Anandamide (AEA) is an endogenous agonist of type 1 cannabinoid receptors (CB1R) that, along with metabolic enzymes of AEA and congeners, compose the “endocannabinoid system (ECS)”. Here, we report the biochemical, morphological and functional characterization of the ECS in human neuroblastoma SH-SY5Y cells, that are an experimental model for neuronal cell damage and death, as well as for major human neurodegenerative disorders. We also show that AEA dose-dependently induced apoptosis of SH-SY5Y cells. Through proteomic analysis, we further demonstrate that AEA-induced apoptosis was paralleled by a ~3 to ~5-fold up-regulation or down-regulation of five genes: BiP/GRP78, stress-induced phosphoprotein-1, and triosephosphate isomerase-1, that were up-regulated, are known to act as anti-apoptotic agents; actin-related protein 2/3 complex subunit 5, and peptidylprolyl isomerase-like protein 3 isoform PPIL3b were down-regulated, and the first is required for actin network formation whereas the second is still function-orphan. Interestingly, only the effect of AEA on BiP was reversed by the CB1R antagonist SR141716, in SH-SY5Y cells as well as in human neuroblastoma LAN-5 cells (that express a functional CB1R), but not in SK-NBE cells (that do not express CB1R). Silencing or over-expression of BiP increased or reduced, respectively, AEA-induced apoptosis of SH-SY5Y cells. In addition, the expression of BiP and of the BiP-related apoptotic markers p53 and PUMA was increased by AEA, through a CB1R-dependent pathway that engages p38 and p42/44 mitogen-activated protein kinases. Consistently, this effect of AEA was minimized by SR141716. In conclusion, we identified BiP as a key-protein in neuronal apoptosis induced by AEA.

Endocannabinoids bind to and activate both type-1 (CB1R) and type-2 (CB2R) cannabinoid receptors, and are widely recognized as important regulators of central and peripheral functions (1-3). The most studied endocannabinoids are anandamide (N-arachidonoyl ethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) (1,3). AEA, unlike 2-AG, binds to and activates also transient receptor potential vanilloid 1 (TRPV1), thus being considered a true “endovanilloid” (4).
The “endocannabinoid system (ECS)” includes the receptors, their ligands AEA and 2-AG and the enzymes that synthesize (N-acyl-phosphatidylethanolamines-hydrolyzing phospholipaseD, NAPE-PLD and diacylglycerol lipase, DAGL), or degrade (fatty acid amide hydrolase, FAAH and monoaoylglycerol lipase, MAGL) AEA and 2-AG respectively (1-3). On the other hand, there is still controversy about the identity and even the existence of purported “endocannabinoid transporters”, that have not yet been cloned, isolated or characterized (5). A growing interest is concerned with the AEA ability to induce apoptosis in neuronal and non-neuronal cells (for comprehensive reviews see 6-9). Such a pro-apoptotic activity of AEA appears to be mediated by divergent pathways, each potentiated, reduced or unaffected by CB or TRPV1 receptors (6-9). They can also be dependent on membrane cholesterol content (10,11), generation of reactive oxygen species (12,13), or even release of ethanolamine from AEA catalyzed by FAAH (14). In this context, it should be stressed that data on ECS and AEA-induced apoptosis in human neuronal cells are still very scant. Furthermore, a proteomic analysis of AEA-induced apoptosis has never been performed, leaving open the question of which proteins (if any) might be modulated at the level of expression upon induction of programmed cell death by this endocannabinoid. On this basis, we sought to characterize through functional and immunochemical assays, and confocal microscopy, the ECS components in human neuroblastoma SH-SY5Y cells. In addition, we performed a proteomic analysis of AEA-induced apoptosis in these cells, that identified five proteins whose expression was modified. Among these proteins, one (BiP/GRP78) was shown to be modulated by AEA in a CB1R-dependent manner, in parallel with apoptosis. Through silencing and over-expression experiments it was found to be a key player in the death programme.

Experimental Procedures

Materials and antibodies - Anandamide (N-arachidonoylethanolamine, AEA), 5-(1,10-dimethylheptyl)-2-[1R,5R-hydroxy-2R-(3-hydroxypropyl)-cyclohexyl]phenol (CP55.940), 4',6-diamino-2-phenylindole (DAPI), SB203580 and PD98059 were purchased from Sigma Chemical Co. (St. Louis, MO), 2-Arachidonoylglycerol (2-AG) and resiniferatoxin (RTX) were from Alexis Corporation (San Diego, CA). N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-3-pyrazole carboxamide (SR141716) and N-[(1S)-endo-1,3,3-trimethy-1-bicyclo [2.2.1]-heptan-2-yl]5-(4-choro-3-methyl-phenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide (SR144528) were kind gifts of Sanofi-Aventis Recherche (Montpellier, France). Iodo-resiniferatoxin (I-RTX) was from Tocris-Cookson (Bristol, UK). [3H]AEA (205 Ci/mmol), [3H]CP55.940 (126 Ci/mmol) and [3H]RTX (43 mCi/mmol) were from Perkin Elmer Life Sciences (Boston, MA). [3H]N-Arachidonoylphosphatidylethanolamine ([3H]NArPE, 200 Ci/mmol) and [3H]2-oleoylglycerol ([3H]2-OG, 20 Ci/mmol) were purchased from ARC (St. Louis, MO). [14C]1-Stearoyl-2-arachidonoyl-sn-glycerol ([14C]DAG, 56 mCi/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden). Rabbit anti-CB1R polyclonal antibodies were purchased from Affinity Bioreagents (Golden, CO). Rabbit anti-CB2R, anti-NAPE-PLD and anti-MAGL polyclonal antibodies were from Cayman Chemical Co. (Ann Arbor, MI). Rabbit anti-DAGL antibodies were from Frontier Science co. ltd (Hokkaido, Japan), and rabbit anti-TRPV1 and anti-β-actin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-BiP polyclonal antibodies were from Abcam (Cambridge, UK). Mouse monoclonal anti-cleaved PARP (Asp214) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Goat Alexa Fluor-conjugates secondary
antibodies, Image-iTTM FX signal enhancer and Prolong antifade kit were from Molecular Probes (Eugene, OR). Goat anti-rabbit and goat anti-mouse antibodies conjugated to horseradish peroxidase (GAR-HRP and GAM-HRP, respectively) were from Santa Cruz Biotechnology.

**Cell culture and determination of apoptosis** - Human neuroblastoma SH-SY5Y cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 15% inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 1 mM sodium pyruvate, 1 mM Hepes and 1 mM non-essential amino acids. Human neuroblastoma SK-NBE cells and human neuroblastoma LAN-5 cells were grown in RPMI medium, supplemented with 20% inactivated FBS, 1 mM sodium pyruvate, 1 mM Hepes. All cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere. SH-SY5Y cells were settled on a glass coverslip at a density of 2x10⁴ cells/cm², and after one day they were treated for 24 h with different concentrations of AEA (in the range 0 - 12 μM), with 2-AG (12 μM), or with vehicle alone (controls). SK-NBE and LAN-5 cells were treated for 24 h with 10 μM AEA under the same experimental conditions. After each treatment, cell viability was measured by Trypan blue exclusion (15).

