re-entering the dark box in the first or second retention test) were expressed as medians. The data were analyzed using the Mann-Whitney U test for non-parametric data. A level of $p < 0.05$ was considered as indicative of statistical significance.

Endocannabinoid extraction and analyses. Rat brains were quickly removed and the cerebral areas (cortex, hippocampus) were obtained within 5 min by regional dissection on ice according to Heffner et al. [46]. In mice, whole brains, and not hippocampi, were analyzed. Tissues were immediately frozen in liquid nitrogen to avoid the postmortem rise in the concentrations of long-chain N-acylethanolamines that starts approximately 30 min after sacrifice, as reported by Schmid et al. [47], and stored at -80°C until used. Tissues were dounce-homogenized with chloroform/methanol/Tris-HCl 50 mM, pH 7.4 (1/1/1 by volume) containing 50 pmol of d_8 -anandamide and 100 pmol of d_5 -2-AG (Cayman Chemicals) as internal standards. The lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel, and analyzed by liquid chromatography-atmospheric pressure chemical ionisation-mass spectrometry (LC-APCI-MS) using a Shimadzu (Duis-

berg, Germany) HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected-ion-monitoring (SIM) mode as described previously [48]. The temperature of the APCI source was 400°C ; the HPLC column was a Phenomenex (5 μm , 150×4.5 mm) reverse-phase column, eluted as described before [48]. Anandamide (retention time: 14.5 min) and 2-AG (retention time: 17.0 min) quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards and their amounts in picamoles normalized per milligram of lipid extract.

RT-PCR and real-time PCR analyses. Rat brains were quickly removed and the hippocampus from both hemispheres (hemisphere ipsilateral to the injection vs contralateral BAP-injected hemisphere) obtained within a few minutes by regional dissection on ice according to Heffner et al. [46]. Tissues were immediately frozen in liquid nitrogen. Total RNAs from these tissues were extracted using the Trizol reagent according to the manufacturer's recommendations (GibcoBRL). Following extraction, RNA was precipitated using ice-cold isopropanol, resuspended in diethyl pyrocarbonate- (Sigma) treated water and its integrity was verified following separation by electrophoresis into a 1% agarose gel containing ethidium bromide. RNA was further treated with RNase-free DNase I (DNA-free kit, Ambion, Austin, Tex.) according to the manufacturer's recommendations to digest contaminating genomic DNA and to subsequently remove the DNase and divalent cations.

The expression of mRNAs for CB_1 , CB_2 , FAAH, monoacylglycerol lipase (MAGL), diacylglycerol lipase α ($\text{DAGL}\alpha$), N-acyl-phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and GAPDH were examined by semiquantitative reverse transcription coupled to RT-PCR. Total RNA was reverse-transcribed using random primers. DNA amplifications were carried out in PCR buffer (Invitrogen, Carlsbad, Calif.) containing 2 μl of cDNA, 500 μM dNTP, 2 mM MgCl_2 , 0.8 μM of each primer and 0.5 U Taq polymerase platinum (Invitrogen). The thermal reaction profile consisted of a denaturation step at 94°C for 1 min, annealing at 52°C (NAPE-PLD), 55°C (GAPDH, MAGL, and $\text{DAGL}\alpha$), 57°C (CB_2) or 60°C (CB_1 and FAAH) for 1 min and an extension step at 72°C for 1 min. A final extension step of 10 min was carried out at 72°C . The PCR cycles observed to be optimal and in the linear portion of the amplification curve were 24 for GAPDH, 27 for NAPE-PLD, 29 for FAAH, 35 for MAGL, CB_1 and CB_2 , and 28 for $\text{DAGL}\alpha$ (data not shown). The reaction was performed in a PE Gene Amp PCR System 9600 (Perkin Elmer, Boston, Mass.). After the reaction, the PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide for UV visualization.

Specific rat oligonucleotides were synthesized on the basis of cloned rat cDNA sequences of CB₁ (GenBank accession number NM_012784.3), CB₂ (GenBank accession number NM_020543), FAAH (GenBank accession number NM_024132.1), NAPE-PLD (GenBank accession number AB112351), MAGL (GenBank accession number NM_138502.1), DAGL α (GenBank accession number NM_001005886.1), and GAPDH (GenBank accession number NM_017008.2). For CB₁, the primers sequences were 5'- GAT GTC TTT GGG AAG ATG AAC AAG C -3' (sense) and 5'- AGA CGT GTC TGT GGA CAC AGA CAT GG -3' (antisense). The CB₂ sense and antisense primers were 5'- TA(C/T) CC(G/A) CCT (A/T)CC TAC AAA GCT C -3' and 5'- C (A/T)GG CAC CTG CCT GTC CTG GTG -3', respectively. For FAAH, the primers sequences were 5'- GTG GTG CT(G/A) ACC CCC ATG CTG G -3' (sense) and 5'- TCC ACC TCC CGC ATG AAC CGC AGA CA -3' (antisense). For NAPE-PLD, the primers sequences were 5'- TGG ACT GGT GGG AGG AG -3' (sense) and 5'- GGT TCA TAA GCT CCG ATG GG -3' (antisense). The MAGL sense and antisense primers were 5'- TCT TCC TCC TGG GCC ACT CCA -3' and 5'- GGA TTG GCA AGG ACC AGA GG -3', respectively. For DAGL α , the primers sequences were 5'- GCT ATC TTC CTC TTC CTG CTA -3' (sense) and 5'- GAG CAC GTA CTG CAT GGA GTC -3' (antisense). The expected sizes of the amplicons were 309 bp for CB₁, 291 bp for CB₂, 300 bp for FAAH, 249 bp for NAPE-PLD, 111 bp for MGL, and 231 bp for DAGL α . In the presence of contaminant genomic DNA, the expected size of the amplicons would be 1351 bp for FAAH and 1062 bp for GAPDH (data not shown). No PCR product was detected when the reverse transcriptase step was omitted.

Data from semi-quantitative RT-PCR on MAGL, DAGL α , FAAH, and NAPE-PLD, were confirmed using real-time PCR. Following extraction, RNA was treated as described above. After DNase digestion, the accurate amount of RNA was evaluated with the RiboGreen-RNA quantitation kit according to the manufacturer's instructions (BioRad). Two micrograms was reverse transcribed in a 25- μ l reaction mixture containing 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM dNTPs, 50 mM Tris-HCl pH 8.3, 20 units RNase inhibitor (Boehringer-Roche, Monza, Italy), 0.125 A₂₆₀ units of hexanucleotide mixture (Boehringer-Roche) for random priming and 200 units of MoMuLV reverse transcriptase (Superscript III; GIBCO, Carlsbad, Calif.). The reaction mixture was incubated in a thermocycler iCycler iQ (BioRad) at 55 °C for 5 min followed by 2 min at 4 °C before the enzyme addition, then at 20 min for 10 min and at 50 °C for 90 min. The reaction was stopped by heating at 95 °C for 5 min. Control samples (no-RT) were prepared by omitting MoMuLV reverse transcriptase in the retrotranscription mixture. Real-time cDNA quantitation was performed by a thermocycler iCycler iQ (BioRad) in a 25- μ l reaction mixture (iQ SYBR Green Super-

mix; BioRad) containing 10–50 ng of cDNA (calculated on the basis of the input RNA) and 300 nM primers. The amplification profile consisted of an initial denaturation of 2 min at 94 °C and 40 cycles of 30 s at 94 °C, annealing for 30 s at TaOpt (optimum annealing temperature – see below) and elongation for 45 s at 68 °C. Fluorescence data were collected at this step. A final extension of 7 min was carried out at 72 °C, followed by melt-curve data analysis. Optimized primers for SYBR Green analysis (and relative TaOpt), were designed by Beacon Designer software, version 4.0 (Biosoft International; Palo Alto, Calif.) and synthesized by MWG-Biotech AG (Ebersberg, Germany). Assays were performed in triplicate (SD of threshold cycle mean <0.5) and a standard curve from consecutive five-fold dilutions (150–0.24 ng) of a cDNA pool representative of all samples was included for each determination. Relative expression analysis corrected for PCR efficiency and normalized with respect to the reference gene (β -actin) was performed by REST software for group-wise comparison and statistical analysis.

