SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Mechanisms underlying 2-AG degradation in the superficial spinal dorsal horn of rodents and COS7 cell culture

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UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF NUROSCIENCES

DEBRECEN, 2018

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The Examination takes place at the library of the Department of Anatomy, Faculty of Medicine, University of Debrecen, 11:00, 12 September 2018

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 13:00, 12 September 2018

INTRODUCTION

"By using a plant that has been around for thousands of years, we discovered a new physiological system of immense importance." These were the words of Raphael Mechoulam, the "father of Cannabis", who opened the doors to the chemistry of cannabis by the discovery of its psychoactive constituent tetrahydrocannabinol (Δ9-THC).

The early studies of THC showed its hydrophobic nature, therefore THC was thought to act unspecifically by influencing the fluidity of the biological membranes rather than via specific interaction with a receptor (Lawrence and Gill 1975). However, it turned out, that its biological activity is stereoselective (Jones et al. 1974) and is mediated through the inhibition of cAMP accumulation (Howlett and Fleming 1984). These findings suggested the existence of a specific receptor, which was identified and cloned in 1990 and named cannabinoid receptor type-1 (CB1) (Matsuda et al. 1990). By the identification of CB_1 it was obvious, that its primary function is not the mediation of the effects of phytocannabinoids, therefore different research groups started to search for the endogenous ligand of this receptor. The first successful group of researchers was Devane and his coworkers, who isolated the first ligand of CB_1 and named it anandamide after the sanskrit word "ananda", meaning inner bliss (Devane et al. 1992). This was followed by the identification of 2-arachidonoylglycerol (2-AG), the second most important endogenous cannabinoid ligand (Mechoulam et al. 1995). These findings encouraged the scientist to broaden the spectrum of their knowledge for not only the molecular components of the endocannabinoid-mediated intercellular communication, but also for the role of the endocannabinoid system in different physiological or pathological processes. Due to this, marijuana was known not only as a symbol of hippies and as a recreational drug but also gained an outstanding role in the treatment of certain pathological conditions, like increased intraocular pressure (Nucci et al. 2007) or atopic dermatitis (Bíró et al. 2009). By different morphological, physiological or pharmacological studies researchers successfully demonstrated the existence of the components of the endocannabinoid system at peripheral, spinal or supraspinal levels of the pain pathway (Agarwal et al. 2007; Drew et al. 2008; Herkenham et al. 1991; Pertwee 2001).

A broad molecular toolbox is responsible for the endocannabinoid-mediated modulation of spinal pain processing pathway, from which the presence of CB_1 is demonstrated at the first relay station of the pain pathway, the dorsal horn of the spinal cord. Hegyi and his coworkers demonstrated the existence of CB_1 and the major synthesizing enzymes of anandamide and 2-AG, the NAPE-PLD and DGLα respectively on many cellular elements of the spinal dorsal

horn. While CB_1 expression can be observed presynaptically mostly on excitatory and inhibitory axon terminals or glial cells (Hegyi et al. 2009), the presence of the NAPE-PLD and DGLα can be observed on the somatodendritic compartments of neurons but also on glial cells (Hegyi et al. 2012). However, the activation and the strength of the cannabinoid modulation is not regulated mainly by the mobilisation of the endocannabinoid ligands, but rather by their concentration, which is regulated mostly by the rate of their synthesis and degradation. At the time of our experiments it was less known about the expression of the degrading enzymes of the endocannabinoid ligands, therefore, considering the importance of 2-AG-mediated signal transduction pathway in the spinal pain processing, we decided to examine two processes responsible for the reduction of the levels of 2-AG. In our experiments we studied the expression of the major degrading enzyme of 2-AG, the monoacylglycerol-lipase (MGL) in the superficial spinal dorsal horn, and also examined the effects of the spontaneous molecular rearrangement of 2-AG on the 2-AG-mediated signal transduction.

MAJOR AIMS

The number of publications dealing with the cannabinoid system has increased rapidly in the last 30 years, and our idea of the role of the endocannabinoids in different physiological or pathological conditions became more and more complex. In spite of the growing number of experimental data in the field of endocannabinoids, our knowledge is still incomplete at many points. For instance the cannabinoid mediated control of spinal pain processing, mainly the 2-AG-mediated modulation of synaptic transmission remains still unclear. At the beginning of our experiments there were almost no data about the termination of 2-AG signalling at the level of spinal cord, therefore our aim was first:

- to examine the distribution of the major degrading enzyme of 2-AG, the MGL in the superficial spinal dorsal horn and its expression on the cellular elements and axon terminals terminating in the superficial laminae.