For quantitation of apoptosis, cells were fixed for 10 min at room temperature with 3% paraformaldehyde (PFA), and then were permeabilized with 0.1% Triton X-100 (TX-100) in phosphate-buffered saline (PBS) for 2 min at 4°C. After a blocking step in Image-iTTM FX signal enhancer for 30 min, cleaved PARP was identified by mouse anti-PARP antibody (diluted 1:500 in Image-iTTM FX signal enhancer), after incubation of cells for 1 hour at room temperature. Secondary antibody conjugated to Alexa Fluor 488 was diluted 1:200 in Image-iTTM FX signal enhancer, and was incubated with the specimens for 30 min at room temperature. Cell nuclei were counterstained with DAPI (0.1 μg/mL in PBS). After washing, the coverslips were mounted using the antifade prolong Gold reagent, and were visualized by Leica TCS SP confocal microscope. Pictures were taken using the LAS AF program (Leica Microsystems, Wetzlar, Germany), and then processed with Adobe Photoshop (Mountain View, CA) for brightness and contrast adjustments. The number of cells with apoptotic morphology was assessed in five randomly chosen fields. For each treatment, 200–300 cells were counted. In SK-NBE and LAN-5 cells, and in BiP-silenced or over-expressing cells, apoptosis was quantified by fluorocytometric analysis. Cells were trypsinized and collected by centrifugation. Pellets were fixed with 70% ethanol for 12 h at -20 °C, were centrifuged again, and were incubated in RNase solution (200 μg/ml) at room temperature for 10 min, and then with DNA-staining solution with propidium iodide (50 μg/ml) for further 10 min. Then, samples were scanned using a Coulter Epics XL (Beckman Coulter, Fullerton, CA) under conditions allowing the measurement of propidium iodide fluorescence. Apoptotic cells appeared as a hypodiploid (sub G1) peak due to nuclear fragmentation and loss of DNA (16).

**Cell transfection** - To construct expression plasmids, total RNA from human neuroblastoma SH-SY5Y cells was extracted and complementary DNA (cDNA) was synthesized by reverse transcription (see the qRT-PCR analysis section below). A full length c-terminal hemagglutinin (HA) epitope-tagged BiP was amplified by using the specific forward and reverse primers, that included NheI and XhoI restriction sites respectively. The polymerase chain reaction (PCR) product was purified and cloned into pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). For transient transfection, SH-SY5Y cells were plated on 12-well plates (Iwaki, Tokyo, Japan) and after 24 h they were transfected with plasmid encoding HA-tagged BiP, or empty vector as control, by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Briefly, 1.6 μg of plasmid and 4 μl of Lipofectamine 2000 were mixed in a total volume of 200 μl, and were added to cells.
After 6 h, 10 μM AEA was added to the medium, alone or in the presence of 1 μM SR141716, and 24 h later cells were harvested and subjected to either apoptosis quantitation or total protein extraction for Western blotting analysis.

Functional assays of ECS: Metabolism of AEA - The synthesis of AEA through NAPE-PLD was assayed in SH-SY5Y cell homogenates (200 μg protein/test), by measuring the release of [³H]AEA from 100 μM [³H]NArPE by reverse phase-high performance liquid chromatography (RP-HPLC), coupled to online scintillation counting (17). The hydrolysis of AEA by FAAH in SH-SY5Y cell homogenates (50 μg protein/test) was evaluated by RP-HPLC, using 10 μM [³H]AEA as substrate (18). Both NAPE-PLD and FAAH activities were expressed as pmol product released per min per mg protein.

Metabolism of 2-AG - To evaluate the synthesis of 2-AG by DAGL, SH-SY5Y cell homogenates (200 μg protein/test) were incubated at 37°C for 30 min with 500 μM [¹⁴C]DAG, as reported (19). Then, a mixture of chloroform/methanol (2:1, v/v) was added to stop the reaction, and the organic phase was dried and fractionated by TLC on silica, using polypropylene plates with chloroform/methanol/NH₄OH (94:6:0.3, v/v/v) as eluent. The release of [¹⁴C]2-AG was measured by cutting the corresponding TLC spots, followed by scintillation counting. The hydrolysis of 2-AG by MAGL was assayed in SH-SY5Y cell supernatants (100 μg protein/test), obtained at 39,000xg and incubated with 10 μM [³H]2-OG at 37°C for 30 min (20). The reaction was stopped with a mixture of chloroform/methanol (2:1, v/v), and the release of [³H]glycerol in the aqueous phase was measured in a β-counter (Perkin Elmer Life Science, Boston, MA). Both DAGL and MAGL activities were expressed as pmol product per min per mg protein.

CBR and TRPV1 receptor binding - For cannabinoid receptor studies, SH-SY5Y, SK-NBE or LAN-5 cells were resuspended in 2 mM Tris-EDTA, 320 mM sucrose, 5 mM MgCl₂ (pH 7.4), and then they were homogenized in a Potter homogenizer and centrifuged as reported (18). The resulting pellet was resuspended in assay buffer (50 mM Tris–HCl, 2 mM Tris-EDTA, 3 mM MgCl₂, 1 mM PMSF, pH 7.4), to a protein concentration of 1 mg/ml. The membrane preparation was divided into aliquots, that were stored at −80°C for no longer than 1 week. These membrane fractions (100 μg protein/test) were used in rapid filtration assays with the synthetic cannabinoid [³H]CP55.940 (400 pM), as described (18). Also binding of the TRPV1 agonist [³H]RTX (500 pM) to SH-SY5Y cells was evaluated by rapid filtration assays (18). Unspecific binding was determined in the presence of cold agonists (1 μM CP55.940 or 1 μM RTX), and was further corroborated by selective antagonists (0.1 μM SR141716 for CB1R, 0.1 μM SR144528 for CB2R, and 1 μM I-RTX for TRPV1), as reported (18).

Fluorescence microscopy studies of ECS - SH-SY5Y cells were plated on collagen-coated glass coverslips and were fixed 24 h later using 3% PFA plus 4% sucrose for 20 min at room temperature. Fixed cultures were permeabilized with 0.1% TX-100 in PBS for 10 min at room temperature. After a blocking step with 5% bovine serum albumin (BSA) in PBS for 45 min at room temperature, cells were incubated for 2 h with the following primary antibodies: anti-CB1R (1:400), anti-CB2R (1:400), anti-TRPV1 (1:100), anti-NAPE-PLD (1:400), anti-FAAH (1:200), anti-DAGL (1:200) or anti-MAGL (1:400), diluted in PBS with 5% BSA. Secondary antibodies conjugated with Alexa Fluor 488 were diluted 1:200 in PBS with 5% BSA, and were incubated with the specimens for 1 h at room temperature. After washing, the coverslips were mounted using the antifade Prolong Gold Reagent, and were visualized by Leica TCS SP confocal microscope (Leica Microsystems, Wetzlar, Germany). Pictures were taken using the LAS AF program (Leica Microsystems), and then were processed with Adobe Photoshop (Mountain View, CA) for adjustments of brightness and contrast.
Construction of plasmids for quantitative PCR - Human CB1R, CB2R, TRPV1, NAPE-PLD, FAAH, DAGL and MAGL genes used as internal standards were prepared by PCR from cDNA derived from SH-SY5Y, SK-NBE or LAN-5 cells, and were cloned into pcDNA 3.1 vector (Invitrogen, Carlsbad, CA). These constructs were checked by sequence analysis, and plasmid concentration was determined by optical density at 260 nm (OD260). Conversion to genome equivalents was based on the following formulas: OD260=1 corresponds to 50 μg/ml, and 1 bp corresponds to 660 g/mol. Standard curves were built up by serial dilutions (in 10-fold steps) of plasmids, that were included in each test. The copy number of each gene was calculated from the corresponding standard curve generated by the real time PCR software operating in the model 7700 sequence detector system (Applied Biosystems, Foster City, CA). This software calculates the standard curve for each PCR run, based on the Ct value for each gene. Then, a regression line is plotted and the corresponding equation is used to calculate the logarithm of the starting quantity and the copy number of the unknown gene.

