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RESEARCH ARTICLE

The endocannabinoid system in rat gliosomes and its role in the modulation of glutamate release

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Abstract The endocannabinoid system and endocannabinoid receptor-driven modulation of glutamate release were studied in rat brain cortex astroglial gliosomes. These preparations contained the endocannabinoids *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol, as well their major biosynthetic (*N*-acyl-phosphatidylethanolamines-hydrolyzing-phospholipase D and diacylglycerol-lipase) and catabolic (fatty acid amide-hydrolase and monoacylglycerol-lipase) enzymes. Gliosomes expressed type-1 (CB1R), type-2 (CB2R) cannabinoid, and type-1 vanilloid (TRPV1) receptors, as ascertained by Western blotting and confocal microscopy. Methanandamide, a stable analogue of anandamide acting as CB1R, CB2R, and TRPV1 agonist, stimulated or inhibited the depolarization-evoked gliosomal [^{3}H]D-aspartate release, at lower and higher concentrations, respectively. Experiments with ACEA (arachidonyl-2′-chloroethylamide), JWH133 ((6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]-pyran) and capsaicin, selective agonists at CB1R, CB2R and TRPV1, respectively, demonstrated that potentiation of [^{3}H]D-aspartate release was due to CB1R while inhibition to CB2R and TRPV1 engagement. These

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findings were confirmed by using selective receptor antagonists. Furthermore, CB1R activation caused increase of intracellular IP3 and Ca^{2+} concentration, suggesting an involvement of phospholipase C.

Keywords Endocannabinoids · Glutamate release · Gliosomes · Cannabinoid receptors · Vanilloid receptors · Neuron-glia communication

Abbreviations

ACEA	Arachidonyl-2′-chloroethylamide
AEA	Anandamide (*N*-arachidonoylethanolamine)
2-AG	2-Arachidonoylglycerol
[^{3}H]D-ASP	[^{3}H]D-aspartate
CB1R	Type-1 cannabinoid receptor
CB2R	Type-2 cannabinoid receptor
DAGL	Diacylglycerol lipase
eCBs	Endocannabinoids
DAG	Diacylglycerol
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
HRP	Horseradish peroxidase
5-IRTX	5-Iodoresiniferatoxin
GFAP	Glial fibrillary acidic protein
JWH133	[(6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]-pyran]
MAGL	Monoacylglycerol lipase
NAPE-PLD	*N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D and diacylglycerol lipase
2-OG	2-Oleoylglycerol
PBS	Phosphate buffered saline
SR141716	*N*-piperidin-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide)
RP-HPLC	Reverse phase-high performance liquid chromatography
RTX	Resinferatoxin
SR144528	*N*-[1(*S*)-endo-1,3,3-trimethylbicyclo[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methyl phenyl)-1-(4-methylbenzyl) pyrazole-3-carboxamide
TRPV1	Transient receptor potential vanilloid-1

Introduction

Endocannabinoids (eCBs) are endogenous ligands of metabotropic type-1 (CB1R) and type-2 (CB2R) cannabinoid receptors, and are widely recognized as being critical regulators of central and peripheral functions [1–3]. The two most studied and best characterized eCBs are anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG). The former also binds to and activates the ionotropic transient receptor potential vanilloid 1 (TRPV1). Therefore AEA, unlike 2-AG, is considered in addition a true "endovanilloid" [4].

The eCBs-binding receptors, their ligands (AEA, 2-AG, and other congeners), and the enzymes that generate (*N*-acyl-phosphatidylethanolamines-hydrolyzing phospholipase D, NAPE-PLD, and diacylglycerol lipase, DAGL), or degrade (fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL) AEA or 2-AG respectively, altogether form the so-called "endocannabinoid system (ECS)" [1–3]. Conversely, there is still controversy about the identity and even the existence of authentic "endocannabinoid transporters", since they have neither been cloned, isolated, nor characterized [5]. A critical role for eCBs within the CNS is neuromodulation, a fundamental process that regulates synaptic transmission, neuronal network activity, and behavior, both in healthy and diseased brain [6, 7].

Emerging evidence demonstrates that astrocytes, a major population of glial cells in the brain, can detect the level of neuronal activity, and can release chemical transmitters to influence neuronal function. One of these transmitters is undoubtedly glutamate: once released from neurones, this excitatory amino acid can activate both ionotropic and metabotropic receptors located on astroglial cells, inducing an increase in intracellular Ca^{2+} [8–10], which is associated with glutamate release [10–12]. Glutamate release from astrocytes can also be observed following other stimuli, including bradykinin [13], prostaglandins [14], chemokines [15], 5-hydroxytryptamine [16], and ATP receptor activation [17]. These observations have led to the concept of "tripartite synapses", where astrocytes are in charge of processing and controlling synaptic information [18].

Also, eCBs can take part in neuron-to-astrocyte communication, demonstrating the existence of an endocannabinoid–glutamate signaling pathway where astrocytes serve as a bridge for nonsynaptic interneuronal cross-talks [19–21]. In this context, it should be stressed that data on ECS and eCBs communication in neuronal and astroglial cells are still very scant.

Most of the studies focused on the release of glutamate from astrocytes have been carried out using cultured neonatal astrocytes [13–15, 22, 23]; only a few investigations have exploited other systems such as astroglioma cells [16]. More recent papers used brain tissue slices isolated from rat hippocampus and electrophysiological measurements: a model that also allows the study of the neuronal responses to glutamate released from astrocytes and vice-versa [20, 24–26]. In the present work, we used a preparation of sub-cellular particles (termed gliosomes) acutely isolated from the brain of the adult rat, which appear well

suited for investigating the functional neurochemistry of mature astrocytes [27, 28]. In a recent paper, we have described that depolarization of gliosomes results in exocytotic glutamate release, driven by reversal of the membrane Na^+/Ca^{2+} exchanger and Ca^{2+} entry [27]. The modulation of this depolarization-evoked exocytotic glutamate release by eCB receptor activation has been investigated in the present work. Unexpectedly, the results obtained indicated that the depolarization-induced release of glutamate is potentiated by CB1R and decreased by CB2R and TRPV1 engagement.

Materials and methods

Animals

Adult Sprague-Dawley rats were used. The animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (lights 7.00 a.m.–7.00 p.m.). Food and water were freely available. All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

Preparation of gliosomes and synaptosomes

After the animals were killed, the brain cortex was quickly removed. Purified gliosomes and synaptosomes were prepared on Percoll gradients essentially according to Nakamura and collaborators [29], with minor modifications [27]. Briefly, the tissue was homogenized in 14 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass–Teflon tissue grinder (clearance 0.25 mm, 12 up-down strokes in about 1 min). The homogenate was centrifuged (1,000 × *g* for 5 min, at 4°C) to remove nuclei and debris, and the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min at 4°C. The layers between 2 and 6% Percoll® (gliosomal fraction) and between 10 and 20% Percoll® (synaptosomal fraction) were collected, washed by centrifugation, and then resuspended in physiological medium.