Because the expression of MGL on the axon terminals of primary afferents and interneurons seemed to be weak, we raised the question if the enzymatic degradation is really the most important mechanism of termination of 2-AG signalling. The ability of 2-AG to isomerize was well known, however, it was less known about the nature of the end product of this process, 1-arachidonoyl-glicerol (1-AG). Therefore we have chosen a simple experimental system, the COS7 cell line, where the CB_1 was overexpressed. Here we wanted to examine:

- the kinetics of the isomerization of 2-AG
- the biological activity of 1-AG
- if 1-AG, the end product of the isomerization of 2-AG has an impact on the 2-AG-CB_1 signal pathway.

MATERIALS AND METHODS

Animals and preparation of tissue sections

Experiments were carried out on nine adult rats (Wistar-Kyoto, 250–300 g, Gödöllő, Hungary), two wild-type and one MGL knock-out (Schlosburg et al. 2010) mice. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen, and were in accordance with the European Community Council Directives and the rules of the Indiana University Institutional Animal Care and Use Committee. The animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with Tyrode's solution (oxygenated with a mixture of 95 % O2 and 5 % CO2), followed by a fixative containing 4 % paraformaldehyde dissolved in 0.1 M phosphate buffer (PB, pH 7.4). After the transcardial fixation, the lumbar segments of the spinal cord were removed, post-fixed in the original fixative for 1–4 h, and immersed into 10 and 20 % sucrose dissolved in 0.1M PB until they sank. In order to enhance reagent penetration the removed spinal cord was freeze-thawed in liquid nitrogen. Fifty micrometre thick transverse sections were cut on a vibratome, and extensively washed in 0.1 M PB.

Immunohistochemistry

A single immunostaining protocol was performed to study the laminar distribution of MGL. Free-floating sections were first incubated in rabbit anti-MGL (diluted 1:30, catalog no.: MGL-Rb-Af200, Frontier Science Co., Ishikari, Hokkaido, Japan) for 48 h at 4 °C, and then were transferred into biotinylated goat anti-rabbit IgG (diluted 1:200, catalog no.: PK-4001, Vector Labs., Burlingame, California, USA) for 12 h at 4 °C. Thereafter, the sections were treated with an avidin biotinylated horseradish peroxidase complex (diluted 1:100, Vector Labs., Burlingame, California, USA) for 5 h at room temperature, and the immunoreaction was completed with a 3,3-diaminobenzidine (catalog no.: D-5637, Sigma, St. Louis, Missouri, USA) chromogen reaction. Before the antibody treatments the sections were kept in 10 % normal goat serum (catalog no.: S-1000, Vector Labs., Burlingame, California, USA) for 50 min. Antibodies were diluted in 10 mM Tris–phosphate-buffered isotonic saline (TPBS, pH 7.4) to which 1 % normal goat serum (catalog no.: S-1000, Vector Labs., Burlingame, California, USA) was added. Sections were mounted on glass slides, dehydrated and covered with Permount neutral medium.