qRT-PCR analysis - RNA was extracted from SH-SY5Y, SK-NBE or LAN-5 cells using the RNeasy extraction kit (Qiagen, Crawley, UK), as suggested by the manufacturer. Quantitative real time reverse transcriptase-polymerase chain reaction (qRT-PCR) assays were performed using the SuperScript III Platinum Two-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA). One μg total RNA was used to produce cDNA with 10 U/μL SuperScript III reverse transcriptase, in the presence of 2 U/μL RNaseOUT, 1.25 μM oligo(dT)20, 1.25 ng/μL random hexamers, 5mM MgCl2, 0.5 mM dNTP mix and DEPC-treated water. The reaction was performed using the following qRT-PCR program: 25°C for 10 min, 42°C for 50 min, 85°C for 5 min, then, after addition of 0.1 U/μL of E. coli RNase H, the product was incubated at 37°C for 20 min. The target transcripts were amplified by means of an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA), using the following primers: human CB1R F1 (5’-CTCTTTGCTGCCTAAATCCAC-3’), human CB1R R1 (5’-CCACTGCTCAAACATCTGAC-3’); human CB2R F1 (5’-TCAACCCTGTACATCTGCTC-3’), human CB2R R1 (5’-AGTCAGTCCCAACACTCACTC-3’); human TRPV1 F1 (5’-TCACCTACATCCCTGCTC-3’); human TRPV1 R1 (AAAGTCTTCAGCTGCTGACC); human NAPE-PLD F1 (5’-TTGTGAAATCCTGGGCAACATGG-3’); human NAPE-PLD R1 (5’-TACTGCAGATGTAGCAGCAGC-3’); human FAAH F1 (5’-CCCAATGGGCTTAAAGGACTG-3’), human FAAH R1 (5’-ATGAACCCGACAGACAAAC-3’); human DAGL F1 (5’-TTCCAAAGGGATTCCGTGACTGC-3’), human DAGL R1 (5’-TTGAAGCCCTTTGTGGTCGCC-3’); human MAGL F1 (5’-ATGCAGAAAAGACTACCTGGGC-3’); human MAGL R1 (5’-TTATTCGAGAGAGCAGC-3’); human BiP F1 (5’-AACATCCCTGTTGTTGGACC-3’); human BiP R1 (5’-TTGTCTTTCCATGACATCTTGGC-3’); human ARP5 F1 (5’-AAGAAACACAGTGTCGTCGG-3’); human ARP5 R1 (5’-ATCCACACCATTCTTGTCC-3’); human PPIL3b F1 (5’-TTGAAGTCTTTGAGAGGAGG-3’); human PPIL3b R1 (5’-AACACTCTACCATGATGC-3’); human STIP1 F1 (5’-ATCGATGATGCCTACAGTCGG-3’); human STIP1 R1 (5’-AAGAACTTAGAGCAGCTGC-3’); human TPI1 F1 (5’-TACTGCCATATCGACCTTGCG-3’); human TPI1 R1 (5’-ATCTGCGATGACCCCTTGTCTGC-3’); human p53 F1 (5’-
TTCAGGTGGCTGGAGTGAG-3');
human PUMA F1 (5'-ACCTCAACGCACAGTACGA-3');
human PUMA R1(5'-CTAATTGGGCTCCATCTCGG-3');
β-actin F1 (5'-TGACCCAGATCATGTTTGAG-3') and
β-actin R1 (5'-TTAATGTCACGCACGATTTCC-3').
β-Actin was used as housekeeping gene for
quantity normalisation. One µl of the first
strand of cDNA product was used for
amplification (in triplicate) in 25 µl
reaction solution, containing 12.5 µl of
Platinum SYBR Green qPCR SuperMix-
UDG (Invitrogen, Carlsbad, CA) and 10
pmol of each primer. The following PCR
program was used: 95°C for 10 min; 40
amplification cycles at 95°C for 30 sec,
56°C for 30 sec, and 72°C for 30 sec.

RNA interference - Synthetic ready-to-use
small (21 nucleotides) interfering RNA
(siRNA), complementary to a region of
BiP, and non-silencing control siRNAs
were custom synthetized by Qiagen
(Tokyo, Japan). SH-SY5Y cells were
transfected with 560 pmol of BiP siRNA
using the Lipofectamine 2000 reagent.
Briefly, 560 pmol siRNA and 28
µl Lipofectamine 2000 were  diluited with
Opti-MEM to a final volume of 3 ml, were
mixed and were added to SH-SY5Y cells
grown to 60% confluency in 10 mm
diameter plates. After 6 h, cells were
washed and cultured in fresh media for 24
h. Then, cells were washed and cultured
for further 24 h in media containing 10 µM
AEA, alone or in the presence of 1 µM
SR141716, and finally qRT-PCR analysis
and Western blotting were performed.

Immunochimical analysis - Western
blotting was performed according to
standard procedures (18). The following
antibody dilutions were used to
immunoelctect specific proteins: anti-CB1R
(1:200), anti-CB2R (1:200), anti-TRPV1
(1:200), anti-NAPE-PLD (1:200), anti-
FAAH (1:500), anti-DAGL (1:200), anti-
MAGL (1:200), anti-BiP (1:1500) and
anti-actin (1:1500). Enzyme-linked
immunosorbert assay (ELISA) of CB1R in
SH-SY5Y, SK-NBE or LAN-5 cells was
performed with anti-CB1R antibodies
diluted 1:200, as reported (18).