Endogenous levels of endocannabinoids

Gliosomes or synaptosomes were subjected to lipid extraction with chloroform/methanol (2:1, v/v), in the presence of d_8-AEA and d_8-2-AG as internal standards. The organic phase was dried and then analyzed by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS), using a single quadrupole API-150EX mass spectrometer (Applied Biosystems, Foster City, CA, USA), in conjunction with a PerkinElmer LC system (PerkinElmer, Waltham, MA, USA). Quantitative analysis was performed by selected ion recording over the respective sodiated molecular ions, as reported [30].

Metabolism of AEA

The synthesis of AEA through NAPE-PLD was assayed in gliosome and synaptosome homogenates (200 μg protein/test), by measuring the release of [^{3}H]AEA from 100 μM [^{3}H]arachidonoyl-phosphatidylethanolamine by reverse-phase high-performance liquid chromatography, coupled to online liquid scintillation counting [31]. The hydrolysis of AEA by FAAH (50 μg protein/test) was evaluated by reverse-phase high-performance liquid chromatography, coupled to online liquid scintillation counting, using 10 μM [^{3}H]AEA as substrate [32]. Both NAPE-PLD and FAAH activities were expressed as pmol product released per minute per milligram protein.

Metabolism of 2-AG

To evaluate the synthesis of 2-AG by DAGL, homogenates (200 μg protein/test) were incubated at 37°C for 30 min with 500 μM [^{14}C]diacylglycerol (DAG), as reported [33]. 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 thin-layer chromatography on silica, using polypropylene plates with chloroform/methanol/NH_4OH (94:6:0.3, v/v/v) as eluent. The release of [^{14}C]2-AG was measured by cutting the corresponding TLC spots, followed by liquid scintillation counting. The hydrolysis of 2-AG by MAGL was assayed in supernatants (100 μg protein/test), obtained at 39,000 × *g* and incubated with 10 μM [^{3}H]2-OG at 37°C for 30 min [34]. The reaction was stopped with a mixture of chloroform/methanol (2:1, v/v), and the release of [^{3}H]glycerol in the aqueous phase was measured by scintillation counting. Both DAGL and MAGL activities were expressed as pmol product per minute per milligram protein.

CBR and TRPV1 binding

For cannabinoid receptor studies, gliosomes or synaptosomes were resuspended in 2 mM Tris–EDTA, 320 mM sucrose, 5 mM $MgCl_2$ (pH 7.4), and then they were homogenized in a Potter homogenizer and centrifuged as reported [32]. The resulting pellet was resuspended in assay buffer (50 mM Tris–HCl, 2 mM Tris–EDTA, 3 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, pH 7.4), to a protein concentration of 1 mg/ml. The membrane

preparations were 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 [^{3}H]CP-55940 (400 pM), as described [32]. Binding of the TRPV1 agonist [^{3}H]RTX (resinferatoxin, 500 pM) was also evaluated by rapid filtration assays [32]. Unspecific binding was determined in the presence of cold agonists (1 μM CP-55940 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 5-IRTX for TRPV1), as reported [32].

Western blot

Gliosome and synaptosome homogenates (20 μg protein/lane) were subjected to SDS-PAGE on a 10% polyacrylamide gel and electroblotted onto a polyvinylidene fluoride membrane. Blots were blocked with 10% non-fat dry milk (Bio-Rad Laboratories, Hercules, CA, USA) and 5% bovine serum albumin for 2 h, and then were incubated with anti-NAPE-PLD (diluted 1:200), anti-FAAH (diluted 1:250), anti-DAGL (diluted 1:1,000), anti-MAGL (diluted 1:200), anti-CB1R (diluted 1:250), anti-CB2R (diluted 1:250) or anti-TRPV1 (diluted 1:1,000) primary antibodies. After washing and incubation with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000, Bio-Rad Laboratories, Hercules, CA, USA), detection was carried out using the West Dura Chemiluminescence System (Pierce, Rockford, IL, USA).

Confocal microscopy

Gliosomes were resuspended in phosphate buffered saline (PBS) and aliquots of the suspension (70 μg protein) were placed onto coverslips pre-treated with poly-lysine and maintained 30 min at room temperature to allow setting and sticking to the substrate. The preparations were fixed with 2% paraformaldehyde (15 min), washed, and incubated (5 min) with 0.05% triton X-100. After washing with PBS containing 0.5% serum albumin, the preparations were incubated for 30 min with the primary antibodies diluted in PBS containing 3% albumin. The following rabbit antibodies were used: anti-glial fibrillary acidic protein (GFAP, 1:100), anti-CB1R (1:100), anti-CB2R (1:100), or anti-TRPV1 (1:100). After washing, the preparations were incubated for 30 min with the appropriate secondary Alexa Fluor 488-tagged or Alexa Fluor 647-tagged antibodies diluted in PBS containing 3% albumin. Fluorescence image acquisition was performed by a three-channel Leica TCS SP2 laser-scanning confocal microscope, and images (512 × 512 × 8 bit) were taken through a plan-apochromatic oil immersion objective (100×/NA = 1.4). Light collection configuration was optimized according to the combination of chosen fluorochromes and sequential channel acquisition was performed to avoid cross-talk phenomena. Leica LCS software package was used for acquisition, storage, and visualization. The on-line Web service "Power Up Your Microscope" (www.powermicroscope.com, Department of Physics, University of Genoa, Italy and Re@lityNET, Genoa, Italy) was employed to perform deconvolution of images, increasing both image resolution and signal-to-noise ratio. Spatial co-localization was analyzed through 2D correlation cytofluorograms accomplished by routines integrated in the Leica LCS software.