Double-immunostaining protocols were performed to study the co-localization of MGL immunoreactivity with various markers of nociceptive primary afferents, axon terminals of putative glutamatergic and GABAergic spinal neurons, astrocytes and microglial cells. Free-floating sections were first incubated with a mixture of antibodies that contained rabbit anti-MGL (diluted 1:30, catalog no.: MGL-Rb-Af200, Frontier Science Co., Ishikari, Hokkaido, Japan) and one of the following antibodies: (1) guinea pig anti-calcitonin gene-related peptide (CGRP) (diluted 1:2,000, catalog no.: T5027, Peninsula Labs, San Carlos, California, USA), (2) biotinylated isolectin B4 (IB4) (1:2,000, catalog no.: I21414, Invitrogen, Eugene, Oregon, USA), (3) guinea pig anti-vesicular glutamate transporter 2 (VGLUT2) (diluted 1:2,000, catalog no.: AB2251, Millipore, Temecula California, USA), (4) guinea pig anti-vesicular GABA transporter (VGAT) (diluted 1:600, catalog no.: 131004, Synaptic Systems, Goettingen, Germany), (5) mouse anti-glial fibrillary acidic protein (GFAP) (diluted 1:1,000, catalog no.: MAB3402, Millipore, Temecula, California, USA), and (6) mouse anti-CD11b (diluted 1:500, catalog no.: MCA275G, AbD Serotec, Oxford, UK). The sections were incubated in the primary antibody solutions for 2 days at 4 ℃ and were transferred for an overnight treatment into the appropriate mixtures of secondary antibodies that were selected from the following: goat anti-rabbit IgG conjugated with Alexa Fluor 488 (diluted 1:1,000, catalog no.: A11034, Invitrogen, Eugene, Oregon, USA), goat anti-guinea pig IgG conjugated with Alexa Fluor 555 (diluted 1:1,000, catalog no.: A21435, Invitrogen, Eugene, Oregon, USA), goat anti-mouse IgG conjugated with Alexa Fluor 555 (diluted 1:1,000, catalog no.: A21422, Invitrogen, Eugene, Oregon, USA), and streptavidin conjugated with Alexa Fluor 555 (diluted 1:1,000, catalog no.: S11225, Invitrogen, Eugene, Oregon, USA). Before the antibody treatments the sections were kept in 10 % normal goat serum (catalog no.: S-1000, Vector Labs., Burlingame, California, USA) for 50 min. Antibodies were diluted in 10 mM TPBS (pH 7.4) to which 1 % normal goat serum (catalog no.: S-1000, Vector Labs., Burlingame, California, USA) was added. Sections were mounted on glass slides and covered with Vectashield (catalog no.: H-1000, Vector Labs, Burlingame, California, USA).

Immunocytochemistry

Transfected COS7 cells were grown on 24-well glass bottom plate and fixed with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer for 10 min. Cells were then treated with 10% normal goat serum for 30 min, followed by an incubation with anti-CB_1 antibody (1:2000, Cayman Chemicals, Ann Arbor, MI, USA, Cat. No: 10006590) for 2 h. After washing, goat

anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:1000, Invitrogen) was applied for 1 h, then cells were covered with Vectashield-DAPI. Antibodies were diluted in PBS (pH 7.4) containing 1% normal goat serum. All incubation steps were carried out at room temperature. Imaging of immunostained COS7 cells was carried out with an Olympus IX-81 inverse microscope attached to a DSD2 an Andor Zyla 5.5 sCMOS camera. Images were acquired using a 60 PlanApo N oil-immersion objective (NA: 1.40) and selecting the "high signal" disk of DSD2, and processed with Adobe Photoshop CS5. The specificity of anti-CB_1 antibody has extensively been characterized earlier in our laboratory (Hegyi et al. 2009). To test the specificity of the immunostaining protocol, transfected COS7 cells were incubated according to the immunostaining protocol described above with primary antibodies omitted or replaced with 1% normal goat serum. No immunostaining was observed under these conditions.

Preembedding immunostaining

A preembedding immunostaining similar to the single immunostaining protocol described above was performed to study the cellular distribution of MGL at the ultrastructural level. Following extensive washes in 0.1 M PB (pH 7.4), free-floating sections were first incubated with rabbit anti-MGL (diluted 1:30, catalog no.: MGL-Rb-Af200, Frontier Science Co., Ishikari, Hokkaido, Japan) for 48 h at 4 ℃, than were transferred into biotinylated goat anti-rabbit IgG (diluted 1:200, catalog no.: PK-4001, Vector Labs., Burlingame, California, USA) for 12 h at 4 ℃. Thereafter, the sections were treated with an avidin biotinylated horseradish peroxidase complex (diluted 1:100, catalog no.: PK-4001, Vector Labs., Burlingame, California, USA) for 5 h at room temperature, and the immunoreaction was completed with a 3,3-diaminobenzidine (catalog no.: D-5637, Sigma, St. Louis, Missouri, USA) chromogen reaction. Before the antibody treatments the sections were kept in 10 % normal goat serum (catalog no.: S-1000, Vector Labs., Burlingame, California, USA) for 50 min. Antibodies were diluted in 10 mM TPBS (pH 7.4). Immunostained sections were treated with 0.5 % osmium tetroxide for 45 min, then dehydrated and flat-embedded into Durcupan ACM resin (catalog no.: 44610, Sigma, St. Louis, Missouri, USA) on glass slides. Selected sections were re-embedded, ultrathin sections were cut and collected on Formvar-coated single-slot nickel grids, and counterstained with uranyl acetate and lead citrate.