Proteomic analysis: Two dimensional gel
electrophoresis and image analysis - SH-
SY5Y cell pellets were sonicated in the
presence of protease inhibitors in 0.1 M
Tris-HCl (pH 7.2); for each sample 100 µg
of proteins were solubilized for 2 h in 350
µl rehydration buffer (4.4 M urea, 1.8 M
thiourea, 3.5% w/v CHAPS, 1.4% carrier
ampholytes (pH 3-10), 85 mM DTT, 0.02
% w/v bromophenol blue). For isoelectric
focusing (IEF), the Ettn IPGphor system
(Amersham Biosciences, Uppsala,
Sweden) and pH 3-10 IPG strips (18 cm,
non linear, Amersham Biosciences) were
used according to the manufacturer's
recommendations. The IPG strips were
rehydrated for 1 h at 0 V and for 8 h at 30
V in rehydration buffer containing protein
samples. Focusing was performed at 18°C,
50 µA/strip, in different steps: 1 h gradient
to 300 V, 1 h at 300 V, 3 h gradient to
3500 V, 3 h at 3500 V, 1.5 h gradient to
8000 V, plateau at 8000 V until 50000 Vh.
The focused gel strips were equilibrated 20
min in equilibration buffer (50 mM Tris-
HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30%
v/v glycerol, 0.02% w/v bromophenol
blue), containing 2% DTT, and for further
20 min in equilibration buffer containing
2.5% iodoacetamide. SDS-PAGE was
performed with gradient gels (9-16%
acrylamide/bisacrylamide), using the
PROTEAN II xi Cell system (Bio-Rad,
Hercules, CA). Each gel (18x20x1.5 cm)
was run at 50 mA and 10°C, until the
bromophenol blue frontier reached the gel
bottom. At least three replicates were
performed for each sample. After fixing,
the protein spots in analytical gels were
visualized by Vorum Silver Staining
(modified for MS) (21) and the stained gels
were scanned with a UMAX Image
Scanner (Amersham Bioscience). Image
analysis was done using the PDQuest
software (version 7.1.1; Bio-Rad). A
reference gel was selected from one of the
experimental gels and unmatched protein
spots of the remaining gels were manually
added to this reference gel to create the
Master Gel, a virtual image comprehensive of all matched spots derived from all analyzed samples. Subsequent to automatic spot detection and spot filtering, the matching of spots between gels was manually reviewed and adjusted as necessary. The abundance of each protein was estimated by the spot intensity. Only those with significant (at least 3-fold) and reproducible changes were considered to be differentially expressed proteins. Statistical analysis was performed applying the Student’s t-test with a confidence level of 95%, in order to estimate the significance of the expression differences of protein spots.

Protein identification - Protein spots of interest were excised from 2-DE gels and in-gel digestion was carried out as reported (22) with a solution of porcine trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate at 37°C for 16–18 h. The reaction was stopped by adding a final concentration of 0.1% trifluoroacetic acid (TFA). For the mass spectrometric analysis of tryptic digests, samples were concentrated and desalted by reverse phase extraction using ZipTip C18 (Millipore, Bedford, MA). Elution and spotting on MTP 384 Ground Steel target plate (Bruker Daltonik GmbH, Bremen, Germany) was obtained using a 0.5 g/l solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA. Samples were analyzed with a Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH), operating in a positive ion mode with a reflectron setup and equipped with a LIFT-cell; fragment ion analysis was performed by tandem mass spectrometry (MS/MS) after laser-induced dissociation. Mass spectra were externally calibrated with peptide standards from Bruker and internally calibrated with autodigest peaks of trypsin. Data obtained from both MS and MS/MS were used to search against the National Center for Biotechnology Information (NCBI) human database, using the peptide search routine MASCOT (Matrix Science, London, UK). Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was allowed as variable modification of the peptides. The query was performed with the maximal tolerance of 100 ppm for peptide mass fingerprinting, and a mass tolerance of 0.5 Da for MS/MS ion search. For positive identification protein scores > 65 and MS/MS ion scores > 35 were considered significant (p<0.05), as calculated by the MASCOT scoring algorithm.

Statistical analysis - Data reported in this paper are the means ± SD of at least three independent experiments, each performed in triplicate. Unless stated otherwise, statistical analysis was performed by the nonparametric Mann-Whitney U test, elaborating experimental data by means of the InStat 3 program (GraphPAD Software for Science, San Diego, CA).

RESULTS

ECS in SH-SY5Y cells. The presence of an endocannabinoid system in human SH-SY5Y cells was demonstrated at transcriptional, translational and functional level. First of all, qRT-PCR analysis demonstrated mRNA transcripts for the main elements of the ECS, including CB1R, CB2R, TRPV1, NAPE-PLD, FAAH, DAGL and MAGL. In particular, TRPV1 and MAGL transcripts were the most abundant, followed by NAPE-PLD, FAAH and DAGL, while both CB receptors were expressed least (Table 1). Next, translation of ECS mRNAs into the corresponding proteins was assessed by Western blotting (Fig. 1A) and immunofluorescence (Figs 1B-H). Western blotting showed that all ECS elements detected by qRT-PCR were also expressed at the translational level, although TRPV1 and MAGL proteins were not abundant as their transcripts (compare Table 1 and Fig. 1A). Confocal microscopy further supported the presence of ECS components in SH-SY5Y cells, where intense and specific immunostaining for
FAAH, NAPE-PLD, TRPV1 and, to a lower extent, for CB1R, CB2R, DAGL and MAGL were seen (Figs 1B-H). Finally, the activity of the ECS elements was tested by biochemical assays, demonstrating that the enzymes and receptors detected in SH-SY5Y cells were all functional (Table 2). In particular, it was found that the binding of the synthetic cannabinoid \[^3H\]CP55,940, a CB1R and CB2R agonist (23), was displaced to ~50% of the controls by either 0.1 µM SR141716, a selective CB1R antagonist, or 0.1 µM SR144528, a selective CB2R antagonist (23). These data, shown in Table 2, indicate that both CB receptor subtypes were functional in human SH-SY5Y cells.

**Induction of apoptosis by AEA.** AEA has been shown to trigger apoptosis at micromolar concentrations (10-15), i.e. those found centrally and peripherally in a number of neurological disorders (24, and references therein). In effect micromolar concentrations of AEA are able to trigger significant neuronal loss (25). Here, treatment of SH-SY5Y cells for 24 h with different amounts of AEA (in the range 0-12 µM) was able to reduce dose-dependently cell viability, to ~60% of controls at 10 µM (not shown). In order to ascertain whether the observed cytotoxicity was indeed due to programmed cell death, PARP cleavage, a hallmark of apoptosis, was measured. Immunofluorescence analysis revealed that AEA induced dose-dependently SH-SY5Y cell apoptosis, with ~40% of apoptotic cells at 10 µM AEA (Fig. 2A). Furthermore, 1 µM SR141716 rescued the apoptosis induced by 10 µM AEA, whereas SR144528 or I-RTX were ineffective at the same concentration (Figs. 2B-C). Interestingly, 2-AG was unable to induce any apoptosis of SH-SY5Y cells even at 12 µM (Fig. 2A), further extending previous data on other cell systems (15).