Release experiments

Gliosomes and synaptosomes were resuspended in HEPES-buffered medium (140 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose; pH 7.4 and incubated in standard HEPES-buffered medium at 37°C for 15 min, in the presence of 0.1 μM [^{3}H]$_D$-ASP. Next, aliquots of suspensions were layered on microporous filters at the bottom of parallel superfusion chambers (Superfusion System, Ugo Basile, Comerio, Varese, Italy) maintained at 37°C [27, 35] and superfusion was started at a rate of 0.5 ml/min. After 36 min of superfusion, to equilibrate the system, the samples were collected according to the following scheme: two 3-min samples (t = 36–39 min and t = 45–48 min; basal release) before and after one 6-min sample (t = 39–45 min; stimulus-evoked release). A 90-s period of depolarization, performed by using 15 mM K^+, substituting for an equimolar concentration of NaCl, was applied at t = 39 min of superfusion. DL-TBOA (10 μM) was present throughout the experiments from t = 30 min. When appropriate, R(+)-methanandamide (0.1–3 μM), ACEA (0.1–1 μM), JWH133 (0.1–1 μM) or capsaicin (1 μM) was introduced concomitantly to KCl; SR141716 (0.1–1 μM), SR144528 (1 μM) or 5-IRTX (1 μM) was added at t = 30 min. [^{3}H]$_D$-ASP radioactivity was determined in each collected sample, as well as in the superfused filters by liquid scintillation counting. [^{3}H]$_D$-ASP efflux was calculated as fractional rate ×100. The stimulus-evoked overflow was estimated by subtracting the transmitter content of the two basal release samples from the release evoked in the 6-min sample collected during and after the depolarization pulse. Drug effects were evaluated as the ratio of the depolarization-evoked overflow in the presence of the drug versus that calculated under control conditions.

Cytosolic Ca^{2+} concentration

Cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ was monitored in gliosomes using the fluorescent dye Fura-3 PE-AM [36]. Gliosomes were incubated for 45 min at 37°C with 10 μM Fura-3 PE-AM. Gliosomal suspensions were then centrifuged at 3,000 × *g* for 2 min to remove the extra-gliosomal dye and resuspended in HEPES-buffered physiological medium (each aliquot containing 200 μg protein). Gliosomes were used within 2 h. Measurements were made at 37°C in a thermostated cuvette under continuous stirring using a RF-5301PC dual wavelength spectrofluorometer (Shimadzu, Kyoto, Japan) by alternating the excitation wavelengths of 340 and 380 nm. Fluorescent emission was monitored at 510 nm. Capsaicin, ACEA, or JWH133 was introduced 2 min before starting of measures. The fluorescent emission was recorded for 2 min before the addition of 15 mM KCl and for another 2 min after depolarization. Calibration of the fluorescent signals was performed at the end of each measure by adding 10 μM ionomycin, to obtain Fmax, followed by 7.5 mM EGTA, to obtain Fmin, and $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [36], using a K_D of 250 nM for the Ca^{2+}/Fura-3 PE-AM complex.

Cytosolic IP3

Suspensions of brain cortex gliosomes (200–300 μg) were equilibrated at 37°C for 15 min. Each sample was stimulated by 15 mM KCl for 3 min in the presence or in the absence of 0.1 μM ACEA or 1 μM capsaicin. The reaction was stopped with ice water and sonication and the cytosolic fraction was obtained by centrifugation (16,000 × *g*, 3 min). Cytosolic IP3 content was determined by a commercially available radioreceptor assay kit (Biotrak TRK 1000, Amersham Biosciences, Buckinghamshire, UK). Under these experimental conditions, the detection limit of the assay was 0.1 pmol/100 μl (standard curve range 0.19–25 pmol/100 μl).

Cytosolic cAMP

Suspensions of brain cortex gliosomes (200–300 μg) were equilibrated at 37°C for 15 min. Each sample was stimulated by 15 mM KCl for 3 min in the presence or in the absence of 0.1 μM ACEA, 1 μM JWH133, or 1 μM capsaicin. The reaction was stopped with ice water and sonication and the cytosolic fraction was obtained by centrifugation (16,000 × *g*, 3 min). Cytosolic cAMP content was determined by a commercially available radioimmunoassay kit (RK-509, Izotop, Budapest, Hungary), using the acetylation protocol. Under these experimental conditions, the detection limit of the assay is 2 fmol/100 μl (standard curve range 2–128 fmol/100 μl).

Statistical analysis

Data reported are the mean ± SEM of at least three independent experiments, each performed in duplicate or triplicate. The two-tailed Student's *t* test was used for the analysis of two population means. Comparisons of more than two means was performed using two-way analysis of variance (ANOVA), followed by Newman–Keuls or Dunnett's test where appropriate. Significant differences were accepted at $p < 0.05$.

Chemicals

All chemicals were of the purest analytical grade. 2-Oleoylglycerol (2-OG), CP-55940 (5-(1,10-dimethyheptyl)-2-[1*R*,5*R*-hydroxy-2*R*-(3-hydroxypropyl)-cyclohexyl]phenol), capsaicin (8-methyl-*n*-vanillyl-6-nomamide), R(+)-methanandamide [R(+)-arachidonyl-1′-hydroxy-2′-propylamide], resinferatoxin (RTX) and GFAP monoclonal antibody were from Sigma Chemical Co. (St. Louis, MO, USA). ACEA (arachidonyl-2′-chloroethylamide), JWH133 (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]-pyran), 5-iodoresiniferatoxin (5-IRTX) and DL-TBOA (DL-Threo-β-benzyloxyaspartic acid) were from Tocris Bioscience (Bristol, UK). Diacylglycerol (1-Stearoyl-2-arachidonoyl-*sn*-glycerol, DAG) was from Enzo Life Sciences AG (formerly Alexis Biochemicals, Lausen, Switzerland). SR141716 (*N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide) and SR144528 (*N*-[1(S)-endo-1,3,3-trimethylbicyclo[2.2.1]-heptan-2-yl]-5-(4-chloro-3-methyl phenyl)-1-(4-methylbenzyl) pyrazole-3-carboxamide) were kind gifts of Sanofi-Aventis Recherche (Montpellier, France). [^{3}H]AEA (205 Ci/mmol), [^{3}H]CP-55940 (126 Ci/mmol) and [^{3}H]RTX (43 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). [^{14}C]DAG (56 Ci/mmol), [^{3}H]2-OG (Ci/mmol), and *N*-[^{3}H]arachidonoyl-phosphatidylethanolamine (200 Ci/mmol) were from ARC (St. Louis, MO, USA). d_8-AEA, d_8-2-AG, rabbit anti-CB1R, anti-CB2R and anti-MAGL polyclonal antibodies were from Cayman Chemicals (Ann Arbor, MI, USA). Rabbit anti-TRPV1 and anti-FAAH polyclonal antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Rabbit anti-NAPE-PLD polyclonal antibody was from Novus Biologicals Inc. (Littleton, CO, USA).