Results

Neuronal damage caused by BAP injection into rat brain

To confirm the hippocampal damage previously observed in several studies of β -amyloid-induced toxicity [see refs. 35, 39, 49 and 50 for some examples], after BAP injection, we performed histological analyses. In agreement with these previous studies, 12 days after the injection of BAP into the ipsilateral cortex, neuronal damage was observed in the CA1, CA2, and CA3 regions of consecutive slices from the ipsilateral versus contralateral hippocampus or versus the ipsilateral hippocampus of vehicle-injected brains (Fig. 1a). The sites of major neuronal loss were localized into foci rather than spread over the whole hippocampal region, as one would expect given the short time from BAP injection, and in agreement with the biochemical data (see Fig. 2). As estimated by counting the missing dots from each slice, about 42 ± 4 missing neurons were observed in three consecutive hippocampal slices (mean \pm SE, $n = 3$ slices from three distinct brains). In fact, significantly increased, albeit limited, DNA fragmentation was also found in the ipsilateral versus contralateral hippocampus or versus the ipsilateral hippocampus of vehicle-injected brains (Fig. 2a and data not shown), whereas Western immunoblots showed a small but significant increase of caspase-3, S100 β and iNOS (Fig. 2b, c). By contrast, COX-2, a very early marker of neuronal toxicity, was more strongly increased, and calbindin was strongly decreased, suggesting that neuronal loss might have been more advanced than suggested by the histological analyses. However, the biochemical analyses were carried out with much more hippocampal tissue than histolog-

ical analyses. No increased histochemical or biochemical damage was found in the cortical region where the BAP was injected with respect to the vehicle-injected ipsilateral cortex (not shown). The finding of neuronal damage and enhanced markers of gliosis (see below) in a brain region far from the injection site confirms that the BAP was perfectly soluble and efficacious and, although we did not assess directly the diffusion of the fragment, suggests that the peptide was capable of diffusing into brain tissue from the moment of injection. In a pilot study, injection of scrambled BAP was shown to cause no histological neuronal damage (Fig. 1c) and no release of caspase-3 from pro-caspase (data not shown). For this reason, in subsequent experiments, we compared the effects observed in the ipsilateral hippocampi with those observed in the contralateral hippocampi or, in select cases, with hippocampi from vehicle-injected rats, and not with hippocampi from scrambled-peptide-injected animals. Including control groups with the scrambled peptide (which requires separate stereotaxic injections) would have meant using many more rats and was deemed to be unethical.

Modulation of the endocannabinoid system in rat brains injected with BAP. To check whether the histological damage was accompanied by changes in the endocannabinoid system we analyzed the hippocampal levels of the two major endocannabinoids, anandamide and 2-AG. An approximately twofold enhancement of 2-AG levels in

the hippocampus dissected from the hemisphere ipsilateral to the injection versus the contralateral hippocampus, was observed only after 12 days from the treatment (Fig. 3a). Anandamide levels in the ipsilateral hippocampus were never higher, and indeed were even lower 20 days after the injection, than those in the contralateral hippocampus (Fig. 3b). However, both 2-AG and anandamide levels also increased in the contralateral hippocampus after 12 days compared with the levels after 5 days. The levels in the ipsilateral cerebral cortex of 2-AG or anandamide were never significantly higher than those in the contralateral cortex (not shown). Identical results were obtained when comparisons were made versus ipsilateral areas from vehicle-injected brains (not shown). When the whole ipsilateral brain was analyzed, we could not see any change in endocannabinoid levels 12 days after injection of BAP (data not shown). The mRNAs encoding the biosynthetic and metabolic enzymes of anandamide and 2-AG were also analyzed by semi-quantitative and real-time RT-PCR. DAGL α and MAGL were found to be significantly elevated 12 days following BAP injections (Fig. 4). By contrast, the levels of the mRNA encoding NAPE-PLD and FAAH were not significantly modified (Fig. 4a). Similar results were obtained when comparisons were made versus ipsilateral hippocampi from vehicle-injected brains (not shown). Exact quantification by real-time PCR revealed a 1.2 ± 0.05 -fold elevation of MAGL expression ($p > 0.05$) and a 1.6 ± 0.2 -fold elevation of DAGL α expression ($p < 0.05$) ($n = 3$, means \pm SE) (Fig. 4b).

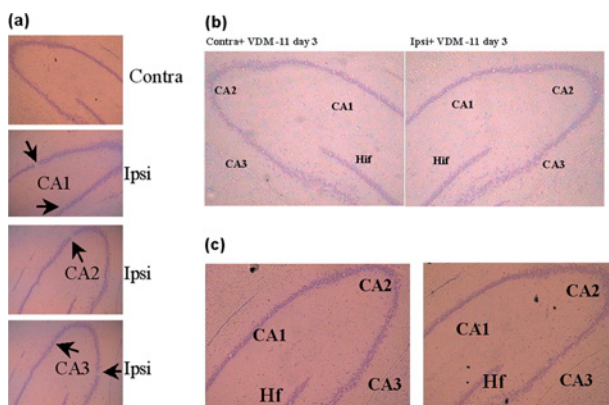


Figure 1. Histological analyses of hippocampal slices from rat brains 12 days after injection of vehicle (a, upper panel), BAP 1–42 (a, lower panels), or BAP plus VDM-11 (b), administered (5 mg/kg, i.p.) sub-chronically starting 3 days after BAP injection. Data are representative of experiments carried out in three different rats per group. Consecutive slices show neuronal loss in different (CA1, CA2, CA3) hippocampal regions of the same brain. Arrows indicate the sites of major neuronal loss, which were localized rather than widespread, as expected given the brief time from BAP injection and in agreement with the biochemical data (see Fig. 2). (c) Histological analyses of rat hippocampus obtained 12 days after injection of a scrambled form of BAP 1–42 (a.a. sequence, H-Lys-Val-Lys-Gly-Leu-Ile-Asp-Gly-Asp-His-Ile-Gly-Asp-Leu-Val-Tyr-Glu-Phe-Met-Asp-Ser-Asn-Ser-Ala-Ile-Phe-Arg-Glu-Gly-Val-Gly-Ala-Gly-His-Val-His-Val-Ala-Gln-Val-Glu-Phe-OH). Two distinct sections from the same rat are shown and are representative of sections from four rats.

Neuroprotection by enhancement of anandamide levels in BAP-injected rats. Enhancement of endocannabinoid levels in various models of brain damage [8, 12, 13, 51] has been shown to exert protection, and therefore we treated the BAP-injected rats, either 3 or 7 days after injection, with the endocannabinoid reuptake inhibitor VDM-11 (5 mg/kg, i.p.). Remarkably, with both administration protocols, the compound caused a statistically significant enhancement of day 12 anandamide levels, but not 2-AG levels, in the hippocampus (Fig. 5a, b) and in the whole ipsilateral brain, but in this case only a $24 \pm 2\%$ elevation was observed ($n = 3$, data not shown). However, only when VDM-11 was administered starting from day 3, was this increase in anandamide levels accompanied by a dramatic reversal of neuronal damage in the ipsilateral hippocampus as assessed by histological analysis (Fig. 1b), DNA fragmentation analysis (Fig. 6a) and Western immunoblotting of caspase-3 (decreased), calbindin (increased), iNOS (decreased), S100 β (decreased), and COX-2 (decreased) (Fig. 6b, c). Identical results were obtained when comparisons were made versus ipsilateral hippocampi from vehicle-injected rat brains (not shown).

The effect of modulation of the endocannabinoid system on memory tasks in mice. No changes in the whole-