**Protein expression patterns of control and AEA-treated SH-SY5Y cells.** In order to map the differential protein expression levels upon 24 h exposure of human SH-SY5Y cells to 10 µM AEA, alone or in the presence of 1 µM SR141716 we performed a proteomic analysis. Proteins were separated by two dimensional gel electrophoresis (2-DE), and visualized by silver staining (Fig. 3). Experiments were performed in triplicate. An average of 372 ± 3 (mean ± SD) spots were resolved on the SH-SY5Y gels, and 326 ± 5 spots and 320 ± 2 spots were visible for AEA- and AEA+SR141716-treated cell maps, respectively. After digital image analysis, the abundance of each single protein spot in gels from different cell preparations was estimated by its intensity, and the results were evaluated in terms of spot units of signal intensity (optical density, OD), expressed as parts per million (ppm). The ratios of the mean normalized spot intensity of treated versus control gels were calculated for spots identified in every three replicates of each sample. Five proteins were chosen for further analysis, because they were present in AEA-treated cells with at least a three-fold (p<0.05) up- or down-regulation of OD values versus controls (Fig. 4). The selected proteins were in-gel digested with trypsin and the extracted peptides were analyzed by either MALDI-TOF-MS mass fingerprint or MALDI-TOF/TOF-MS/MS peptide sequencing. For both approaches, the acquired spectral data were compared to the theoretical molecular weight and fragmentation pattern of peptide sequence in a database, to identify the proteins by means of probabilistic assignments (p<0.05). This analysis identified BiP (GRP78), stress-induced phosphoprotein-1 (STIP1) and triosephosphate isomerase-1 (TPI1) as the three proteins whose expression was up-regulated by more than 3-fold over controls; actin-related protein 2/3 complex subunit 5 (ARP5) and peptidylprolyl isomerase-like protein 3 isoform PPIL3b (PPIL3b) as the two proteins whose expression was down-regulated by more than 3-fold to the controls (Table 3). We performed a literature search, in order to find out the function(s) of these proteins, and the results are summarized in Table 4. Remarkably, we found that only the effect of AEA on BiP protein expression was significantly reduced by SR141716 (Fig. 4).
mRNA expression of proteins modulated by AEA in apoptotic cells. In order to corroborate the proteomic analysis data, we evaluated the transcriptional abundance of the five proteins modulated upon AEA-induced apoptosis of SH-SY5Y cells. We found that mRNA levels followed the same trend of protein expression (Fig. 5). In particular, only the increase of BiP mRNA induced by AEA was significantly reduced by SR141716, in parallel with the reduced apoptosis. To further extend these data, we used two other human neuroblastoma cell lines, SK-NBE and LAN-5, after checking for the presence of functional CB1 receptors. While LAN-5 cells express the receptor at the mRNA and protein level, and show CB1R binding comparable to that of SH-SY5Y cells, SK-NBE cells do not (Fig. 6A). After treatment with 10 μM AEA, in the presence or absence of 1 μM SR141716, apoptosis (Fig. 6B) and BiP mRNA expression (Fig. 6C) in LAN-5 cells but not in SK-NBE cells were found to be similar to that observed in SH-SY5Y cells, further supporting that AEA-induced apoptosis and up-regulation of BiP were both mediated by CB1R.

Involvement of BiP in AEA-induced apoptosis

Thus a role of BiP in the AEA-induced apoptosis of human neuronal cells seems likely. Therefore we further explored whether the modulation of BiP upon AEA treatment was linked by a cause-effect relationship to the induction of programmed cell death, by silencing or over-expressing BiP gene in SH-SY5Y cells. Transfection of these cells with BiP-siRNA or with a plasmid encoding HA-tagged BiP efficiently reduced or enhanced, respectively, endogenous BiP mRNA (Fig. 7A) and protein level (Fig. 7B). The modulation of BiP expression was specific, as suggested by the lack of effect on the amount of β-actin in the same cells (Fig. 7B). In addition, non-silencing siRNAs, used as a control, did not affect neither mRNA nor protein levels of BiP, under the same experimental conditions (data not shown). Treatment with 10 μM AEA significantly enhanced BiP mRNA level in SH-SY5Y cells where BiP had been silenced, while 1 μM SR141716 counteracted this effect of AEA (Fig. 7A). Instead 10 μM AEA did not increase any further the BiP mRNA level in SH-SY5Y cells that over-expressed it (data not shown), possibly due to a saturating effect. Next, we investigated the effect of BiP silencing or over-expression on apoptosis, by fluorocytometric analysis (Fig. 7C). We found that, after the administration of 4 μM AEA in cells where BiP was silenced (siBiP), the apoptosis was almost ~4-fold higher than that in untreated siBiP cells (~20% versus ~5%), whereas the same amount of AEA only doubled apoptosis (~4% versus ~2%) in control cells. Administration of 1 μM SR141617 minimized AEA-induced apoptosis in both cases (Fig. 7C). On the other hand, 4 μM AEA failed to induce apoptosis in cells that overexpressed BiP (data not shown). We further extended our study to two BiP-related apoptotic markers: p53 and PUMA. As shown in Table 5, the level of these gene products increased after treatment of SH-SY5Y cells with 10 μM AEA, and again this effect of AEA was reversed by 1 μM SR141716.

Mechanism of AEA-induced apoptosis and BiP expression through CB1R. CB1R activation by AEA triggers two common signalling pathways that engage p38 and p42/44 mitogen-activated protein kinase (MAPK) (26,27). Therefore, we used selective inhibitors of p38 and p42/44 MAPKs at concentrations known to inhibit their target enzymes (28,29). Our results show that SB203580, a selective inhibitor of p38 MAPK (28), and PD98059, a selective inhibitor of p42/44 MAPKs at concentrations known to inhibit their target enzymes (28,29). Our results show that SB203580, a selective inhibitor of p38 MAPK (28), and PD98059, a selective inhibitor of p42/44 MAPKs (29), were able to fully prevent the AEA-induced increase of mRNA expression of BiP and of the BiP-related apoptotic markers p53 and PUMA (Table 5). Altogether, these data suggest that the apoptotic activity of AEA is mediated by CB1R, through a signalling cascade that triggers p38 and p42/44, leading to
increased expression of the ER-stress sensor BiP, and hence of the apoptotic markers p53 and PUMA.

**DISCUSSION**

In this investigation we demonstrate the presence of a fully functional endocannabinoid system in human neuroblastoma SH-SY5Y cells. In addition, we report the first proteomic analysis of the protein expression modulation in these cells upon induction of apoptosis by anandamide, that identifies BiP as a key-protein in the apoptotic programme induced by AEA in human neuronal cells.

Human SH-SY5Y cells were chosen for this study, because they have been widely used to investigate neuronal cell damage and death. For instance, they served as an *in vitro* model for Parkinson’s disease and associated neuronal apoptosis (30-35); to evaluate the protective effects of anti-apoptotic substances in a cellular model of Alzheimer’s disease (36); and to replicate *in vitro* some pathological changes observed in the brain of mice affected by the latter disease (37,38). In short, human SH-SY5Y cells are largely employed to evaluate neurotoxic conditions that lead to apoptosis (39-42). Remarkably, endocannabinoid signalling has been shown to play a central role in both Parkinson’s and Alzheimer’s diseases, as well as in other major human neurodegenerative or neuroinflammatory disorders (for a recent review see 43). In addition, the ECS tightly controls the cell choice between life and death (6,7,8), with a therapeutic impact for the treatment of cancer (44). Therefore, the demonstration of a complete and functional ECS should allow a better understanding of the role of ECS-related compounds and ECS-targeted drugs, in models of neurotoxicity based on SH-SY5Y cells.