Results

The endocannabinoid system in gliosomes and synaptosomes

The presence of the endocannabinoids AEA and 2-AG could be detected in gliosomes and synaptosomes, as control, to comparable levels (Fig. 1a, b). Consistently, gliosomes and synaptosomes were found to possess active enzymes for the synthesis of AEA and 2-AG (Fig. 1c, d), and for their degradation (Fig. 1e, f). There were no significant differences between the two preparations, apart from an increased DAGL in gliosomes versus synaptosomes (Fig. 1d). In keeping with the enzymatic activity data, Western-blot analysis showed that specific anti-NAPE-PLD, anti-FAAH, anti-DAGL, and anti-MAGL antibodies recognized a single immunoreactive band of the expected molecular size, both in gliosomes and synaptosomes (Fig. 2). In addition, Western-blot analysis showed that anti-CB1R, anti-CB2R, and anti-TRPV1 antibodies recognized single immunoreactive bands in gliosomes and synaptosomes, of the expected molecular size (Fig. 2). The presence of active CB1R, CB2R, and TRPV1 receptors was also demonstrated by binding data using the CB1R and CB2R ligand [^{3}H]CP-55940, or the TRPV1 ligand [^{3}H]RTX (Fig. 3a, b), and by the effect of selective CB1R, CB2R, or TRPV1 antagonists. In fact, [^{3}H]CP-55940 was displaced to a similar extent (i.e., down to ~30% of controls) by 0.1 μM SR141716 or 0.1 μM SR144528, CB1R, and CB2R antagonists, respectively, whereas the TRPV1 antagonist 5-IRTX (1 μM) fully displaced [^{3}H]RTX from TRPV1 receptors (data not shown).

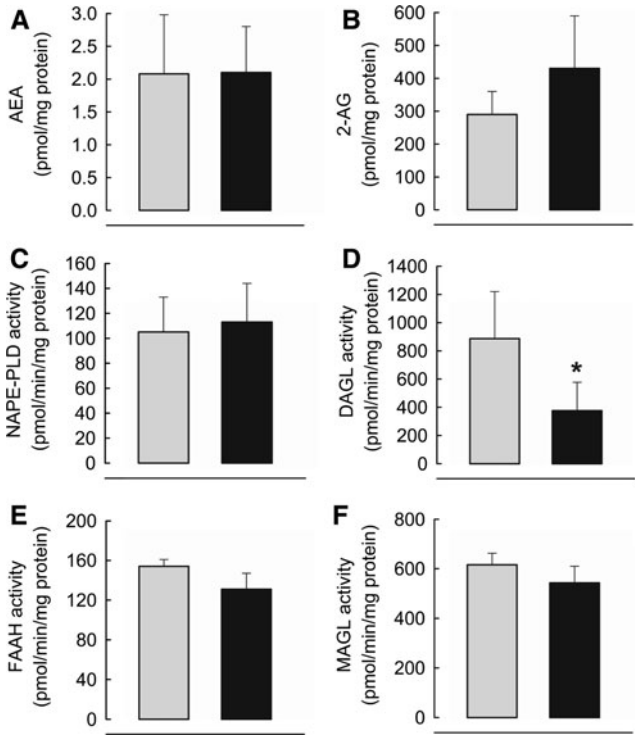

Fig. 1 Endocannabinoid system in gliosomes (*grey bars*) and synaptosomes (*black bars*). Endogenous levels of AEA (**a**) and 2-AG (**b**). Metabolism of AEA and 2-AG, which includes synthesis by NAPE-PLD activity for AEA (**c**) and by DAGL activity for 2-AG (**d**); hydrolysis by FAAH activity for AEA (**e**) and by MAGL activity for 2-AG (**f**). Results are expressed as pmol/mg for endogenous levels and pmol/min/mg protein for the enzymatic activities. Reported data are mean ± SEM of at least three independent experiments, each performed in duplicate or triplicate. In panel **d**, $*p < 0.05$ versus gliosomes

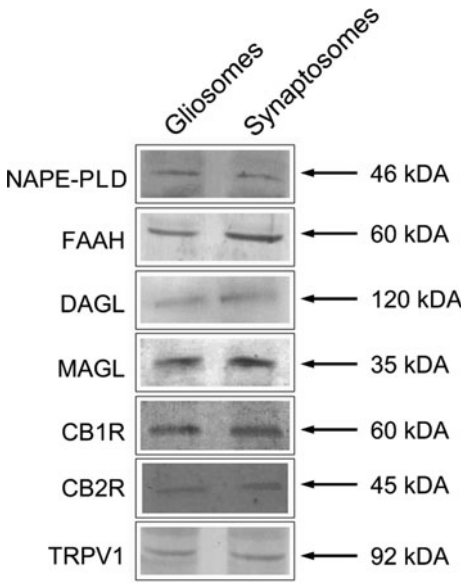

Fig. 2 Western-blot analysis of the elements of the endocannabinoid system in gliosomes and synaptosomes. Gliosome and synaptosome homogenates were reacted with anti-NAPE-PLD, anti-FAAH, anti-DAGL, anti-MAGL, anti-CB1R, anti-CB2R, and anti-TRPV1 polyclonal antibodies. Expected molecular mass values are reported on the *left-hand side*

In keeping with binding experiments, confocal microscopy confirmed the presence of the eCBs-binding receptors CB1R, CB2R and TRPV1 in gliosomal particles. In a previous study, we already checked the purity of gliosomes isolated from rat cerebral cortex as described above, demonstrating that more than 80% of the gliosomal particles originate from astrocytes [27]. In the present experiments (Fig. 3c), gliosomes were labeled with an anti-GFAP antibody (i, v, ix) as well as with anti-CB1R (ii), anti-CB2R (vi) or anti-TRPV1 (x) antibodies. The preparation efficiently stained for all the antibodies tested. Co-localization of GFAP and CB1R (iii), GFAP and CB2R (vii) and GFAP and TRPV1 (xi) was analyzed. Merging of the appropriate image pair of 3–4 experiments revealed that 60.5 ± 7.9% of the CB1R-positive particles (iv), 49.2 ± 5.5% of the CB2R-positive particles (viii), and 42.8 ± 8.2% of the TRPV1-positive particles (xii) were also GFAP positive.