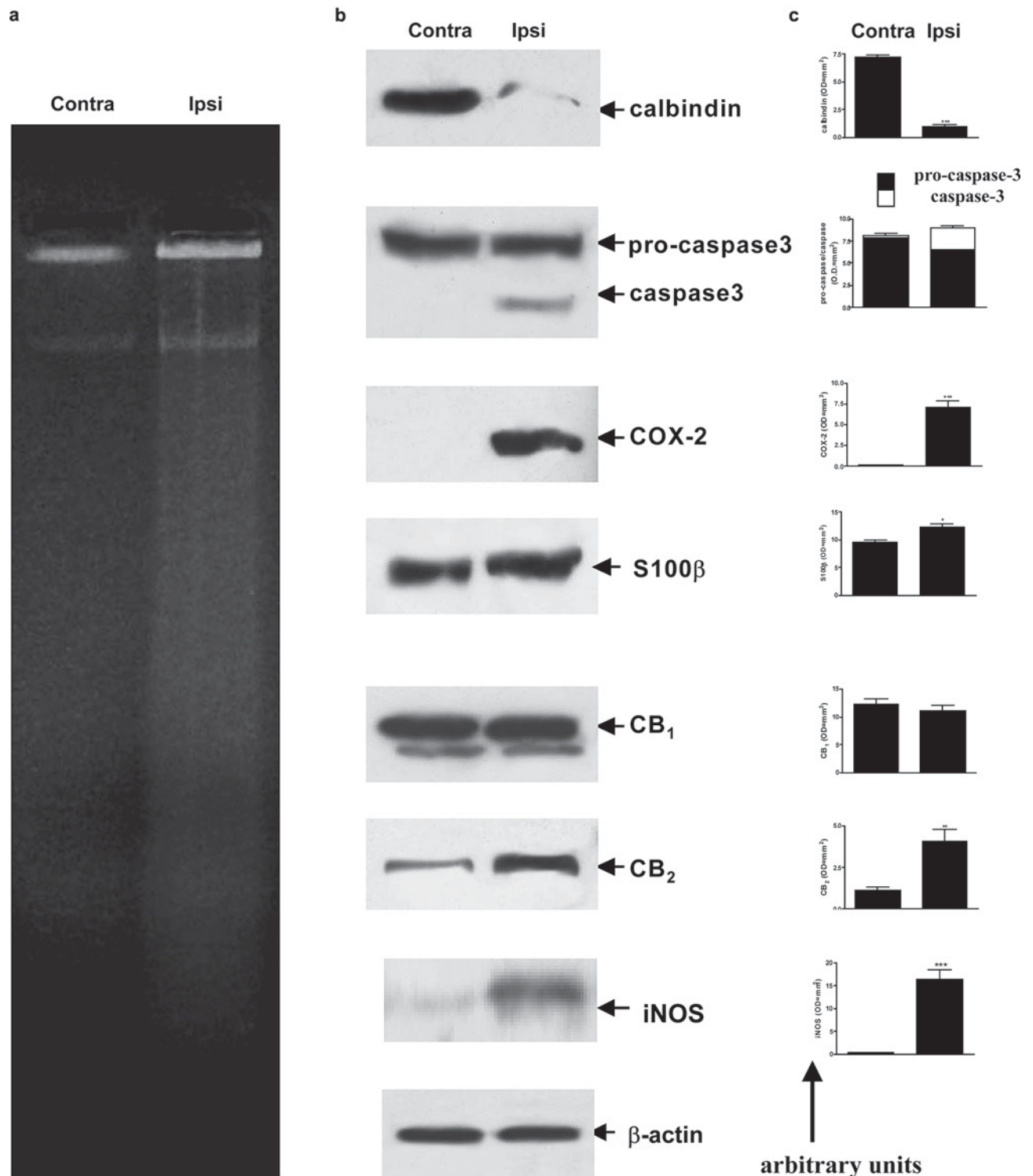


Figure 2. Hippocampal damage at day 12 after stereotaxic injection of BAP 1–42 into the rat cortex. (a) Fragmentation of the DNA extracted from the ipsilateral hippocampus (Ipsi) as compared with the contralateral one (Contra). Representative of three separate experiments in corresponding distinct animals. (b, c) Change in the expression of several markers of neuronal loss and/or glial cell activation, and of cannabinoid CB₁ and CB₂ receptors, as determined by Western blot (b) carried out with the respective antibodies on the proteins extracted from the ipsilateral (Ipsi) and contralateral (Contra) hippocampus. Data were compared by densitometry scanning (c) of the corresponding gel bands after normalization to β -actin, and are means \pm SE of three experiments in corresponding distinct animals. * $p < 0.05$, *** $p < 0.005$ vs Contra by ANOVA followed by Bonferroni's post hoc test. From top to bottom, blots and scans of calbindin, pro-caspase-3/caspase-3, COX-2, S100 β , CB₁, CB₂, and iNOS are shown.

brain levels of either 2-AG or anandamide could be detected in mice 14 days following i.c.v. administration of BAP versus vehicle (Fig. 7a). However, i.p. administration of VDM-11 (5 mg/kg) either 3 or 7 days after BAP or

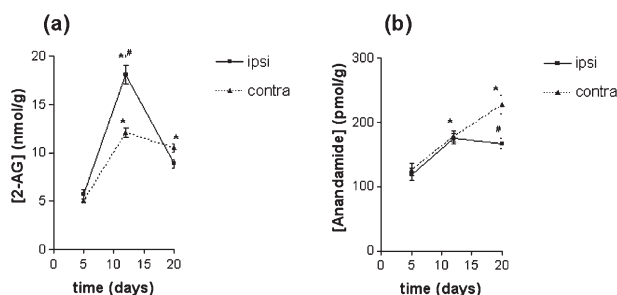


Figure 3. Changes in 2-AG (a) and anandamide (b) levels, determined by isotope-dilution LC-MS, in the ipsilateral hippocampus (ipsi) as compared with the contralateral one (contra), 5, 12 and 20 days after the stereotaxic injection of BAP 1–42 into the rat cortex. Data are means \pm SE of four experiments in corresponding distinct animals, and were compared using ANOVA followed by Bonferroni's post hoc test. *p < 0.05 vs 5 days; #p < 0.05 between 'contra' and 'ipsi'.

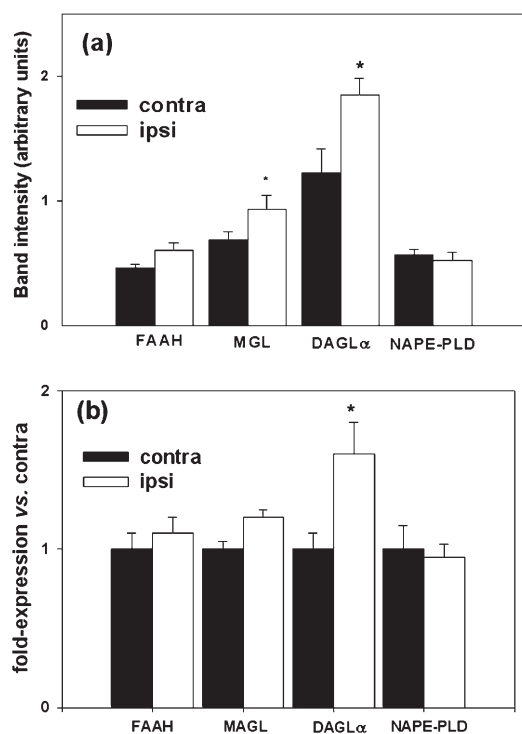


Figure 4. Changes in the levels of mRNA encoding for endocannabinoid enzymes, as determined by semi-quantitative RT-PCR (a) and real-time PCR (b), in the ipsilateral hippocampus (ipsi) as compared with the contralateral one (contra), 12 days after the stereotaxic injection of BAP 1–42 into the rat cortex. (a) Data represent densitometry scans of the amplicon bands obtained after semi-quantitative RT-PCR, subtracted of background and normalized to the housekeeping gene amplicon (GAPDH), and are means \pm SE of three experiments in corresponding distinct animals, and were compared by using ANOVA followed by Bonferroni's post hoc test. (b) Data represent fold-expression as compared with expression in 'contra' (see Materials and methods for details on the real-time PCR technique). *p < 0.05 vs 'contra'.

vehicle injection caused a dramatic enhancement of both anandamide and 2-AG levels in whole brain (Fig. 7a, b). I.p. administration of AA-5-HT (5 mg/kg) either 3 or 7 days after BAP or vehicle injection caused a significant enhancement of 2-AG levels in mouse whole brain (Fig. 8a, b), although this effect was much smaller than that observed with VDM-11. Anandamide levels were increased only when AA-5-HT was administered 7 days after BAP or vehicle injection (Fig. 8a, b).

VDM-11 (5 mg/kg, i.p.) administered 3 days after i.c.v. injection of BAP in mice produced a significant amelioration in task retention times observed after both the first and second passive-avoidance test. No effect was seen in control, healthy mice injected with vehicle instead of BAP (Fig. 9a), thus indicating that the changes induced by VDM-11 in AD mice were due to the disease and not to other possible behavioral actions of the uptake inhibitor. By contrast, when VDM-11 was administered 7 days after i.c.v. injection of BAP or vehicle, it caused a significant reduction of memory retention in diseased mice after the first test and no effect after the second test. The

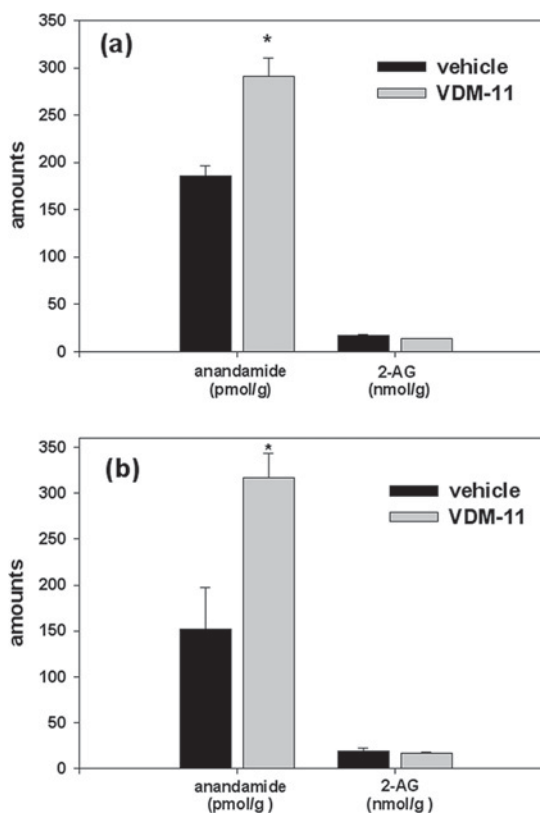


Figure 5. Changes in 2-AG and anandamide levels, determined by isotope-dilution LC-MS, in the rat hippocampus 12 days after injection of BAP 1–42 (by stereotaxis) alone or with VDM-11 administered (5 mg/kg, i.p.) sub-chronically starting 3 days after BAP injection (a), or BAP 1–42 (by stereotaxis) alone or with VDM-11 administered (5 mg/kg, i.p.) sub-chronically starting 7 days after BAP injection (b). Data are means \pm SE of four experiments in distinct animals, and were compared by using ANOVA followed by Bonferroni's post hoc test. *p < 0.05 vs vehicle.

task retention of control, healthy mice was decreased after both the first and second test (Fig. 9b).