Another major outcome of this study is the identification of five different proteins, whose expression is up-regulated (BiP, STIP1, TPI1) or down-regulated (ARP5 and PPIL3b) more than ~3-fold over controls in AEA-induced apoptotic SH-SY5Y cells. This is the first proteomic approach to the understanding of AEA pro-apoptotic activity. By the way, the first ever proteomic analysis on the effects of the cannabis-derived Δ⁹-tetrahydrocannabinol in chronic users (45), and in mouse cerebellum (46), appeared during the preparation of this manuscript. It seems noteworthy that the three proteins that are up-regulated by AEA are known to act as anti-apoptotic agents. In fact, BiP/GRP78 is a molecular chaperone of the heat shock protein 70 (Hsp70) family, and belongs to a defence mechanism against endoplasmic reticulum (ER) stress induced by accumulation of unfolded protein aggregates (unfolded protein response, UPR). BiP normally leads to a general inhibition of protein translation, to an increase in chaperone transcription and translation and to a cell cycle arrest; these processes allow rapid restoration of protein homeostasis, and avoid cell death due to the initiation of apoptotic pathways (47,48). In addition, up-regulation of BiP is mediated by the extracellular signal-regulated kinase (ERK)1/2 pathway (49), and it stabilizes mitochondrial permeability in cells treated with ER stress inducers (50,51). These observations suggest that BiP may inhibit apoptosis via a mitochondria-dependent route (52). In line with this, AEA induces apoptosis in neuronal cells through an ERK1/2- and mitochondria-dependent pathway (11). Here we have shown that programmed cell death and BiP expression are both dependent on p38 and p42/44 MAPK signalling triggered through CB1R. Therefore, the increase of BiP in apoptotic SH-SY5Y cells might represent a neuroprotective mechanism, aimed at counteracting AEA-induced cell death. For instance, BiP is upregulated in neurons of Alzheimer’s disease brains, where this protein has been indeed shown to play a neuroprotective role (53,54). Consistently, here we observed that over-expression of BiP confers resistance to AEA-induced apoptosis in human neuronal cells.
Conversely, programmed cell death induced by AEA was paralleled by the increased expression of two marker genes that are strictly related to ER stress-induced apoptosis: p53 and PUMA. The latter is known to be significantly up-regulated after prolonged exposure to classical ER stress inducers (55), and it has been previously identified as a p53 target (56,57). Human cells deficient in PUMA expression show a dramatically reduced apoptosis in response to ER stress (55), and induction of PUMA has been observed also in primary neurons and in response to cerebral ischemia in vivo, altogether suggesting that regulation of PUMA expression may be a common pathway during ER stress-induced cell death in mammals. Therefore the death programme triggered by AEA seems to engage typical executioners of ER stress-induced cell death (p53 and PUMA), along with the specific defence mechanism that counteracts it through BiP. This effect seems to extend well beyond a single cell-type and to be true also for human neuroblastoma cells that express CB1R.

On the other hand, other four proteins were affected by AEA at the transcriptional and translational level, although their modulation did not depend on CB1R, at variance with AEA-induced apoptosis. STIP1 is an Hsp70/Hsp90-organising protein (58), and it regulates protein folding and proteasomal degradation upon induction of neuronal apoptosis by staurosporine (42). Therefore, the upregulation of STIP1 in apoptotic cells seems to be neuroprotective similarly to that of the chaperone BiP. TPII is a homodimeric enzyme, which catalyses the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (59), and has an important role in glycolysis, gluconeogenesis, fatty acid synthesis, and the pentose cycle (60). Several reports have shown that TPII can be induced as a stress protein in mouse brain capillary endothelial cells (61) and can be up-regulated through an activator protein-1 (AP-1)-dependent pathway (62). It seems noteworthy that inhibition of TPII induces progressive and selective death of cortical and striatal neurons (63), and that in an in vitro model of acute pancreatitis it was overexpressed, suggesting an anti-apoptotic role (64). Therefore, also the up-regulation of this protein might contribute to neuroprotection against cell death induced by AEA. On the other hand, it remains to be clarified the role of the down-regulation of ARP5 and PPIL3b. In fact, the ARP complex is required for the formation of branched networks of actin filaments in the cell cortex (65). It has been implicated in abnormal cellular signal transduction, abnormal morphology of cancer cells, and even cancer development and progression (66). No information is yet available on the functional role of PPIL3b, but that this protein arises from a splicing variant of the cyclophilin-like gene PPIL3, whose mRNA was found to be ubiquitously transcribed in adult human tissues, low-expressed in human brain tissues and over-expressed in human glioma tissues (67,68). Recently, a direct binding between PPIL3 and the Apoptin protein was reported in cancer cells, where a PPIL3-controlled nuclear-cytoplasmic distribution of Apoptin suggested a possible role in apoptosis (69).

In conclusion, we have demonstrated the presence of a complete and fully functional endocannabinoid system in human SH-SHYSY cells, a widely used model for the study of neuronal cell damage and death, as well as for major human neurological disorders. In addition, we performed a proteomic analysis that identifies BiP as a key-protein in AEA-induced apoptosis of human neuronal cells. Incidentally, a recent study has documented the proapoptotic and potential antitumoral action of (endo)cannabinoids in glioma stem-like cells (70), that is in line with our work. We believe that an investigation on the regulation of cell survival and death by endocannabinoids in stem-derived neuronal cells is worthwhile also for therapeutic exploitation. In fact, endocannabinoid-targeted drugs are been considered for the treatment of neurodegenerative diseases (43), and
cancer (44), where apoptosis plays a central role and stem cells hold the promise of opening novel avenues of intervention.

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FOOTNOTES

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3The abbreviations used are: AEA, N-arachidonoylethanolamine (or anandamide); 2-AG, 2-arachidonylglycerol; ARP5, actin-related protein 2/3 complex subunit 5; BSA, bovine serum albumin; CB1/2R, type-1/2 cannabinoid receptor; CP55,940, 5-(1,10-dimethylheptyl)-2-[1R,5R-hydroxy-2R-(3-hydroxypropyl)-cyclohexyl]phenol; DAG, 1-stearoyl-2-arachidonoyl-sn-glycerol; DAGL, sn-1-specific diacylglycerol lipase; DAPI, 4’,6-diamino-2-phenylindole; 2-DE, two dimensional gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiotreitol; ECS, endocannabinoid system; FAAH, fatty acid amide hydrolase; FBS, fetal bovine serum; IEF, isoelectric focusing; I-RTX, iodo-resiniferatoxin; MAGL, monoacylglycerol lipase; MS, mass spectrometry; NAPE-PLD, N-acylphosphatidylethanolamine phospholipase D; NArPE, N-arachidonoyl-phosphatidylethanolamine; 2-OG, 2-oleoyl-glycerol; o/e, over-expression; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PPIL3b, peptidylprolyl isomerase-like protein 3 isoform PPIL3b; ppm, parts per million; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RP-HPLC, reverse phase-high performance liquid chromatography; RTX, resiniferatoxin; siRNA, small interfering RNA; STIP1, stress-induced phosphoprotein-1; SR141716, N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; SR144528, N-[(1S)-endo-1,3,3-trimethy-1-bicyclo[2.2.1]-heptan-2-yl]5-(4-choro-3-methyl-phenyl)-1-(4-methyl-benzyl)-pyrazole-3-carboxamide; TFA, trifluoroacetic acid; TPI1, triosephosphate isomerase-1; TRPV1, transient receptor potential vanilloid 1; TX-100, Triton X-100.

FIGURE LEGENDS

Fig. 1. The endocannabinoid system in human SH-SY5Y cells. (A) Western blotting of the ECS elements in SH-SY5Y cells. The expected molecular mass (Mr) of each protein is shown on the right-hand side. (B-H) Characterization of the ECS elements by immunofluorescence. SH-SY5Y cells were grown on glass coverslips to 40–50% confluence for 24 h, then were stained with the indicated antibodies for confocal laser-scanning microscopy. Non-specific immunofluorescence in these cells was negligible for all tested antibodies (data not shown). The subcellular localization of the receptors revealed weak cytoplasmic and nuclear staining for CB1R (B) and CB2R (C), and to a larger extent for
TRPV1 (D). Immunoreactivities for the enzymes NAPE-PLD (E), FAAH (F), DAGL (G) and MAGL (H) were mainly restricted to internal membranes, possibly those of the endoplasmic reticulum. Pictures were taken using a confocal microscope equipped with a 63X objective (numerical aperture = 1.4). Images are representative of at least three independent experiments, and five fields were examined for each treatment. Bars, 10 µm.