Confocal microscopy and analysis

Series of 1 μm thick optical sections with 0.5 μm separation in the Z axis were scanned with an Olympus FV1000 confocal microscope. Scanning was carried out using a 60 oil-immersion lens (NA: 1.42). The confocal settings (laser power, confocal aperture and gain) were identical for all sections, and care was taken to ensure that no pixels corresponding to immunostained puncta were saturated. The scanned images were processed by Adobe Photoshop CS5 software. The co-localization of MGL with the investigated markers was quantitatively analyzed in the double-stained sections. A 10x10 standard square grid in which the edge-length of the unit square was 4 μm (the whole grid was 40 μmx40 μm in size) was placed onto the regions of confocal images corresponding to laminae I–II of the superficial spinal dorsal horn. The proper placement of the grid was based on the following criteria: (a) The border between the dorsal column and the dorsal horn was easily identified on the basis of the intensity of immunostaining. (b) The border between laminae II and III was approximated on the basis of previous ultrastructural observations (Molander et al. 1984). Thus, immunoreactivities and co-localizations were investigated in the most superficial 150 μm thick zone of the dorsal horn that had earlier been identified as a layer of the gray matter corresponding to laminae I and II in the lumbar segments of the spinal dorsal horn. Profiles that showed immunoreactivity for MGL over the edges of the standard grid were counted in the intermediate compartments of laminae I and II. The selected profiles were then examined whether they were also immunoreactive for the axonal or glial markers. Since MGL is regarded to be a cytoplasmic enzyme associating with cell membranes, to define the co-localization values we counted only those MGL immunolabeled puncta that were located within the confines of the areas immunostained for the marker. The co-localization for all investigated markers was analyzed in three animals. The quantitative measurement was carried out in three sections that were randomly selected from each animal. Thus the calculation of quantitative figures, mean values and standard error of means (SEM), was based on the investigation of nine sections.

Controls and Western Blot analysis

To test the specificity of the antibody raised against MGL, free-floating sections obtained from MGL knock-out (Schlosburg et al. 2010) and wild type mice were immunostained according to the single immunostaining protocol described above. Sections of MGL knock-out mice were negative for MGL, whereas wild-type mice showed a characteristic immunostaining identical to that observed in rats. To obtain a more global view about the specificity of the anti-MGL

antibody a Western-blot analysis was performed on rats. While the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.), the lumbar segments of the spinal cord were dissected. The dorsal horn was sonicated in 20 mM Tris lysis buffer (pH 7.4) containing the following protease inhibitors (mM): EDTA (4.0), EGTA (2.5), PMSF (0.002) benzamidine (0.013), pepstatin A (0.004), soybean trypsin inhibitor (0.001), leupeptin (0.001) and aprotinin (0.001). After removing cell debris from the sonicated samples with centrifugation (1,500 rcf for 10 min at 4 ℃), the supernatant was centrifuged again (12,000 rcf for 20 min at 4 ℃). The pellet was re-suspended in lysis buffer containing 1 % Triton X-100 and 0.1 % SDS, and the samples were run on 10 % SDS–polyacrylamide gels according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA, USA), and the membranes were immunostained according to the single immunostaining protocol described above. The immunostaining revealed only one immunoreactive band at molecular weight of 33 kDa corresponding to the molecular weight of MGL (Dinh et al. 2002). To test the specificity of the immunostaining protocol, free-floating sections were incubated according to the single immunostaining protocol described above with primary antibodies omitted or replaced with 1 % normal goat serum. No immunostaining was observed in these sections.