AA-5-HT (5 mg/kg, i.p.) administered 3 days after i.c.v. injection of BAP or vehicle in mice did not produce any significant change in task retention times, either after the

first or the second passive-avoidance test (Fig. 10a). By contrast, when AA-5-HT was administered 7 days after i.c.v. injection of BAP or vehicle, it caused a significant reduction of memory retention in diseased mice after the first test and no effect after the second test. The task re-

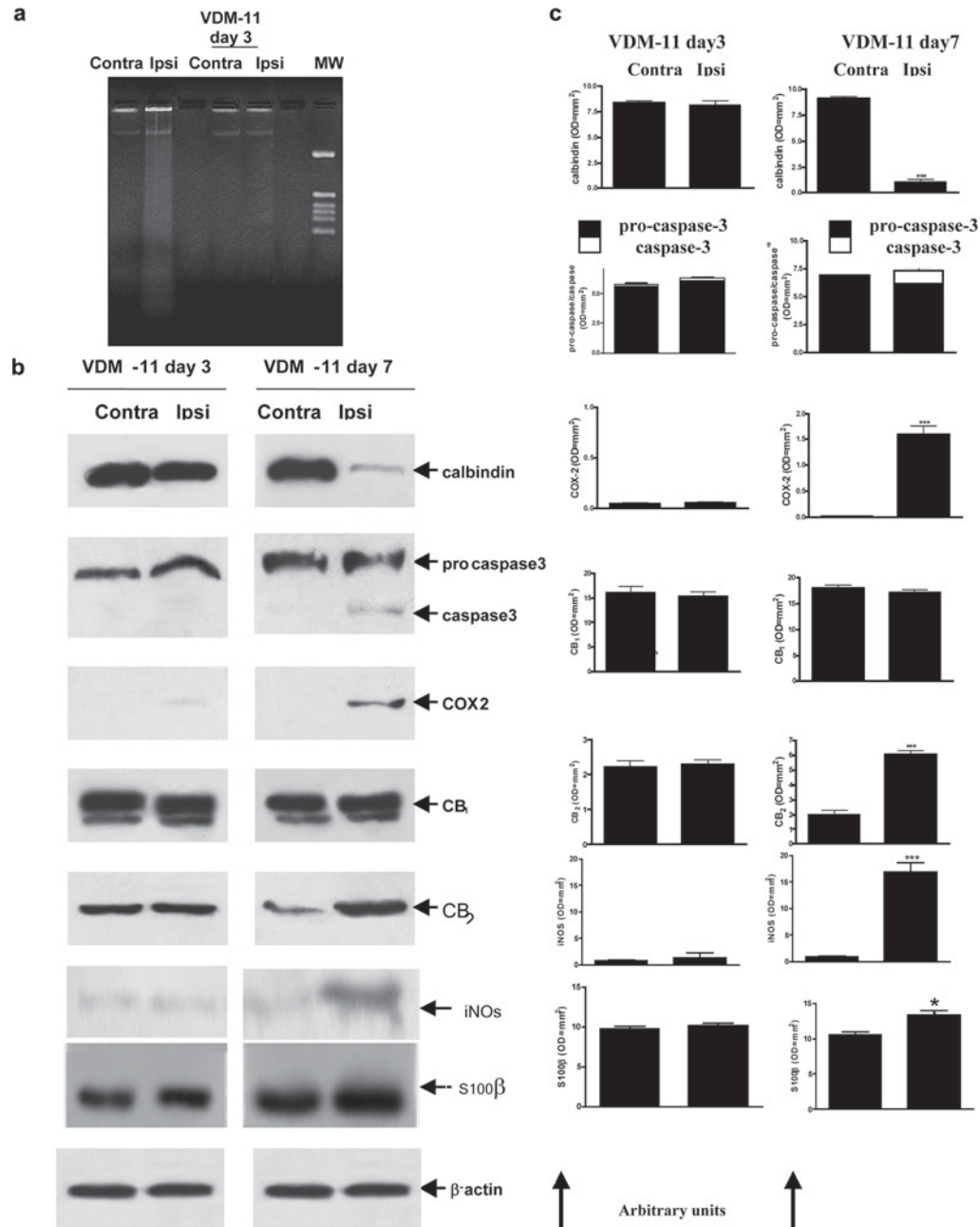


Figure 6. Hippocampal damage at day 12 after stereotaxic injection of BAP 1-42 into the ipsilateral rat cortex, in the presence of VDM-11 administered (5 mg/kg, i.p.) sub-chronically starting 3 or 7 days after BAP injection. (a) Fragmentation of the DNA extracted from the ipsilateral hippocampus (Ipsi) as compared with the contralateral one (Contra). This experiment was carried out only with tissues from animals treated with VDM-11 starting from 3 days, and is representative of three separate experiments with distinct animals. Data in the hippocampus from vehicle-treated BAP-injected animals are the same as in Figure 1. MW, molecular-weight ladder. (b) VDM-11, administered starting on day 3, restores normal (i.e. contralateral) levels of biochemical markers of neuronal loss and/or glial cell activation, as well as cannabinoid CB₁ and CB₂ receptors, in the rat hippocampus, as determined by Western blot carried out with the respective antibodies on the proteins extracted from the ipsilateral (Ipsi) and contralateral (Contra) hippocampus. (c) Data were compared by densitometry scanning of the corresponding gel bands after normalization to β -actin, and are means \pm SE of three experiments in distinct animals (see also Fig. 1). *** p < 0.005 vs contra by ANOVA followed by Bonferroni's post hoc test. From top to bottom, blots and scans of calbindin, pro-caspase-3/caspase-3, COX-2, CB₁, CB₂, iNOS and S100 β are shown. Compare also with the data obtained with ipsilateral hippocampi from animals treated only with BAP (Fig. 1).

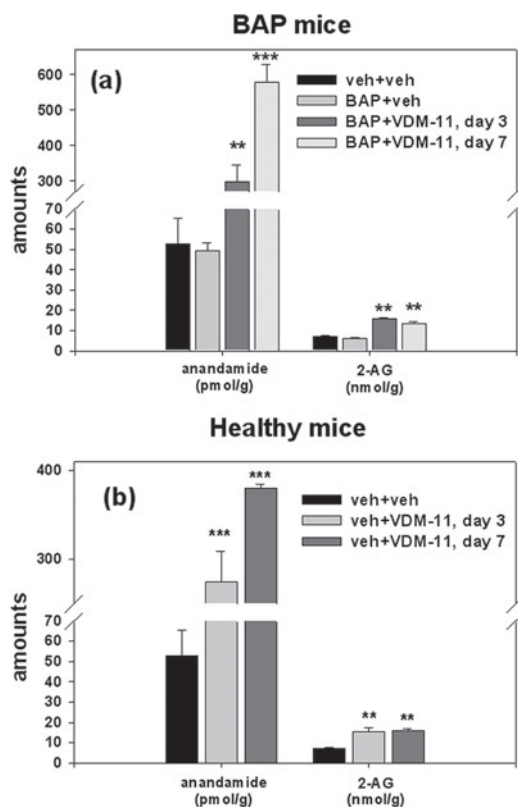


Figure 7. Changes in 2-AG and anandamide levels, determined by isotope-dilution LC-MS, in whole mouse brain 14 days after treatment with vehicle (i.c.v.), BAP 1–42, (i.c.v.), BAP plus VDM-11 administered (5 mg/kg, i.p.) sub-chronically starting 3 days after BAP injection, and BAP plus VDM-11 administered (5 mg/kg, i.p.) sub-chronically starting 7 days after BAP injection (a), or vehicle (i.c.v.), VDM-11 administered (5 mg/kg, i.p.) sub-chronically from 3 days after vehicle injection, and VDM-11 (5 mg/kg, i.p.) administered sub-chronically from 7 days after vehicle injection (b). Data are means \pm SE of $n = 4$ experiments in distinct animals, and were compared using ANOVA followed by Bonferroni's post hoc test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ vs vehicle.

tention of control, healthy mice were decreased after both the first and second test (Fig. 10b).