**Fig. 2.** AEA-induced apoptosis in SH-SY5Y cells. (A) Dose-dependent effect of endocannabinoids treatment (24 h) on PARP cleavage in SH-SY5Y cells, a marker of apoptotic nuclei. Induction of apoptosis was expressed as % of PARP staining. (B) Effect of 10 µM AEA, alone or in combination with 1 µM SR141716 (SR1), 1 µM SR144528 (SR2) or 2.5 µM Iodo-Resiniferatoxin (I-RTX) on apoptosis. (C) Effect of 10 µM AEA on PARP staining (green), and DAPI staining (blue), that was used to counterstain all nuclei. Data are shown as fold induction over controls (means ± SD) of four independent experiments. In panel A, *denotes p<0.05, and **p<0.01 versus controls. In panel B, *denotes p<0.01 versus controls; #denotes p< 0.01 versus AEA-treated cells. In panel C, pictures were taken using a confocal microscope equipped with a 40X objective (numerical aperture = 0.75). Reported images are representative of triplicate experiments. Bars, 75 µm.

**Fig. 3.** Representative 2-DE gels of SH-SY5Y cells, used for protein profiling. Cell lysates (100 µg protein) were fractionated over immobilized non-linear pH gradients (from 3 to 10) in the horizontal dimension, followed by fractionation by SDS-PAGE (9-16% gradient gels) in the vertical dimension. The gels were visualized by Silver Staining. The synthetic Master Gel was obtained from the image analysis (PDQuest). Labeled protein spots (circle: up-regulation; square: down-regulation) were differentially expressed in SH-SY5Y cells treated for 24 h with 10 µM AEA alone (AEA) or in the presence of 1 µM SR141716, versus controls (Ctrl).

**Fig. 4.** Histogram plot of statistically significant protein spot differences between SH-SY5Y cells untreated (Ctrl), or treated with 10 µM AEA alone (AEA) or in the presence of 1 µM SR141716. Polypeptide quantities were calculated in parts per million (ppm) of the total integrated optical density (OD). Mean and standard deviation (vertical bars) of each differentially regulated protein spot are shown. In all panels, *denotes p<0.01, and ** p<0.05 versus controls; #denotes p<0.05 versus AEA-treated cells.

**Fig. 5.** Effect of AEA on mRNA expression in human SH-SY5Y cells. Exposure to 10 µM AEA for 24 h induced a significant increase of the mRNA levels of BiP (~3-fold), STIP1 (~2-fold) and TPI1 (~7-fold) over controls. Instead, mRNA levels of ARP5 and PPIL3b were reduced by less than a half under the same experimental conditions. In the presence of 1 µM SR141716 the effect of AEA on BiP mRNA was minimized, whereas the expression of the other proteins was not significantly affected. Gene levels were measured by qRT-PCR, using β-actin as housekeeping gene to normalize the data. Values represent the means of at least four qRT-PCR experiments, each performed in triplicate. *Denotes p<0.01, and **p<0.05 versus controls; # denotes p<0.01 versus AEA-treated cells.

**Fig. 6.** Expression of CB1R in human SH-SY5Y, LAN-5 and SK-NBE cells, and effect of AEA on apoptosis and BiP mRNA expression. (A) Analysis of CB1R expression at the mRNA (by qRT-PCR) and protein level (by ELISA), and binding assays demonstrated a functional receptor in SH-SY5Y and LAN-5 cells, but not in SK-NBE cells. (B) Exposure to 10 µM AEA for 24 h induced apoptosis in LAN-5 cells (~4-fold over controls), but not in SK-NBE cells, and 1 µM SR141716 minimized this effect. (C) Exposure to 10 µM AEA for 24 h induced a significant increase of the mRNA levels of BiP (~2-fold) over controls in the case of LAN-5 cells, whereas SK-NBE cells did not show any significant change under the
same conditions. In the presence of 1 μM SR141716 the effect of AEA on BiP mRNA was minimized only in the case of LAN-5 cells. Gene levels were measured by qRT-PCR, using β-actin as housekeeping gene to normalize the data. In both panels, values represent the means of at least three independent experiments, each performed in triplicate. In panel B, *denotes p<0.001 versus controls; # denotes p<0.001 versus AEA-treated cells. In panel C, * denotes p<0.05 versus controls; # denotes p<0.05 versus AEA-treated cells.

Fig. 7. Effect of silencing and over-expression of BiP on its mRNA and protein content, and on AEA-induced apoptosis. (A) Modulation of BiP mRNA by RNA interference (siBiP) or over-expression (o/e BiP), and effect of 10 μM AEA, alone or in the presence of 1 μM SR141716. (B) SH-SY5Y cells were transfected with either BiP-siRNA or BiP-pcDNA3.1, and 48 h later cells were lysed, collected and subjected to Western blotting using anti-BiP or anti-β-actin antibodies. Control cells were transfected with non-silencing oligos or empty pcDNA3.1, as controls of si BiP or o/e BiP, respectively. Blots are representative of three independent experiments. (C) Apoptosis of SH-SY5Y cells, treated with 4 μM AEA for 24 h. In panels A and C, data are shown as fold induction over controls (means ± SD) of four independent experiments. In panel A, * denotes p<0.001 versus controls; # denotes p<0.001 versus si BiP; § denotes p<0.001 versus si BiP+AEA. In panel C, * denotes p<0.05 versus controls; ** denotes p<0.01 versus controls; # denotes p<0.05 versus AEA; § denotes p<0.01 versus si BiP; ^ denotes p<0.01 versus si BiP+AEA.
Table 1. Comparison of the qRT-PCR standard curves of the ECS elements in human SH-SY5Y cells.

| Genes   | Slope | $r^2$ | $C_t$     | Copies number* | mRNA copies ** |
|---------|-------|-------|-----------|----------------|----------------|
| CB1R    | -1.56 | 0.99  | 37.30±0.17| 2.03±0.01      | 3.64E-06       |
| CB2R    | -1.237| 0.98  | 34.60±0.22| 2.60±0.03      | 4.66E-06       |
| TRPV1   | -1.56 | 0.99  | 20.30±0.07| 112362.69±392.16| 2.02E-01       |
| NAPE-PLD| -1.454| 0.98  | 23.60±0.09| 998.48±3.99    | 1.79E-03       |
| FAAH    | -1.695| 0.99  | 26.20±0.06| 434.56±0.94    | 7.80E-04       |
| DAGL    | -1.113| 0.97  | 24.70±0.16| 5246.57±63.74  | 9.42E-03       |
| MAGL    | -1.734| 0.97  | 20.80±0.14| 14575.81±371.74| 2.62E-02       |

*Per 50 ng cDNA.
**Normalized to gene copies of the housekeeping gene β-actin.
Table 2. Activity of the ECS elements in human SH-SY5Y cells.