The COS7 samples were sonicated in 20 mM TRIS (pH 7.4) lysis buffer supplemented with protease inhibitors (4 mM EDTA, 2.5 mM EGTA, 2 mM PMSF, 26 mM benzamidine, 8 mM pepstatin A, 2 mg/ml soybean trypsin inhibitor, 2 mg/ml leupeptin, 2 mg/ml aprotinin). The cell debris was removed by centrifugation (10 min at 1500 g and 4C), then the supernatant was again centrifuged (20 min at 12,000 g and 4℃). The pellet was resuspended in lysis buffer containing 1% TRITON X-100 and 0.1% SDS. The samples were stored at -70℃ until use. The protein concentration of the samples was determined using the detergent compatible BCA assay (Pierce, Rockford, IL, USA). The samples were dissolved in reducing sample buffer (50 mg protein/lane) and run on 10% SDS-polyacrylamide gels (Laemmli, 1970). The separated proteins were electrophoretically transferred onto PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with 10% bovine serum albumin (Sigma) in TTBS solution (20 mM TRIS, 500 mM NaCl, pH 7.5, 0.05% Tween-20). Membranes were incubated with anti-CB_1antibody (1:1000, Cayman Chemicals, Ann Arbor, MI, USA, Cat. No: 10006590) for 2 h at room temperature. After extensive washing with TTBS the membranes were incubated with anti-rabbit IgG-HRP secondary antibody (DakoCytomation,
Glostrup, Denmark). The labeled protein bands were visualized with 3, 3-diaminobenzidine (Sigma).

Decomposition of 2-AG in Aqueous Solutions

The endocannabinoid 2-AG and 1-AG were prepared and extracted as described earlier (Higuchi et al., 2010; Zhang et al., 2010; Zoerner et al., 2012) with some modifications. Briefly, 250 ml HBSS containing 0.625 mg/mL anandamide was spiked with either 2-AG (Cayman Chemical) or 1-AG (Cayman Chemical) dissolved in acetonitrile in a final concentration of 0.25 mg/mL in test tubes, and incubated at 37°C for 1.25, 2.5, 5, or 10 min. Thereafter, the samples were frozen in liquid nitrogen and kept there until further processing. Zero time samples were spiked with 2-AG after freezing in liquid nitrogen. During sample preparation, 10 ml trifluoroacetic acid and 1000 ml hexane was added to the frozen samples, which were allowed to melt during constant vigorous shaking at 1400 rpm. This allowed the endocannabinoids to be transferred to the organic phase immediately after melting. TFA was added to stop the base-catalyzed isomerization. Then, the phases were separated by centrifugation at 13000 rpm for a minute. An 800 ml aliquot of the hexane layer was evaporated to dryness, redissolved in 40 ml acetonitrile containing 0.1% formic acid. Then, 20 ml of this solution was injected into the LC/MS. The experiment was performed in three replicates for each time point.

Quantification of 1-AG and 2-AG with LC-MS

Changes in the amounts and ratio of 2-AG and 1-AG were determined on a YMC-Triart C18 (100 mm 3.0 mm, 1.9 mm, 12 nm, YMC Co., Ltd, Kyoto 600-8106, Japan) column, using an Accela HPLC system (Thermo Electron Corp., San Jose, CA, USA) eluted with a gradient of acetonitrile (A) and water (B) containing 0.1%(V/V) formic acid each. The gradient was from 60% of A (hold for 2 min) to 90% A over 7 min, hold for 6 min and return to initial conditions and hold for 5 min to equilibrate the column. The LC system was coupled with a Thermo LTQ XL mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) using positive-ion ESI mode as a method of ionization. The ion injection time was set to 100 ms. ESI parameters were as follows: spray voltage: 5 kV, source heater temperature: 280°C, capillary temperature of: 300°C, sheath gas flow: 25 units N_2, auxiliary gas flow: 8 units N_2. The tray temperature was set to 12°C and the column oven was set to 30°C to perform the optimal retention of the compounds in the reaction mixtures. MS2 product-ion scans were obtained after collision induced dissociation with helium as the target gas. Compound identification was based on their retention times (tR), HESI mass spectra and MS2 with authentic compounds as references. 1-

AG and 2-AG levels were determined by LC-ESI-MS/MS in SRM mode and calibration with solutions of known concentrations of the analytes extracted for analyses. As an internal standard, anandamide was added before each sample extraction. SRM transitions were 379–287 for 1AG and 2AG and 348–287 for ANA, respectively. For all analytes of interest, recovery was calculated to be above 85% which is comparable to that published by Zoerner et al. (2012).