Discussion

The rationale behind this study was based on previous observations that endocannabinoids, by acting through several possible molecular mechanisms (i.e., via cannabinoid CB₁ or CB₂ receptors, TRPV1 channels or non-cannabinoid non-vanilloid receptor-mediated pathways), exert a protective function against both acute and chronic neuronal damage in a variety of pathological conditions, including β -amyloid-induced neuronal toxicity *in vitro* and *in vivo* [22, 23]. Therefore, these compounds, irrespective of their molecular mode of action, are ideal candidates to play a protective role against the hippocampal damage caused by β -amyloid accumulation. For this neu-

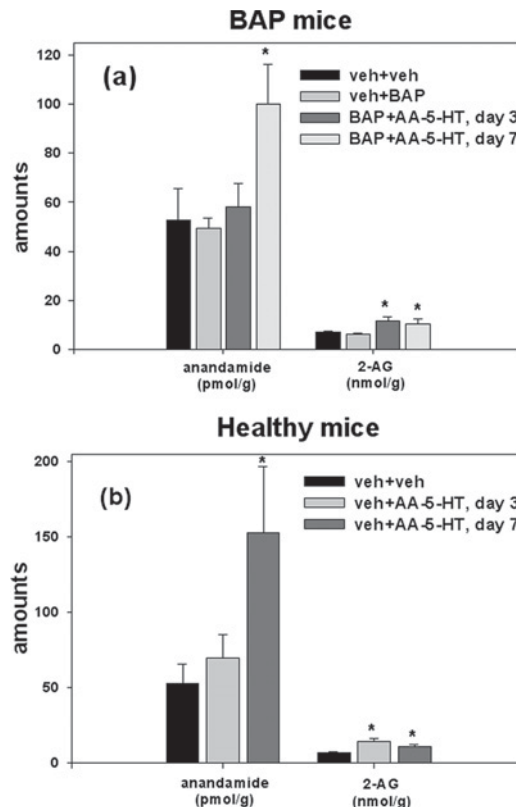


Figure 8. Changes in 2-AG and anandamide levels, determined by isotope-dilution LC-MS, in whole mouse brain 14 days after treatment with vehicle (i.c.v.), BAP 1–42 (i.c.v.), BAP plus AA-5-HT administered (5 mg/kg, i.p.) sub-chronically starting 3 days after BAP injection, and BAP plus AA-5-HT administered (5 mg/kg, i.p.) sub-chronically starting 7 days after BAP injection (a), or vehicle (i.c.v.), AA-5-HT administered (5 mg/kg, i.p.) sub-chronically from 3 days after vehicle injection, and AA-5-HT (5 mg/kg, i.p.) administered sub-chronically from 7 days after vehicle injection (b). Data are means \pm SE of $n = 4$ experiments in distinct animals, and were compared using ANOVA followed by Bonferroni's post hoc test. * $p < 0.05$ vs vehicle.

roprotective function to be exerted, endocannabinoid levels need to be increased, and so we analyzed the levels of anandamide and 2-AG in the rat cortex and hippocampus 5, 12 and 20 days after BAP 1–42 injection. Several authors have used other fragments, e.g. β -amyloid 1–40 [22, 34, 39, 49] or β -amyloid 25–35 [23, 35], which appear to be more efficacious at inducing neurotoxicity, whereas others have found that β -amyloid 1–42 in the aggregated form produces stronger effects [50]. We used the soluble form of β -amyloid 1–42 because its neurotoxic effects both *in vitro* and *in vivo* have been well characterized in our [32, 37, 38, 42] and other [52, 53] laboratories. We show that already 12 days after injection of this BAP fragment there is neuronal loss in the CA1, CA2, and CA3 regions of the hippocampus (Fig. 1), accompanied by the appearance of typical markers of apoptosis and gliosis, i.e., caspase-3, iNOS, S100 β and COX₂, and by loss of the neuronal marker calbindin (Fig. 2). These neurotoxic

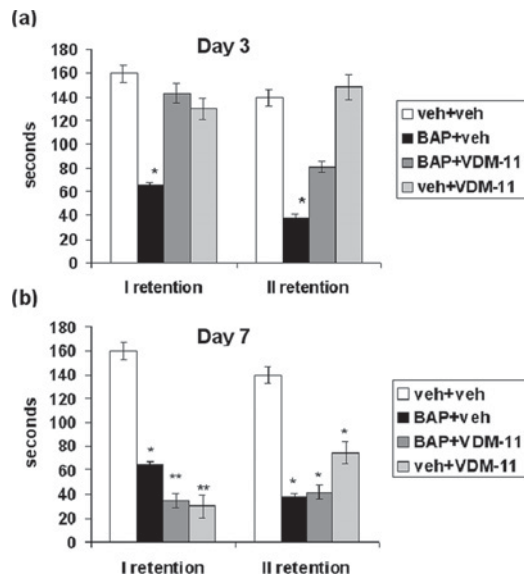


Figure 9. Time of memory retention in the passive-avoidance test carried out in mice at day 15 (I retention) and day 22 (II retention) after the injection (i.c.v.) of BAP 1–42, i.e., 1 and 7 days after the learning trial. Different groups of animals were treated with vehicle (i.c.v.), BAP (i.c.v.), VDM-11 (5 mg/kg, i.p.) or BAP+VDM-11. VDM-11 was administered sub-chronically starting 3 days (a) or 7 days (b) after BAP injection. The results (time in seconds taken for re-entering the dark box in the first or second retention test) are expressed as medians of $n = 10$ animals. The data were analyzed using the Mann-Whitney U test for non-parametric data. * $p < 0.05$; ** $p < 0.01$ vs veh-veh. For each median, the semi-inter-quartile range (IQR/2) is shown.

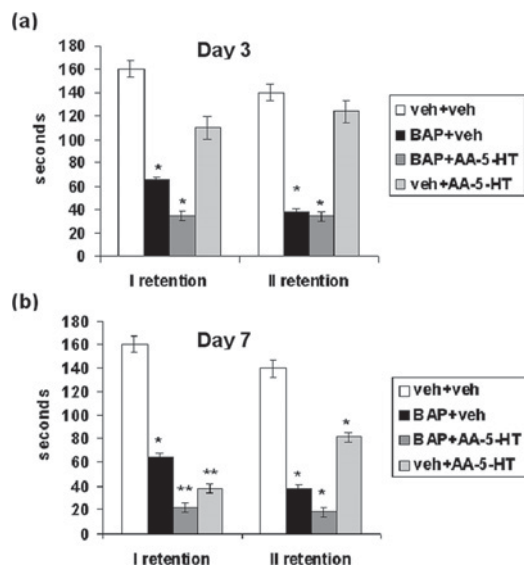


Figure 10. Time of memory retention in the passive avoidance test carried out in mice at day 15 (I retention) and day 22 (II retention) after the injection (i.c.v.) of BAP 1–42, i.e., 1 and 7 days after the learning trial. Different groups of animals were treated with vehicle (i.c.v.), BAP (i.c.v.), AA-5-HT (5 mg/kg, i.p.) or BAP + AA-5-HT. AA-5-HT was administered sub-chronically starting 3 days (a) or 7 days (b) after BAP injection. The results (time in seconds employed for re-entering the dark box in the first or second retention test) are expressed as medians of $n = 10$ animals. The data were analyzed using the Mann-Whitney U test for non-parametric data. * $p < 0.05$, ** $p < 0.01$ vs veh-veh. For each median, the semi-inter-quartile range (IQR/2) is shown.

effects were not related to the injection procedure used, as they were not observed in vehicle-injected rats and were not observed in the injection area (i.e., the cortex).

The first new finding of the present study was that BAP also causes a selective approximately twofold enhancement of 2-AG levels in the rat hippocampus dissected from the hemisphere ipsilateral to the injection versus the contralateral hippocampus or versus the ipsilateral hippocampus of vehicle-treated brains, again only after 12 days from the treatment (Fig. 3). Anandamide levels in the ipsilateral hippocampus were not higher than those in the contralateral hippocampus. It is worth noting that both 2-AG and anandamide levels also increased in the contralateral hippocampus after 12 days as compared with the initial levels, suggesting that some non-specific event, possibly due to stress [54], was affecting, in part, the measurement of endocannabinoid levels in both hemispheres. On the other hand, as with markers of neuronal loss, the levels in the ipsilateral cerebral cortex of 2-AG and anandamide were never significantly higher than those in the contralateral cortex (data not shown). These data suggest that: (i) the changes in 2-AG levels observed in the ipsilateral hippocampus at the 12-day time point were specifically due to BAP-induced damage and not to an artifact arising from the stereotaxic injection, which was carried out in the cortex, nor to other environmental factors, and (ii) BAP-induced 2-AG elevation in the rat brain is an early and time-limited event. The enhancement of 2-AG levels was probably due to its enhanced synthesis, since we observed that the hippocampal levels of the mRNA encoding the 2-AG-biosynthesizing enzyme DAGL α [55] were also elevated following BAP injections (Fig. 4). By contrast, the levels of the mRNA encoding the anandamide-biosynthesizing and -degrading enzymes, NAPE-PLD and FAAH, respectively, were not changed, in agreement with the finding of no significant changes in anandamide levels following BAP injection. Our finding of elevated DAGL α expression following BAP injection is in agreement with the previous report of elevated DAGL enzymatic activity in the hippocampus of patients with AD [56], which suggests that a tissue-selective up-regulation of 2-AG levels also occurs during this disorder in humans. In view of previous findings in postmortem brain from AD patients [21, 23], we also analyzed the levels of CB $_1$ and CB $_2$ in the hippocampus of BAP-treated rats. Western blot (Fig. 2b, c) confirmed the increase in expression of the CB $_2$ receptor protein, but not of CB $_1$, as previously reported in AD brains [21]. If endocannabinoids are elevated at a certain time after BAP-induced hippocampal damage to exert a neuroprotective function, the pharmacological elevation of their levels should produce beneficial effects on this damage [12, 51], irrespective of the receptor type and mechanism involved in endocannabinoid neuroprotective effects. In fact, if endocannabinoids use more than one mechanism