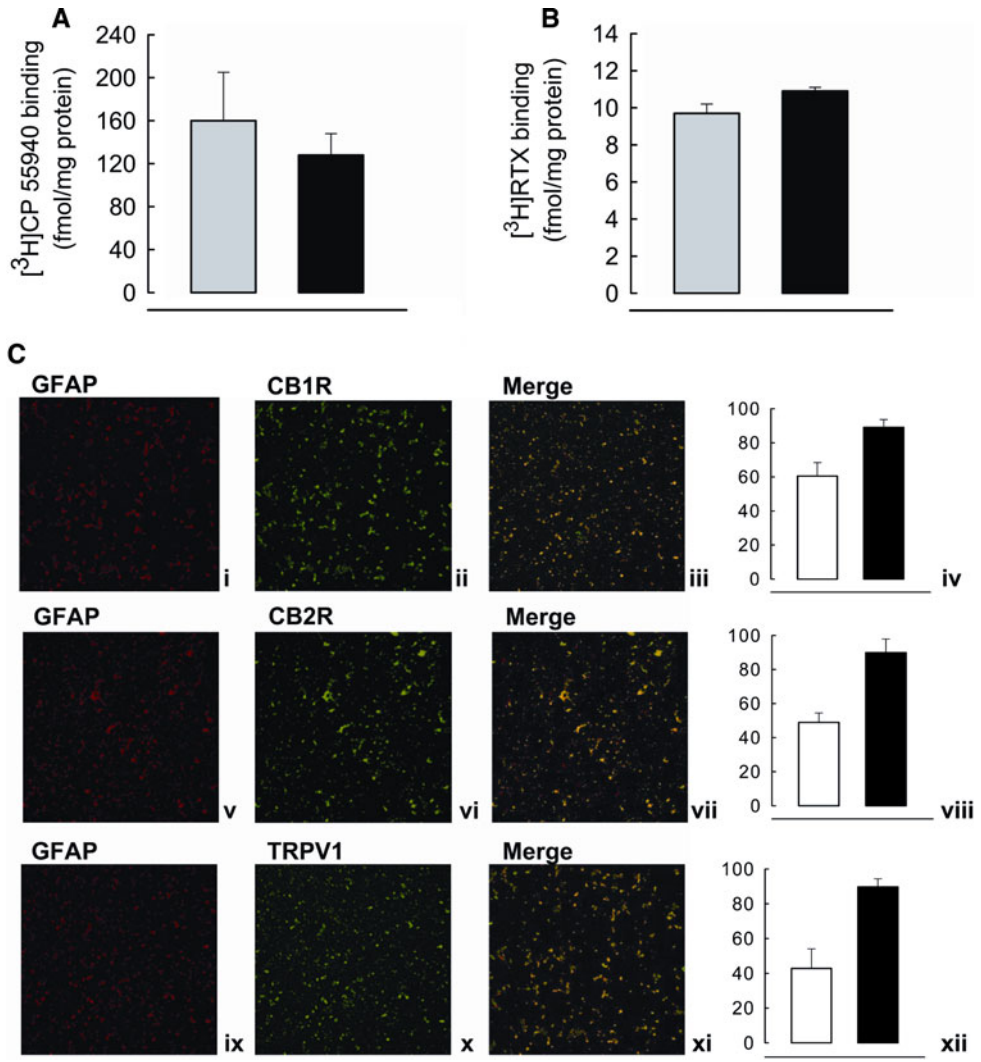

Fig. 3 Binding to cannabinoid (**a**) and vanilloid (**b**) receptors in gliosomes (*grey bars*) and synaptosomes (*black bars*). Binding of [^{3}H]CP-55940 for CBR, and of [^{3}H]resinferatoxin (RTX) for TRPV1 are expressed as fmol/mg of protein. **c** Identification by immunocytochemistry of the glial fibrillary acidic protein (GFAP), of the type-1 cannabinoid receptor (CB1R), of the type-2 cannabinoid receptor (CB2R) and of the type-1 vanilloid receptor (TRPV1) in gliosomes purified from rat cerebral cortex. Samples were analyzed by laser confocal microscopy. Images show immune-positivity for the Alexa Fluor 647-tagged anti-GFAP (panels *i*, *v*, *ix*), for the Alexa Fluor 488-tagged anti-CB1R (panel *ii*), or anti-CB2R (panel *vi*), or anti-TRPV1 (panel *x*). The *yellow color* represents co-expression of GFAP and CB1R (panel *iii*), of GFAP and CB2R (panel *vii*) and of GFAP and TRPV1 (panel *xi*) in merged images. *Bar plots* show the co-localization of red in green (*empty bars*) and green in red (*solid bars*) of the couples GFAP/CB1R (panel *iv*), GFAP/CB2R (panel *viii*) and GFAP/TRPV1 (panel *xii*). Percent values are mean ± SEM of 3–4 independent experiments

Endocannabinoid-mediated modulation of glutamate release

Gliosomes were labeled with [^{3}H]D-ASP, a non-metabolizable analogue of glutamate, stratified on microporous filters and exposed in superfusion to depolarizing concentrations of KCl and to drugs active at eCB receptors. As reported previously [28], also in the present experiments exposure to 15 mM KCl produced a significant increase of the spontaneous release of the radioactive tracer. Since we have previously observed that the 15 mM KCl-induced [^{3}H]D-ASP and Glu release were almost halved by omission of Ca^{2+}, suggesting exocytosis as a mechanism, and by reversal of transporters [28], the present experiments were performed in the presence of DL-TBOA, a non transportable blocker of glutamate transporters [37]. Under these conditions, we could analyze only the exocytotic component of release.

Figure 4 illustrates the modification of the depolarization-induced [^{3}H]D-ASP release by increasing concentrations (0.1–3 μM) of methanandamide. The effects observed were not univocal, as 0.1 μM drug concentration increased the KCl-induced Ca^{2+}-dependent overflow of [^{3}H]D-ASP, while this effect was not seen at higher methanandamide

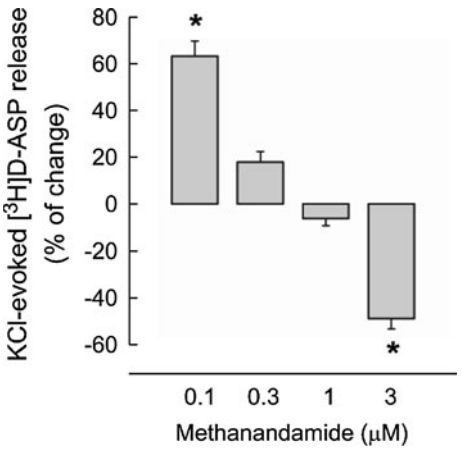

Fig. 4 Effect of methanandamide on the KCl-induced release of [^{3}H]D-ASP from rat brain cortex gliosomes. Gliosomes were labeled with the radioactive tracer and exposed in superfusion to a 90-s pulse of 15 mM KCl. Methanandamide was introduced together with KCl. The experiments were conducted in the presence of DL-TBOA (10 μM) to abolish the transporter-mediated component of the KCl-induced neurotransmitter release in gliosomes. DL-TBOA was introduced 9 min before KCl. The data are mean ± SEM of 5–8 separate experiments in triplicate; $*p < 0.05$ when compared to corresponding control overflow

concentrations; it even turned into inhibition at 3 μM of the drug. On the other hand, methanandamide did not modify the spontaneous release of [^{3}H]D-ASP at any tested concentration (not shown).