Plasmid Construction

The mammalian expression vector pcDNA3 CB_1 was used to overexpress CB_1 receptor in COS7 cell line [generous gift from Mary Abood, Addgene plasmid # 13391, (Abood et al., 1997)]. To verify the successful transfection, the red fluorescent protein (RFP) coding expression vector CMV-Brainbow-1.0 H (a gift from Joshua Sanes, Addgene plasmid # 18720) was co-expressed in the cells under the control of the same CMV promoter as the pcDNA3-CB1 plasmid (Livet et al., 2007).

Cell Culture and Transfection

COS7 cells (originated from ATCC, kindly provided by the Department of Biophysics, University of Debrecen) were grown to 90% confluence (104 cell/cm^2) in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine (Gibco, USA) under 5% CO_2 at 37°C. Prior to electroporation, cells were detached from 75 cm^2 culturing flasks by incubation with Trypsin-EDTA (Sigma-Aldrich, USA), pelleted by centrifugation (900 x g for 10 min) and resuspended in DMEM (1.9 105 cell/ml). This suspension was used for CB_1/RFP co-transfection (20 mg/ml CB_1 plasmid and 4 mg/ml RFP plasmid) and for control RFP transfection (4 mg/ml RFP plasmid). Electroporation was carried out by ECM830 electroporator (BTX, Harvard Apparatus, USA) using disposable 2 mm Gap Cuvette (Model No. 620, BTX, Harvard Apparatus, USA). The electroporation protocol was as follows: 220 V, two 500 ms width pulses with 1 s intervals.

Ca^{2+} Measurements

Before Ca^{2+} imaging experiments, transfected COS7 cells were loaded with 1 mM Fluo-8-AM in the presence of 0.01% pluronic at room temperature for 30 min. Ca^{2+} imaging was carried

out with an Andor Zyla 5.5 sCMOS camera attached to a differential spinning disk (DSD2, Andor Technology) built on an Olympus IX-81 inverse microscope. Using a 10x objective (NA:0.25), images of 540 pixels x 306 pixels (corresponding to 1400 mm 790 mm field of view, which contained around 200 to 250 cells) were acquired at 15 frames per second with Andor iQ3 software. Fluo-8 filled cells were excited at 488 nm and emission was collected at 520 nm. Acquisition parameters (illumination intensity, exposure time, readout time, frame rate) were identical for all experiments. Changes in fluorescence intensities were measured over the entire COS7 cell surface by drawing freehand ROIs around single transfected COS7 cells with sharp RFP signal, that identified unequivocally the entire contour of the transfected cell. Ca^{2+} variations were estimated as changes of the fluorescence signal over baseline 11(ΔF/F0, where F0 was the average initial fluorescence). A region of interest was considered to respond to the application of a compound if ΔF/F0 three times the standard deviation of the baseline for at least five consecutive images. Experimental data were analyzed with Microsoft Excel 2013 (Microsoft), and FFT filtering to reduce noise and calculation of area under the curve (AUC) were performed with Origin Pro 8.0 (Originlab, Northampton, MA, USA). Statistical analysis was performed with two-tailed non-parametric Mann–Whitney U test. The differences were considered significant when the p level was < 0.05.

RESULTS

Distribution of MGL immunoreactivity in the superficial spinal dorsal horn

To elucidate the distribution of the MGL protein in laminae I–II of the spinal dorsal horn, immunostaining for MGL with an antibody directed against a 35 amino acid long segment of the enzyme (residues 1–35) was carried out in the rat lumbar spinal cord. Peroxidase-based single immunostaining revealed an abundant immunoreactivity for MGL in the lumbar spinal cord of rats that was mostly confined to the superficial spinal dorsal horn, laminae I and II of the spinal gray matter. Within this area, the inner layer of lamina II (lamina IIi) was even more densely stained than the rest of the superficial spinal dorsal horn. Immunostained elements appeared as punctate profiles both in the densely and sparsely stained zones.