of action to protect neurons from damage, inhibitors of endocannabinoid inactivation should be even more efficacious than 'direct' agonists of each receptor type potentially involved in this action. Therefore, the next step in our investigation was to assess the effect of either early or late pharmacological elevation of brain endocannabinoid levels on neuronal damage in the rat hippocampus. The selective inhibitor of anandamide cellular uptake, VDM-11, was administered i.p. to BAP-treated rats every other day, starting 3 or 7 days from BAP injection, at a dose previously shown to enhance some of the typical pharmacological effects of anandamide in normal rats without influencing locomotor behaviour and nociception per se [45]. With both administration schedules, VDM-11 significantly elevated anandamide, but not 2-AG, levels in the ipsilateral hippocampus (Fig. 4). The lack of effect on 2-AG levels might be due to the fact that the concentration of this endocannabinoid in the hippocampus, unlike that of anandamide, was already maximally enhanced following BAP treatment. Nevertheless, the enhancement of total endocannabinoid levels caused by VDM-11 may have been enough to explain its inhibition of the neuronal damage induced in the hippocampus by BAP administration, as we found that the hippocampal concentration of anandamide was enhanced from ~180 to ~290 nM, and there is evidence that such an increase of anandamide concentration can inhibit BAP-induced neurotoxicity in neurons *in vitro* with ~50% more efficacy [22]. Accordingly, VDM-11 administered 3 days after BAP treatment entirely reversed the histological damage and the biochemical markers of neuronal loss and gliosis induced by the peptide, as well as the increase in CB₂ receptor protein (Fig. 6). By contrast, when the inhibitor was administered 7 days after BAP treatment, i.e. only 5 days from the day of sacrifice, no significant amelioration of the histological and biochemical changes induced by BAP was observed even in the presence of enhanced anandamide levels, suggesting that endocannabinoid elevation must be induced early to achieve neuroprotection.

The next aim of our study was to investigate whether VDM-11 causes amelioration of overt signs of β -amyloid-induced neurotoxicity, such as loss of memory retention. The answer to this question was not at all obvious because endocannabinoids not only have neuroprotective actions but, mostly by acting via CB₁ receptors, they can also impact negatively on memory consolidation in several experimental paradigms in healthy rodents [28]. Indeed, a previous study showed that acute blockade of endocannabinoid actions at CB₁ receptors produces alleviation of the amnesia caused in mice by the same BAP fragment used in the present study [32]. In this previous study, a passive-avoidance test was used to monitor memory retention. This is a test that is carried out in mice much more often than rats when the effect of β -amyloid-induced neurotoxicity needs to be assessed. In mice, the

hippocampal damage and the subsequent amnesia secondary to defective hippocampal cholinergic signaling are induced by BAP within 2 weeks of i.c.v. injection [42 and references cited therein], exactly as shown above for rats. For these reasons, we decided to study here the effect of VDM-11 in the same experimental paradigm in mice. In BAP-treated mice, VDM-11, again administered i.p. every other day, and starting 3 days from BAP injection, caused a striking enhancement of brain endocannabinoid levels. Although, unlike in the rat, 2-AG levels were also elevated, once again the effect was more marked with anandamide (sixfold enhancement) (Fig. 7). We found that VDM-11 also produced a significant amelioration in task retention times of BAP-treated mice, measured after both the first and second passive-avoidance test when administered starting 3 days from BAP injection (Fig. 9). However, although VDM-11 still very potently enhanced endocannabinoid levels when administered only after 7 days from BAP treatment, this was not sufficient to reverse the amnesic effect of BAP, and caused, instead, a further decrease in retention times as assessed after the first passive-avoidance test. Notably, VDM-11 also produced a strong enhancement of both anandamide and 2-AG brain levels in healthy mice, irrespective of the timing of its administration (Fig. 7), and in these mice this effect was accompanied by a worsening of memory retention only in those animals that had been treated starting 7 days from vehicle injection. The selective FAAH inhibitor AA-5-HT [43] also elevated endocannabinoid levels when administered starting either 3 or 7 days after administration (Fig. 8), but was much less effective than VDM-11 (compare Figs. 7 and 8). However, AA-5-HT, irrespective of the time of administration, and unlike VDM-11, never ameliorated amnesia in BAP-treated mice, although, like VDM-11, it did inhibit the performance of normal and BAP-injected mice in the passive-avoidance tests when administered 7 days after vehicle or BAP administration (Fig. 10).

These findings in mice suggest that: (i) in order to ameliorate BAP-induced memory retention loss, the pharmacological elevation of endocannabinoid levels must not only be strong, as in the case of VDM-11 and not AA-5-HT, but it must also occur early in the disorder, and (ii) pharmacological elevation of endocannabinoid levels may inhibit memory consolidation in healthy as well as BAP-injected mice if it is effected only for a few days, possibly due to the previously described development of tolerance to the memory-disrupting effects of prolonged activation of CB₁ receptors [31]. These two hypotheses are supported by the observation that acute administration of either VDM-11 or AA-5-HT immediately before the first passive-avoidance test did not affect in either way the performance in this test of both BAP-injected and healthy mice (data not shown). Furthermore, the worsening effect on memory retention in healthy animals, observed with

both VDM-11 and AA-5-HT when administered starting 7 days after vehicle, significantly decreased when passing from the first to the second retention test (i.e. from 1 to 8 days after the last administration of the compounds) (Figs. 9, 10), thus indicating that this effect, also when observed in BAP-treated mice, is due to a direct action on mnemonic processes rather than to a negative and long-lasting effect on neuronal damage. On the other hand, the beneficial action on memory retention, observed with VDM-11 when administered 3 days after BAP, was also less efficacious when assessed after the second test (Fig. 9), probably because of both the progress of β -amyloid-induced neurotoxicity (deduced from the decrease of retention in BAP+vehicle-treated mice at the second retention test) and loss of efficacy of the compound.

Other possible explanations for the observed bi-phasic effects of VDM-11 on memory retention should also be taken into consideration. For example, we observed that the elevation of mouse brain anandamide levels was significantly higher when this inhibitor was administered starting 7 days after BAP injection, whereas the opposite seems to occur with 2-AG levels, which are higher when VDM-11 is administered 3 days after BAP (Fig. 7a). As this phenomenon was observed also with AA-5-HT (Fig. 8a), one could also hypothesize that the elevation of anandamide, rather than 2-AG, levels was responsible for the memory-impairing actions of inhibitors of endocannabinoid inactivation. This possibility is supported by the fact that, unlike 2-AG, anandamide also activates the vanilloid TRPV1 receptor [17], which is expressed in the hippocampus, where it appears to enhance GABAergic neurotransmission [57]. However, it should be noted that in the rat hippocampus, neuroprotection from BAP-induced neuronal damage corresponded to elevation of anandamide levels (Figs. 5 and 6).

Several studies have been performed previously in hippocampal neurons *in vitro* to identify the molecular mechanisms through which endocannabinoids and cannabinoids exert their neuroprotective effects. For example, stimulation of CB₁ receptors was shown to reduce NMDA-receptor-induced excitotoxicity by reducing [Ca²⁺]_i spiking and cell death [58]. More recent results suggest that cannabinoids prevent cell death by initiating a time- and dose-dependent inhibition of adenyl cyclase that is capable of reducing [Ca²⁺]_i via a cAMP/PKA-dependent process during the neurotoxic event [59]. These inhibitory effects on [Ca²⁺]_i might underlie the neuroprotective effects of endocannabinoids observed here, since BAP 1–42 was recently found to increase dendritic Ca²⁺ influx and loss of Ca²⁺ homeostasis in hippocampal neurons, two events that initiate neuronal degenerative processes in the hippocampus [60].