In order to identify the receptors producing this composite effect of methanandamide, experiments were performed with selective agonists of the AEA-binding receptors, namely CB1R, CB2R and TRPV1. The results are reported in Fig. 5a and show that the selective CB1R agonist ACEA (0.1–1 μM) mimicked the potentiating effect of 0.1 μM methanandamide; instead, JWH133 (0.1–1 μM) and capsaicin (1 μM), selective CB2R and TRPV1 agonists, respectively, inhibited the KCl-induced release of [^{3}H]D-ASP. These agonists did not modify per se the spontaneous release of [^{3}H]D-ASP (not shown).

Control experiments testing the effects of selective antagonists of CB1R, CB2R, and TRPV1 were also performed. Figure 5b shows that the potentiation of the depolarization-induced release of [^{3}H]D-ASP brought about by 0.1 μM methanandamide was abolished by the selective CB1R antagonist SR141716 (0.1 μM), but was unaffected by 1 μM of the selective CB2R antagonist SR144528 or of the TRPV1 antagonist 5-IRTX. These data suggest that the effect of low methanandamide concentrations was due exclusively to activation of CB1R. Figure 5b also shows that the lack of effect observed at 1 μM methanandamide turned into inhibition in the presence of 1 μM SR141716 or into potentiation using 1 μM 5-IRTX, suggesting the presence of an equilibrium between enhancing and inhibitory effects of methanandamide. No changes of [^{3}H]D-ASP release were observed when using the CB2R antagonist SR144528 (Fig. 5b). These results suggest that the inhibitory effect of high doses of methanandamide could be ascribed to TRPV1 receptor activation.

Experiments were also performed with synaptosomes prepared from rat cerebral cortex, and exposed in superfusion to methanandamide and to the other selective CB1R and CB2R agonists. Figure 6 shows that either CB1R activation, by 0.1 μM methanandamide or 0.1–1 μM ACEA, and CB2R activation, by 0.1–1 μM JWH133,

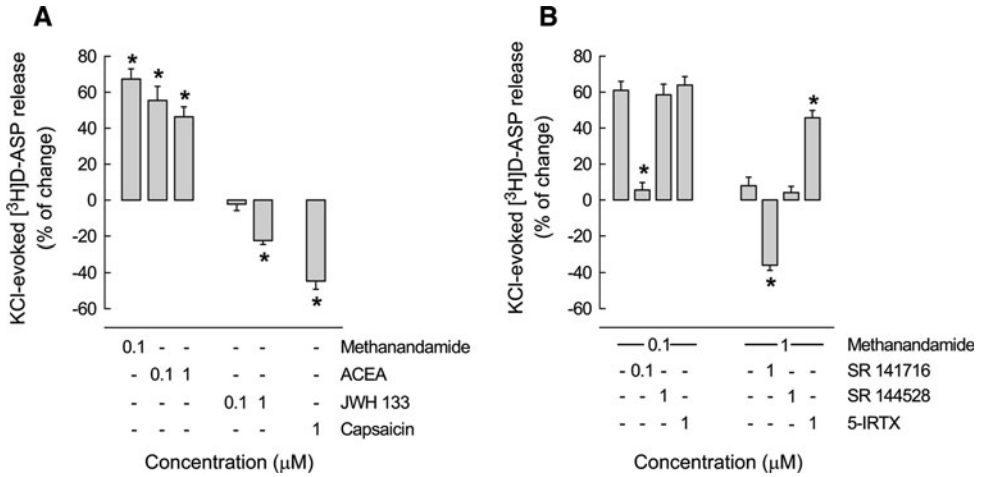

Fig. 5 **a** Effect of methanandamide, ACEA, JWH133, or capsaicin on the KCl-induced release of [^{3}H]D-ASP from rat brain cortex gliosomes, and **b** antagonism by SR141716, SR144528, or 5-IRTX. Gliosomes were pre-labeled with the radioactive tracer and exposed in superfusion to a 90-s pulse of 15 mM KCl. Methanandamide, ACEA, JWH133, or capsaicin were added concomitantly to KCl. SR141716, SR144528, or 5-IRTX were introduced 9 min before methanandamide. The experiments were conducted in the presence of DL-TBOA (10 μM), introduced 9 min before KCl. The data are mean ± SEM of 5–7 separate experiments in triplicate; $*p < 0.05$ when compared to corresponding control overflow

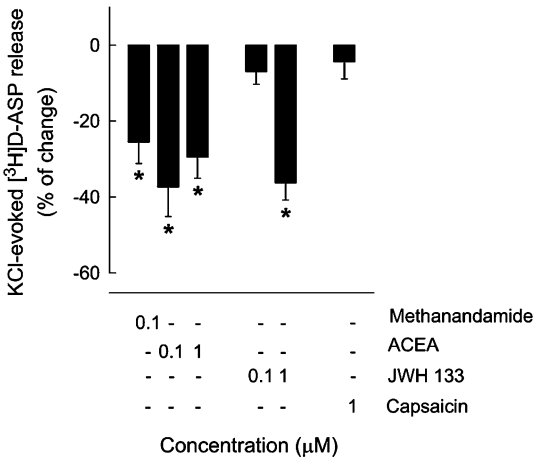

Fig. 6 Effect of methanandamide, ACEA, JWH133, or capsaicin on the KCl-induced release of [^{3}H]D-ASP from rat brain cortex synaptosomes. Synaptosomes were pre-labeled with the radioactive tracer and exposed in superfusion to a 90-s pulse of 15 mM KCl. Methanandamide, ACEA, JWH133, or capsaicin were added concomitantly to KCl. The data are mean ± SEM of 5–7 separate experiments in triplicate; *$p < 0.05$ when compared to corresponding control overflow

inhibited the KCl-induced release of [^{3}H]D-ASP. Instead, the same figure shows that activation of TRPV1 by capsaicin (up to 1 μM) did not affect the release of [^{3}H]D-ASP induced by depolarization.