| ECS element                  | Activity   |
|------------------------------|------------|
| CBR                          | 31 ± 3a    |
| CBR + 0.1 µM SR141716        | 14 ± 2a,*  |
| CBR + 0.1 µM SR144528        | 16 ± 2a,*  |
| TRPV1                        | 45 ± 5a    |
| NAPE-PLD                     | 49 ± 4b    |
| FAAH                         | 86 ± 9b    |
| DAGL                         | 13 ± 3b    |
| MAGL                         | 574 ± 36b  |

*aValues were expressed as fmol per mg protein.
bValues were expressed as pmol/min per mg protein.
*Denotes p<0.01 versus CBR.
Table 3. Related information of the five proteins differentially expressed in AEA-treated SH-SY5Y cells, and identified on the 2-DE gels by MALDI-TOF/TOF-MS and/or MS/MS.

| Spot no. | Accession no. a) | Protein identified | pI b) | \( M_r \) c) | Mascot Score | Cov. d) | No. of peptides matched | MS/MS sequence | Ions score | FC e) |
|----------|------------------|-------------------|-------|-------------|--------------|--------|------------------------|----------------|-----------|-------|
| 1902     | gi|6470150          | BiP/GRP78         | 5.23  | 71002       | 105     | 20%                    | 10             | GFESPSDNNSSAMLQWHEK; VDVDEYDENKFVDEEDG GDGQAGPDEGEVDSCLR | 101 | ↑ 4.60 |
| 3102     | gi|5031593          | Actin-related protein 2/3 complex subunit 5 (ARP5) | 5.47  | 16367       | 127     | 92%                    | 13             |                                           | 195 | ↓ 3.03 |
| 6002     | gi|19557636         | Peptidylprolyl isomerase-like protein 3 isoform PPIL3b (PPIL3b) | 6.29  | 18371       | 112     | 68%                    | 10             | DITIHANPFAQ; TYRPLNDVHIIK; GFMVQTGDPTGTGR       | 84  | ↓ 3.69 |
| 6904     | gi|5803181          | Stress-induced phosphoprotein-1 (STIP1)     | 6.40  | 63227       | 127     | 30%                    | 18             |                                           | 35  | ↑ 3.51 |
| 7201     | gi|4507645          | Triosephosphate isomerase-1 (TPI1)          | 6.45  | 26938       | 164     | 79%                    | 15             | KFFVGGNWK; VPADTEVVCAAPPTAYIDFAR                    | 77  | ↑ 3.31 |

a) Accession number according to NCBI nr database.
b)-c) Theoretical isoelectric point (pI) and molecular mass (\( M_r \)) calculated by the MASCOT software.
d) Percentage of amino acid sequence coverage by measured peptides.
e) Fold change calculated by taking the averaged ratio of spot intensity between AEA-treated and untreated SH-SY5Y cells.
Table 4. Functions of the proteins modulated by AEA in apoptotic SH-SY5Y cells.

| Protein                                      | Description                                                                 | Function                                                                                           | References                              |
|----------------------------------------------|----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|-----------------------------------------|
| BiP/GRP78                                    | Molecular chaperone of the Hsp70 family located in the lumen of the ER    | It promotes folding of unfolded proteins                                                            | Kohno et al., 1993; Cox and Walter, 1996 |
|                                             |                                                                           | In the presence of ER stress inducers, it reduces apoptosis by stabilizing mitochondrial permeability | Yu et al., 1999; Ranganathan et al., 2006          |
| Actin-related protein 2/3 complex subunit 5  | Controller of actin polymerization                                        | It is required for the formation of branched networks of actin filaments at the cell cortex         | Vadlamudi et al., 2004                   |
| (ARP5)                                       |                                                                           |                                                                                                    |                                         |
| Peptidylprolyl isomerase-like protein 3 isoform PPIL3b (PPIL3b) | Member of the cyclophilin family | Not available                                                                                       | Zhou et al., 2001                        |
| Stress-induced phosphoprotein-1 (STIP1)      | Co-chaperone, Hsp70/Hsp90-organising protein                              | In the case of apoptosis, it regulates protein folding and proteasomal degradation                  | Takayama et al., 2003; Short et al., 2007 |
|                                             |                                                                           | It catalyses the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate     | Gracy, 1982                              |
| Triosephosphate isomerase-1 (TP11)           | Glycolytic enzyme                                                        | Its inhibition induces death of cortical and striatal neurons                                       | Sheline and Choi, 1998                  |
|                                             |                                                                           | It is overexpressed in acute pancreatitis, where it might have an anti-apoptotic action            | Yu et al., 2008                         |
Table 5. mRNA expression of BiP and of two BiP-related apoptotic markers in human SH-SY5Y cells treated with 10 μM AEA, alone or in the presence of 1 μM SR141716 (SR1), 10 μM SB203580 (SB), or 10 μM PD98059 (PD).

| mRNA Level (Fold over Ctrl) | Ctrl    | AEA     | AEA+SR1 | AEA+SB  | AEA+PD  |
|-----------------------------|---------|---------|---------|---------|---------|
| BiP                         | 1.00±0.10 | 3.20±0.20* | 1.10±0.10# | 1.48±0.30## | 1.00±0.10# |
| p53                         | 1.00±0.01 | 5.20±0.13* | 2.50±0.15## | 1.45±0.12# | 1.51±0.04# |
| PUMA                        | 1.00±0.08 | 3.60±0.07* | 2.30±0.04## | 1.00±0.10# | 1.91±0.15## |

*Denotes p<0.01, and ** p<0.05 versus Ctrl.
#Denotes p<0.01, and ## p<0.05 versus AEA.
Figure 1

A

B

C

D

E

F

G

H

SH-SY5Y cells

CB1R

CB2R

TRPV1

NAPE-PLD

FAAH

DAGL

MAGL

Mr

66 kDa

45 kDa

110 kDa

36.5 kDa

57 kDa

115 kDa

23 kDa

CB1R

CB2R

TRPV1

NAPE-PLD

FAAH

DAGL

MAGL

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Figure 2

A

% of Apoptosis

Endocannabinoid concentration (µM)

B

Apoptosis (Fold over Ctrl)

Ctrl  AEA  AEA+SR1  AEA+SR2  AEA+1-RTX

C

anti-Cleaved PARP

DAPI
Figure 4

Spot no. 1902

Spot no. 3102

Spot no. 6002

Spot no. 6904

Spot no. 7201
Figure 5

A

B

C

D

E

Figure 5

A

B

C

D

E

Figure 5

A

B

C

D

E

Figure 5
Figure 6

A

CBIR Levels

Fold over SH-SY5Y cells

mRNA
Protein
Binding

SH-SY5Y
LAN-5
SK-NBE

B

% Apoptosis

CTRL
AEA
SR141716
CTRL
AEA
SR141716

LAN-5 cells
SK-NBE cells

C

BIP mRNA expression/Fold Over Ctrl

CTRL
AEA
SR141716
CTRL
AEA
SR141716

LAN-5 cells
SK-NBE cells
Figure 7

A

BIP mRNA expression (Fold over Ctrl)

Ctrl  si BIP  o/e BIP  si BiP+AEA  si BiP+AEA+SR141716

B

BiP

β-actin

C

% apoptosis

Ctrl  AEA  AEA+SR141716  si BiP  si BiP+AEA  si BiP+AEA+SR141716
Characterization of the endocannabinoid system in human neuronal cells, and proteomic analysis of anandamide-induced apoptosis

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