In conclusion, we have provided evidence that both early and strong pharmacological elevation of brain endocannabinoid concentrations can provide protection against BAP-induced neuronal damage or memory loss in ro-

dens. By contrast, when it is exerted at a later phase of β -amyloid-induced neurotoxicity (or when it is not strong enough, as with AA-5-HT), boosting of brain endocannabinoid levels has no effect on neuronal damage and worsens memory loss in BAP-treated rats and mice, respectively. This resembles in some ways what has previously been described in animal models of Parkinson's disease, where both the enhancement of endocannabinoid signaling [61] or its counteraction [61–64] can reduce the consequences of this disorder. We did not investigate here the mechanisms through which endocannabinoids exert neuroprotection, because this was not the aim of our study and we would have had to test antagonists and inhibitors of all the possible receptors and proteins through which endocannabinoids, and anandamide in particular, have been reported to produce their effects to date (i.e., CB₁, CB₂ and TRPV1 receptors, ion channels, etc.) [see ref. 65 for a recent review on this issue] with subsequent use of an unethical number of laboratory animals and no guarantee of a successful outcome. Yet, our findings suggest that either agents that block [32] or prolong (this study) the action of endocannabinoids might be used in the future as novel therapeutic drugs against β -amyloid-induced neurotoxicity.

Acknowledgements. The authors are grateful to V. Guglielmotti, P. Orlando and M. Valenti for their valuable help, to the Italian Ministry of Education, University and Research (MIUR-FIRB to V. D. M., and PRIN 2004, to L. S. and T. I.) and the Volkswagen Stiftung (to V. D. M.) for support.

- 1 Di Marzo, V., Bifulco, M. and De Petrocellis, L. (2004) The endocannabinoid system and its therapeutic exploitation. *Nat. Rev. Drug Discov.* 3, 771–784.
- 2 Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., Gibson, D., Mandelbaum, A., Etinger, A. and Mechoulam, R. (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258, 1946–1949.
- 3 Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., Gopher, A., Almog, S., Martin, B. R., Compton, D. R., Pertwee, R. G., Griffin, G., Bayewitch, M., Barger, J. and Vogel, Z. (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50, 83–90.
- 4 Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A. and Waku, K. (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 215, 89–97.
- 5 Hampson, R. E. and Deadwyler, S. A. (1999) Cannabinoids, hippocampal function and memory. *Life Sci.* 65, 715–723.
- 6 Shen, M. and Thayer, S. A. (1999) Delta9-tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. *Mol. Pharmacol.* 55, 8–13.
- 7 Nagayama, T., Sinor, A. D., Simon, R. P., Chen, J., Graham, S. H., Jin, K. and Greenberg, D. E. (1999) Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J. Neurosci.* 19, 2987–2995.
- 8 Panikashvili, D., Simeonidou, C., Ben-Shabat, S., Hanus, L., Breuer, A., Mechoulam, R. and Shohami, E. (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature* 413, 527–531.

- 9 van der Stelt, M., Veldhuis, W. B., van Haften, G. W., Fezza, F., Bisogno, T., Bar, P. R., Veldink, G. A., Vliegthart, J. F., Di Marzo, V. and Nicolay, K. (2001) Exogenous anandamide protects rat brain against acute neuronal injury *in vivo*. *J. Neurosci.* 21, 8765–8771.
- 10 Hansen, H. H., Schmid, P. C., Bittigau, P., Lastres-Becker, I., Berrendero, F., Manzanares, J., Ikonomidou, C., Schmid, H. H., Fernandez-Ruiz, J. J. and Hansen, H. S. (2001) Anandamide, but not 2-arachidonoylglycerol, accumulates during *in vivo* neurodegeneration. *J. Neurochem.* 78, 1415–1427.
- 11 Veldhuis, W. B., van der Stelt, M., Wadman, M. W., van Zadelhoff, G., Maccarrone, M., Fezza, F., Veldink, G. A., Vliegthart, J. F., Bar, P. R., Nicolay, K. and Di Marzo, V. (2003) Neuroprotection by the endogenous cannabinoid anandamide and arvanil against *in vivo* excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases. *J. Neurosci.* 23, 4127–4133.
- 12 Marsicano, G., Goodenough, S., Monory, K., Hermann, H., Eder, M., Cannich, A., Azad, S. C., Cascio, M. G., Gutierrez, S. O., van der Stelt, M., Lopez-Rodriguez, M. L., Casanova, E., Schutz, G., Zieglansberger, W., Di Marzo, V., Behl, C. and Lutz, B. (2003) CB1 cannabinoid receptors and on-demand defense against excitotoxicity. *Science* 302, 84–88.
- 13 Wallace, M. J., Blair, R. E., Falenski, K. W., Martin, B. R. and DeLorenzo, R. J. (2003) The endogenous cannabinoid system regulates seizure frequency and duration in a model of temporal lobe epilepsy. *J. Pharmacol. Exp. Ther.* 307, 129–137.
- 14 Guzman, M. (2003) Neurons on cannabinoids: dead or alive? *Br. J. Pharmacol.* 140, 439–440.
- 15 Walter, L. and Stella, N. (2004) Cannabinoids and neuroinflammation. *Br. J. Pharmacol.* 141, 775–785.
- 16 van der Stelt, M., Hansen, H. H., Veldhuis, W. B., Bar, P. R., Nicolay, K., Veldink, G. A., Vliegthart, J. F. and Hansen, H. S. (2003) Biosynthesis of endocannabinoids and their modes of action in neurodegenerative diseases. *Neurotox. Res.* 5, 183–200.
- 17 Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Sorgard, M., Di Marzo, V., Julius, D. and Hogestatt, E. D. (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400, 452–457.
- 18 Pegorini, S., Braidia, D., Verzoni, C., Guerini-Rocco, C., Consalez, G. G., Croci, L. and Sala, M. (2005) Capsaicin exhibits neuroprotective effects in a model of transient global cerebral ischemia in Mongolian gerbils. *Br. J. Pharmacol.* 144, 727–735.
- 19 Fernandez-Ruiz, J., Lastres-Becker, I., Cabranes, A., Gonzalez, S. and Ramos, J. A. (2002) Endocannabinoids and basal ganglia functionality. *Prostaglandins Leukot. Essent. Fatty Acids* 66, 257–267.
- 20 Baker, D. and Pryce, G. (2003) The therapeutic potential of cannabis in multiple sclerosis. *Expert Opin. Invest. Drugs* 12, 561–567.
- 21 Benito, C., Nunez, E., Tolon, R. M., Carrier, E. J., Rabano, A., Hillard, C. J. and Romero, J. (2003) Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *J. Neurosci.* 23, 11136–11141.
- 22 Milton, N. G. (2002) Anandamide and noladin ether prevent neurotoxicity of the human amyloid-beta peptide. *Neurosci. Lett.* 332, 127–130.
- 23 Ramirez, B. G., Blazquez, C., Gomez del Pulgar, T., Guzman, M. and de Ceballos, M. L. (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J. Neurosci.* 25, 1904–1913.
- 24 Lichtman, A. H., Varvel, S. A. and Martin, B. R. (2002) Endocannabinoids in cognition and dependence. *Prostaglandins Leukot. Essent. Fatty Acids* 66, 269–285.
- 25 Davies, S. N., Pertwee, R. G. and Riedel, G. (2002) Functions of cannabinoid receptors in the hippocampus. *Neuropharmacology* 42, 993–1007.
- 26 Castellano, C., Cabib, S., Palmisano, A., Di Marzo, V. and Puglisi-Allegra, S. (1997) The effects of anandamide on memory consolidation in mice involve both D1 and D2 dopamine receptors. *Behav. Pharmacol.* 8, 707–712.
- 27 Hernandez-Tristan, R., Arevalo, C., Canals, S. and Leret, M. L. (2000) The effects of acute treatment with delta9-THC on exploratory behaviour and memory in the rat. *J. Physiol. Biochem.* 56, 17–24.
- 28 Castellano, C., Rossi-Arnaud, C., Cestari, V. and Costanzi, M. (2003) Cannabinoids and memory: animal studies. *Curr. Drug Targets CNS Neurol. Disord.* 2, 389–402.
- 29 Lichtman, A. H. (2000) SR 141716A enhances spatial memory as assessed in a radial-arm maze task in rats. *Eur. J. Pharmacol.* 404, 175–179.
- 30 Wolff, M. C. and Leander, J. D. (2003) SR141716A, a cannabinoid CB1 receptor antagonist, improves memory in a delayed radial maze task. *Eur. J. Pharmacol.* 477, 213–217.
- 31 Hampson, R. E., Simeral, J. D., Kelly, E. J. and Deadwyler, S. A. (2003) Tolerance to the memory disruptive effects of cannabinoids involves adaptation by hippocampal neurons. *Hippocampus* 13, 543–556.
- 32 Mazzola, C., Micale, V. and Drago, F. (2003) Amnesia induced by beta-amyloid fragments is counteracted by cannabinoid CB1 receptor blockade. *Eur. J. Pharmacol.* 477, 219–225.
- 33 Flood, J. F., Morley, J. E. and Roberts, E. (1991) Amnesic effects in mice of four synthetic peptides homologous to amyloid beta protein from patients with Alzheimer disease. *Proc. Natl. Acad. Sci. USA* 88, 3363–3366.
- 34 Emre, M., Geula, C., Ransil, B. J. and Mesulam, M. M. (1992) The acute neurotoxicity and effects upon cholinergic axons of intracerebrally injected beta-amyloid in the rat brain. *Neurobiol. Aging* 13, 553–559.
- 35 Harkany, T., Hortobagyi, T., Sasvari, M., Konya, C., Penke, B., Luiten, P. G. and Nyakas, C. (1999) Neuroprotective approaches in experimental models of beta-amyloid neurotoxicity: relevance to Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 23, 963–1008.
- 36 Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., Moriello, A. S., Davis, J. B., Mechoulam, R. and Di Marzo, V. (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *Br. J. Pharmacol.* 134, 845–852.
- 37 Iuvone, T., Esposito, G., Esposito, R., Santamaria, R., Di Rosa, M. and Izzo, A. A. (2004) Neuroprotective effect of cannabidiol, a non-psychoactive component from *Cannabis sativa* on β -amyloid-induced toxicity in PC12 cells. *J. Neurochem.* 89, 134–141.
- 38 Esposito, G., De Filippis, D., Carnuccio, R., Izzo, A. A. and Iuvone, T. (2005) The marijuana component cannabidiol inhibits beta-amyloid-induced tau protein hyperphosphorylation through Wnt/beta-catenin pathway rescue in PC12 cells. *J. Mol. Med.* 84, 253–258.
- 39 Kowall, N. W., Beal, M. F., Busciglio, J., Duffy, L. K. and Yankner, B. A. (1991) An *in vivo* model for neurodegenerative effects of β -amyloid and protection by substance, P. *Proc. Natl. Acad. Sci. USA* 88, 7247–7251.
- 40 De Petrocellis, L., Bisogno, T., Davis, J. B., Pertwee, R. G. and Di Marzo, V. (2000) Overlap between the ligand recognition properties of the anandamide transporter and the VR1 vanilloid receptor: inhibitors of anandamide uptake with negligible capsaicin-like activity. *FEBS Lett.* 483, 52–56.
- 41 Chan, P. H., Kawase, M., Murakami, K., Chen, S. F., Li, Y., Calagui, B., Reola, L., Carlson, E. and Epstein, C. J. (1998) Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after global cerebral ischemia and reperfusion. *J. Neurosci.* 18, 8292–8299.
- 42 Maurice, T., Lockhart, B. P. and Privat, A. (1996) Amnesia induced in mice by centrally administered beta-amyloid peptides involves cholinergic dysfunction. *Brain Res.* 706, 181–193.