Transduction mechanisms

Finally, experiments were performed in the attempt to clarify the mechanisms underlying the modulation of glutamate release mediated by CB1, CB2, and TRPV1 receptors in gliosomes. As shown in Fig. 7a, KCl-depolarization increased the basal level of IP3 by more than 70%, and the IP3 gliosomal content under depolarizing conditions was even higher in the presence of 0.1 μM ACEA. Surprisingly, 1 μM capsaicin, which reduced the depolarization-evoked [^{3}H]D-ASP release, also increased IP3 formation. Figure 7b shows the modification of cytosolic Ca^{2+} concentration induced by 15 mM KCl and by the CB1R, CB2R, or TRPV1 agonists used in the release experiments. The resting $[Ca^{2+}]_i$ was almost doubled after exposure of gliosomes to depolarization, and this value was significantly increased by 0.1 μM ACEA or 1 μM capsaicin, but not by 1 μM JWH133. Finally, also cAMP basal content in gliosomes was increased by 15 mM KCl (Fig. 7c). This value was significantly reduced by JWH133 (1 μM), but it was unaffected by 0.1 μM ACEA nor by 1 μM capsaicin. ACEA or JWH133 did not modify the basal IP3, $[Ca^{2+}]_i$ and cAMP content in gliosomes under non-depolarizing conditions; instead, capsaicin produced an about 70% increase of $[Ca^{2+}]$, leaving unchanged both IP3 and cAMP (not shown).

Discussion

In the present paper we studied the ECS in astrocytes and the modulation of glutamate release by receptors which are targets for ECBs. Experiments were carried out by using a sub-cellular preparation (termed gliosomes), acutely purified from the brain cortex of the adult rat. These particles have been purified from synaptosomes by differential migration in a discontinuous Percoll gradient, according to a technique set up several years ago [29, 38], and recently revisited by our laboratory [27, 28, 39–43]. We have characterized biochemically and morphologically rat brain cortex gliosomes, in comparison to concomitantly purified synaptosomal fractions [27]. Western-blot results, as well as confocal and electron microscopy images showed that gliosomes represent a preparation highly purified from neuronal particles, and vice-versa, and that they do not show microglia or oligodendrocyte contamination. Similar results have been obtained in hippocampus and cerebellum [39] or spinal cord [43]. Published functional experiments support the morphological evidence that gliosomes are a preparation with very limited neuronal contamination, and that they may be useful to study specific features of astrocytes. As a matter of fact, we have reported that the high mobility box 1 cytokine efficiently stimulated the release of glutamate from hippocampal or cerebellar gliosomes, but it was totally inactive in purified synaptosomes [39]. In addition, glycine potently increased the release of [^{3}H]D-ASP from mouse spinal cord synaptosomes through a mechanism involving glycine transporters located on glutamate-releasing nerve endings; on the contrary, glycine did not increase significantly [^{3}H]D-ASP release from gliosomes [41]. Finally, activation of α7-containing nicotine receptors stimulate synaptosomal glutamate release only when the nerve terminal membrane is depolarized, while the same receptors increase glutamate release from gliosomes also under resting conditions [40].

One main characteristic of gliosomes is that they are acutely prepared from in-situ astrocytes of the adult brain that have matured in the presence of neurons. For this reason, they are likely to strictly mirror astrocyte features. In fact, we recently found that gliosomes, unlike cultured astrocytes prepared from newborn rats, release glutamate after membrane depolarization and Ca^{2+} entry through the membrane Na^+/Ca^{2+} exchanger, working in the reverse mode under depolarizing conditions [28]. Interestingly, the ability to release glutamate upon depolarization, absent in new-born rat astrocytes, was shared by astrocytes cultured

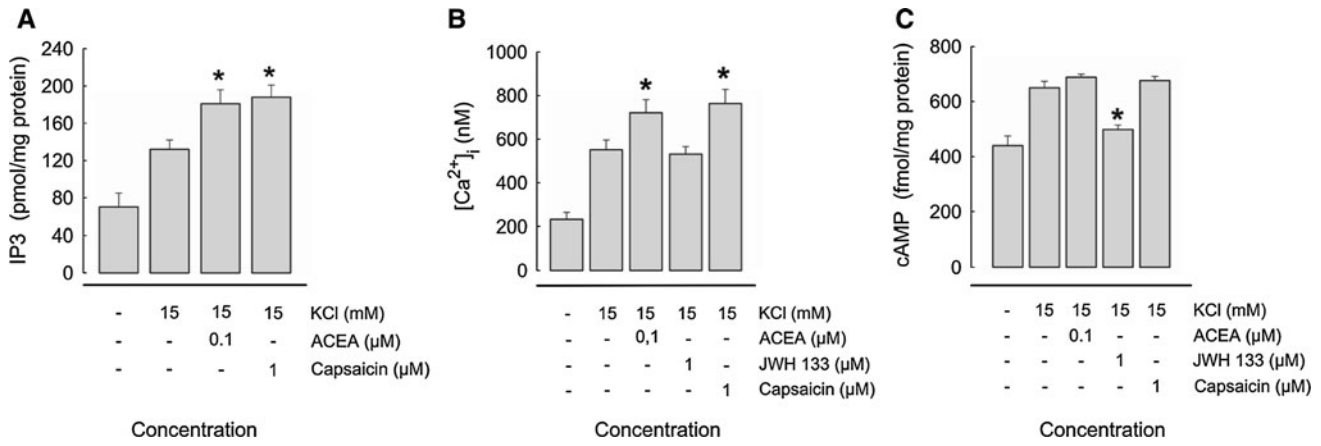

Fig. 7 **a** Effect of ACEA or capsaicin on IP3 cytoplasmic content in rat cerebral cortex gliosomes. Gliosomes were equilibrated at 37°C for 15 min and stimulated by KCl in the presence or in the absence of ACEA or capsaicin. IP3 was determined by a commercially available radioreceptor assay. Data are mean ± SEM of five separate experiments in duplicate, $*p < 0.01$ when compared to the corresponding control. **b** Effect of ACEA, JWH133, or capsaicin on the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat cerebral cortex gliosomes. Gliosomes were loaded with Fura-2AM before fluorometric measurements and stimulated by 15 mM KCl in the presence or in the absence of ACEA, JWH133, or capsaicin. Data are mean ± SEM of five separate experiments in duplicate; $*p < 0.01$ when compared to the corresponding control. **c** Effect of ACEA, JWH133, or capsaicin on cAMP cytoplasmic content in rat cerebral cortex gliosomes depolarized with KCl. Gliosomes were equilibrated at 37°C for 15 min and stimulated by 15 mM KCl, in the presence or in the absence of ACEA, JWH133, or capsaicin. cAMP was assayed by a commercially available radioimmunoassay. Data are mean ± SEM of five separate experiments in duplicate; $*p < 0.01$ when compared to the corresponding control

from the adult rat, especially when adult astrocytes were co-cultured in-vitro with neurons.