Co-localization of MGL with markers of nociceptive primary afferents

Laminae I–II of the spinal dorsal horn receives peripheral sensory inputs mostly from nociceptive primary afferents (Ribeiro da Silva and De Korninck 2009), which release glutamate as synaptic neurotransmitter. A population of nociceptive primary afferents, however, also releases neuropeptides in addition to glutamate (Willis and Coggeshall 2004). It is well established that most of the peptidergic nociceptive primary afferents express calcitonin gene-related peptide (CGRP), whereas non-peptidergic axon terminals selectively binds isolectin-B4 (IB4) (Willis and Coggeshall 2004; Ribeiro da Silva and De Korninck 2009). Thus, to study the expression of MGL on central axon terminals of peptidergic and non-peptidergic nociceptive primary afferents we investigated the co-localization of the enzyme with CGRP immunoreactivity and IB4-binding. In agreement with previous studies, we observed a strong immunostaining for CGRP in laminae I–IIo (Traub et al. 1989; Nasu 1999). Investigating the co-localization between MGL and CGRP immunoreactivity, we collected 225 and 387 profiles immunostained for CGRP and MGL, respectively, and found that 10.0 ± 2.5 % of MGL immunoreactive puncta were also stained for CGRP, whereas 17.0 ± 3.5 % of CGRP immunoreactive axon terminals proved to be immunoreactive also for MGL. Confirming earlier observations, IB4-binding labeled a large number of axon terminals in lamina IIi (Guo et al. 1999). Despite the strong immunostaining and the substantial spatial overlap between the investigated profiles, the co-localization between axon terminals labeled with IB4-binding and puncta immunoreactive for MGL was very low. After the investigation of 964 IB4-binding and

1090 MGL immunostained profiles, it was found that 1.1 ± 0.3 % of MGL immunoreactive puncta were also positive for IB4-binding, whereas 1.1 ± 0.3 % of axon terminals that were positive for IB4-binding proved to be immunoreactive also for MGL.

Co-localization of MGL with markers of axon terminals of glutamatergic and GABAergic spinal neurons.

Since dorsal rhizotomy leaves VGLUT2 immunoreactivity of the spinal gray matter unaffected, it is generally accepted that in immunostaining protocols VGLUT2 can be used as a marker for axon terminals of intrinsic spinal excitatory neurons (Alvarez et al. 2004; Brumovsky et al. 2007). There is also general agreement that vesicular inhibitory amino acid transporter (VGAT) is expressed in axon terminals of GABAergic and glycinergic neurons (Chaudhry et al. 1998). Therefore, to study the expression of MGL on central axon terminals of putative glutamatergic and GABAergic spinal neurons we investigated the co-localization between the enzymes and VGLUT2 as well as VGAT immunoreactivities. Confirming results of previous studies (Li et al. 2003; Oliveira et al. 2003; Todd et al. 2003; Alvarez et al. 2004; Brumovsky et al. 2007), we have revealed a strong punctuate immunostaining for VGLUT2 in laminae I–II. Evaluating 751 and 1251 profiles immunostained for VGLUT2 and MGL, respectively, we found that 6.3 ± 0.5 % of MGL immunoreactive puncta were also immunostained for VGLUT2, whereas 10.7 ± 1.0 % axon terminals that were positive for VGLUT2 were also immunoreactive for MGL. As it has been reported earlier (Polgar and Todd 2008), VGAT immunoreactive axon terminals showed a dense and homogeneous distribution in the superficial spinal dorsal horn. Investigating the co-localization between MGL and VGAT immunoreactivity, we collected 609 and 524 profiles immunostained for VGAT and MGL, respectively, and found a minimal overlap between VGAT and MGL. Only 2.9 ± 0.8 % of MGL immunoreactive puncta were also stained for VGAT, and similarly only 2.4 ± 0.7 % of VGAT immunoreactive axon terminals proved to be immunoreactive also for MGL.