- 43 Bisogno, T., Melck, D., De Petrocellis, L., Bobrov, M. Yu., Gretskaya, N. M., Bezuglov, V. V., Sitachitta, N., Gerwick, W. H. and Di Marzo, V. (1998) Arachidonoylserotonin and other novel inhibitors of fatty acid amide hydrolase. *Biochem. Biophys. Res. Commun.* 248, 515–522.
- 44 Venault, P., Chapouthier, G., de Carvalho, L. P., Simiand, J., Morre, M., Dodd, R. H. and Rossier, J. (1986) Benzodiazepine impairs and beta-carboline enhances performance in learning and memory tasks. *Nature* 321, 864–866.
- 45 de Lago, E., Ligresti, A., Ortas, G., Morera, E., Cabranes, A., Pryce, G., Bifulco, M., Baker, D., Fernandez-Ruiz, J. and Di Marzo, V. (2004) *In vivo* pharmacological actions of two novel inhibitors of anandamide cellular uptake. *Eur. J. Pharmacol.* 484, 249–257.
- 46 Heffner, T. G., Hartman, J. A. and Seiden, L. S. (1980) A rapid method for the regional dissection of the rat brain. *Pharmacol. Biochem. Behav.* 13, 453–456.
- 47 Schmid, P. C., Krebsbach, R. J., Perry, S. R., Dettmer, T. M., Maasson, J. L. and Schmid, H. H. (1995) Occurrence and post-mortem generation of anandamide and other long-chain N-acyl-ethanolamines in mammalian brain. *FEBS Lett.* 375, 117–120.
- 48 Di Marzo, V., Goparaju, S. K., Wang, L., Liu, J., Batkai, S., Jarai, Z., Fezza, F., Miura, G. I., Palmiter, R. D., Sugiura, T. and Kunos, G. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature* 410, 822–825.
- 49 Scali, C., Proserpi, C., Giovannelli, L., Bianchi, L., Pepeu, G. and Casamenti, F. (1999) Beta(1–40) amyloid peptide injection into the nucleus basalis of rats induces microglia reaction and enhances cortical gamma-aminobutyric acid release *in vivo*. *Brain Res.* 831, 319–321.
- 50 Richardson, R. L., Kim, E. M., Shephard, R. A., Gardiner, T., Cleary, J. and O'Hare, E. (2002) Behavioural and histopathological analyses of ibuprofen treatment on the effect of aggregated A β (1–42) injections in the rat. *Brain Res.* 954, 1–10.
- 51 de Lago, E., Fernandez-Ruiz, J., Ortega-Gutierrez, S., Cabranes, A., Pryce, G., Baker, D., Lopez-Rodriguez, M. and Ramos, J. A. (2006) UCM707, an inhibitor of the anandamide uptake, behaves as a symptom control agent in models of Huntington's disease and multiple sclerosis, but fails to delay/arrest the progression of different motor-related disorders. *Eur. Neuropsychopharmacol.* 16, 7–18.
- 52 Ramirez, G., Toro, R., Dobeli, H. and von Bernhardi, R. (2005) Protection of rat primary hippocampal cultures from A β cytotoxicity by pro-inflammatory molecules is mediated by astrocytes. *Neurobiol. Dis.* 19, 243–254.
- 53 Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagan, T. and Ichijo, H. (2005) Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Differ.* 12, 19–24.
- 54 Patel, S., Roelke, C. T., Rademacher, D. J., Cullinan, W. E. and Hillard, C. J. (2004) Endocannabinoid signaling negatively modulates stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Endocrinology* 145, 5431–5438.
- 55 Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M. G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E. J., Gangadharan, U., Hobbs, C., Di Marzo, V. and Doherty, P. (2003) Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *J. Cell. Biol.* 163, 463–468.
- 56 Farooqui, A. A., Liss, L. and Horrocks, L. A. (1988) Neurochemical aspects of Alzheimer's disease: involvement of membrane phospholipids. *Metab. Brain Dis.* 3, 19–35.
- 57 Al-Hayani, A., Wease, K. N., Ross, R. A., Pertwee, R. G. and Davies, S. N. (2001) The endogenous cannabinoid anandamide activates vanilloid receptors in the rat hippocampal slice. *Neuropharmacology* 41, 1000–1005.
- 58 Shen, M. and Thayer, S. A. (1999) Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol. Pharmacol.* 54, 459–462.
- 59 Zhuang, S. Y., Bridges, D., Grigorenko, E., McCloud, S., Boon, A., Hampson, R. E. and Deadwyler, S. A. (2005) Cannabinoids produce neuroprotection by reducing intracellular calcium release from ryanodine-sensitive stores. *Neuropharmacology* 48, 1086–1096.
- 60 Chen, C. (2005) Beta-amyloid increases dendritic Ca²⁺ influx by inhibiting the A-type K⁺ current in hippocampal CA1 pyramidal neurons. *Biochem. Biophys. Res. Commun.* 338, 1913–1919.
- 61 Maccarrone, M., Gubellini, P., Bari, M., Picconi, B., Battista, N., Centonze, D., Bernardi, G., Finazzi-Agrò, A. and Calabresi, P. (2003) Levodopa treatment reverses endocannabinoid system abnormalities in experimental parkinsonism. *J. Neurochem.* 85, 1018–1025.
- 62 Di Marzo, V., Hill, M. P., Bisogno, T., Crossman, A. R. and Brotchie, J. M. (2000) Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. *FASEB J.* 14, 1432–1438.
- 63 Fernandez-Espejo, E., Caraballo, I., de Fonseca, F. R., El Banoua, F., Ferrer, B., Flores, J. A. and Galan-Rodriguez, B. (2005) Cannabinoid CB1 antagonists possess antiparkinsonian efficacy only in rats with very severe nigral lesion in experimental parkinsonism. *Neurobiol. Dis.* 18, 591–601.
- 64 van der Stelt, M., Fox, S. H., Hill, M., Crossman, A. R., Petrosino, S., Di Marzo, V. and Brotchie, J. (2005) A role for endocannabinoids in the generation of parkinsonism and levodopa-induced dyskinesia in MPTP-lesioned non-human primate models of Parkinson's disease. *FASEB J.* 19, 1140–1142.
- 65 Di Marzo, V. and De Petrocellis, L. (2006) Non-CB1, non-CB2 receptors for endocannabinoids. In: *Endocannabinoids* (Onaivi E., Sugiura T. and Di Marzo V., Eds.), pp. 151–174. CRC-Press/Taylor & Francis, Boca Raton.