Summarizing, gliosomes represent a viable preparation that allows to study mechanisms of transmitter release and its regulation in adult astrocytes, and may have a number of advantages over other preparations, such as cultured astrocytes: they can be rapidly prepared and, most importantly, they originate directly from mature brain astrocytes. Due to their characteristics, gliosomes can be obtained from adult animals acutely or chronically treated with drugs, from knock-out or knock-down animals, from animals that are models of brain diseases, and from fresh human brain samples of surgical origin.

In the present investigation, we addressed the possibility that the depolarization-induced release of glutamate from rat brain cortex gliosomes could be modulated by CB1R, CB2R, and TRPV1 activation. We found that CB1R increased, while CB2R and TRPV1 decreased, the stimulus-induced release of gliosomal glutamate. This finding is at variance with the neuronal counterpart (i.e., synaptosomes), where we found that both CB1R and CB2R inhibited glutamate release and TRPV1 was ineffective.

The technique used here to monitor release from gliosomes is well suited to identify targets located on a given family of releasing particles, as experienced in the last three decades using nerve terminals. To study the effects of substances added to the superfusion medium on the release of a given transmitter, indirect effects have to be minimized. This can be obtained by stratifying gliosomes (or synaptosomes) on microporous filters in such an amount that, given the size of these organelles, the particles constitute a monolayer [44]. Many studies have demonstrated that, when such a monolayer is up-down superfused, as in the present experiments, any compound released is removed before it can activate targets (transporters, receptors, etc.) located on the releasing particles [35]. Under these conditions, changes of the release of a given transmitter produced by a compound added to the superfusion medium are due to the direct action of that compound at the particles releasing the transmitter in point. As a consequence, we can conclude that eCB receptors that modulate glutamate release in the present experiments are directly located on glutamate-releasing gliosomes.

This assumption is corroborated also by binding, Western-blot, and confocal microscopy experiments, the latter showing that a substantial portion of GFAP-positive gliosomes also express cannabinoid and vanilloid receptors. Whether CB1R, CB2R, and TRPV1 are concomitantly expressed on the same gliosomal particle is difficult to say on the basis of the available results, even though the percentages of GFAP-positive gliosomes that express CB1R (~60%), CB2R (~50%) or TRPV1 (~40%) suggest a significant degree of coexistence. These data also suggest that not all astrocytes express these receptors and, therefore, that there may exist different subpopulations with respect to eCB-signaling.

Beside eCB-binding receptors, astrocyte-derived gliosomes express AEA and 2-AG, the two most prominent eCBs, as well as their metabolic enzymes (see Ref. 45 and references therein). It has been proposed that astrocyte can

inactivate AEA by uptake and subsequent hydrolysis [46, 47], but their ability to produce eCBs remains largely unexplored. Our results underscore the existence of a complete eCB signaling system in astrocytes, thus enlarging the role played by these cells in the eCB-mediated synaptic transmission. It can be envisaged, for instance, that astrocytes can release eCBs upon cytosolic Ca^{2+} increase, as they do for glutamate, to signal between each other or with neighboring neurons.

In a recent excellent paper, published while our work was in progress, Navarrete and Araque [20] showed that hippocampal astrocytes express CB1 receptors that, upon activation, lead to phospholipase C-and thapsigargin-dependent Ca^{2+} mobilization from internal stores. These receptors are activated by eCBs released from neurons after their membrane depolarization, which activate also CB1R in neighboring astrocytes. As a consequence, their intracellular Ca^{2+} concentration is elevated and release of glutamate is induced; the latter compound in turn serves as a feedback signal that activates NMDA receptors in pyramidal neurons. Taken together, these results demonstrate the existence of eCB-mediated neuron–astrocyte signaling, and suggest intercellular communication pathways mediated by endocannabinoid–glutamate signaling, where astrocytes may serve as a bridge for interneuronal communication. The present results extend these observations to cerebral cortex and confirm that also in this brain area CB1R may warrant stimulation of the astrocytary glutamate release. Remarkably, astrocyte-derived cortical gliosomes also express CB2R and TRPV1 receptors that are known to be present in astrocytes [45, 48]. Unexpectedly, in gliosomes TRPV1 were found to be inhibitory.

As in the above-mentioned paper [20], stimulatory CB1R works through phospholipase C activation and phosphoinositide hydrolysis, leading to mobilization of intracellular Ca^{2+} by IP3, as demonstrated by direct determination of Ca^{2+} levels and IP3 formation in gliosomes. Recent studies have evidenced that CB1R, beside influencing adenylyl cyclase, voltage-sensitive Ca^{2+} and K^+ channels, can stimulate Ca^{2+} recruitment from internal stores [49], by activating PLC and increasing IP3 [50]. Canonically, the inhibition of glutamate release by CB2R in gliosomes is supported by reduction of cytoplasm cAMP. On the contrary, TRPV1 activation, which inhibits glutamate release, as CB2R, does not affect cAMP but augments IP3 formation and Ca^{2+} mobilization by high KCl, two activities that hardly cope with neurotransmitter release impairment. Therefore, other as-yet-unclear mechanisms should support the described activity of these receptors. Which mechanism(s) may be actually involved is difficult to say. Capsaicin modified neither IP3 formation nor glutamate release under basal conditions; it only produced a modest increase of the basal level of cytosolic Ca^{2+}. It seems, therefore, that the inhibitory effect of TRPV1 is correlated to depolarization of gliosomes and, possibly, to the depolarization-linked signaling cascade. It can be speculated, for instance, that the rapid desensitization of the receptor may play a role. As an alternative, the surplus of Na^+ entry throughout the TRPV1 channel could modify the efficiency of the Na^+/Ca^{2+} exchanger, which plays a pivotal role in the depolarization-evoked gliosomal glutamate release [28]. TRPV1 directly or indirectly interacts with plasma membrane or soluble proteins, modifying cell functions [51–53]. Again, it can be speculated that, in our experimental paradigm, the interaction with (or phosphorylation of) proteins involved in the modulation of exocytosis can be a cause of the observed inhibition. Activation of TRPV1 is known to inhibit voltage-gated calcium channels in dorsal horn root ganglia through Ca^{2+}-dependent or independent mechanisms [54, 55]. Voltage-gated calcium channels are present also in astrocytes, although they seems to be not directly connected with glutamate exocytosis [56]; as a consequence, it might be proposed that modifications of the spatial distribution of the cytosol concentrations of Ca^{2+} can influence the release probability.

In conclusion, this investigation demonstrates that gliosomes are fully equipped with a functional endocannabinoid system, and support the existence of a neuron–astrocyte communication route mediated by endocannabinoid–glutamate signaling in the cerebral cortex.

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