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Co-localization of MGL with markers of astrocytes and microglial cells

The existence of bidirectional molecular communication pathways between glial cells and neurons has been generally accepted (Araque et al. 2001; Volterra and Bezzi 2002; Nedergaard et al. 2003; Haydon and Carmingnoto 2006; Suter et al. 2007; Zhang et al. 2008). It has also been demonstrated that CB_1s are expressed in microglial cells and astrocytes in various parts

of the central nervous system including the dorsal horn of the spinal cord (Rodriquez et al. 2001; Salio et al. 2002; Cabral and Marciano-Cabral 2005; Navarrate and Araque 2008; Hegyi et al. 2009, 2012). Navarrate and Araque (2008) provided direct experimental evidence that CB_1s on astrocytes can be activated by endocannabinoids released by neurons. MGL is also expressed by astrocytes in the dentate gyrus (Uchigasima et al. 2011) and by Bergman glial cells in the cerebellum (Tanimura et al. 2012). Thus, because of their potential importance in pain processing we investigated the localization of MGL on astrocytes and microglial cells by using GFAP and CD11b as markers for astrocytes and microglial cells, respectively. We have obtained strong immunolabeling in the superficial spinal dorsal horn for both GFAP and CD11b that were identical to that reported earlier (Garrison et al. 1991; Eriksson et al. 1993; Molander et al. 1997). Investigating the co-localization between MGL and GFAP immunoreactivity, we collected 674 and 794 profiles immunoreactive for GFAP and MGL, respectively, and found that 17.4 ± 1.1 % of MGL immunoreactive puncta were also stained for GFAP, whereas 20.2 ± 0.9 % of GFAP immunoreactive profiles proved to be immunoreactive also for MGL. In contrast to GFAP, we revealed almost a complete segregation between MGL and CD11b immunoreactivities. After the investigation of 455 and 1245 profiles immunostained for CD11b and MGL, respectively, the co-localization analysis showed that only 1.0 ± 0.4 % of MGL immunoreactive puncta were also stained for CD11b, whereas 2.3 ± 1.4 % of CD11b immunoreactive profiles proved to be immunoreactive also for MGL in the spinal dorsal horn.

Ultrastructural localization of MGL immunoreactivity

After revealing a remarkably selective co-localization between MGL and various axonal and glial markers, we intended to define the sub-cellular localization of MGL both in axon terminals and glial profiles. Furthermore, since in immunofluorescence studies we recovered MGL immunoreactive puncta in neural perikarya but not in dendrites, an extensive search for MGL immunolabeling in the somato-dendritic compartment of neurons was also carried out. In agreement with the results obtained from the colocalization studies, peroxidase reaction precipitates labeling MGL were recovered primarily in axons and glial-like processes, but were also found in neural perikarya. Regardless of whether the labeled profile was an axon terminal or a glia-like profile, immunolabeling was revealed in close association to the plasma membrane in a way that the immunoprecipitates always extended into the cytoplasm. In axon terminals, the immunolabeling was always recovered in the vicinity of synaptic contacts. Some

of the labeled axons contained spheroid, others were full of pleomorphic synaptic vesicles. In glia-like processes, the labeling was abundant for MGL. Immunolabeled glia-like processes were frequently observed. It was a general finding that the membrane compartment immunoreactive for MGL was restricted only to a segment of the glial profile, while the adjacent part of the profile was free of labeling. Unfortunately we were not able to define whether the detected immunoprecipitates were in astrocytes or microglial cells, since we cannot make any distinction between astrocytic and microglial profiles in the electron microscope. In perikarya of neurons, immunoprecipitates were associated with cytoplasmic membranes resembling membranes of endoplasmic reticulum and Golgi apparatus.

2-AG Rearranges Rapidly to 1-AG in HBSS

Many studies reported rearrangement of 2-AG into 1-AG under ambient conditions. In order to simulate the conditions of our in vitro experiments to the highest possible extent, Hank's Balanced Salt Solution (HBSS), a commonly used cell medium (HBSS) was spiked with 2-AG and the decomposition curve at 37°C was determined using LC-MS. The other well-known endocannabinoid anandamide, which remains stable during the analytical procedure (Zoerner et al., 2012), was successfully used as internal standard to manage inaccuracies of liquid–liquid extraction and injections. The peak of anandamide showed no decrease over time. In accordance with other publications employing RPMI medium (Rouzer et al., 2002), we found that 2-AG is rapidly converted to 1-AG also in HBSS. The half-life of conversion was 16.16 ± 3.74 min (without serum, extrapolated) and 8.81 ± 2.51 min (with serum), which is within the same order of magnitude as reported for other physiological solutions. Differences may come from the differences in the media, and from the different initial concentration of 2-AG, since Rouzer et al. (2002) used 2 mg/mL (equal to 5 mM), while in our experimental settings the concentration was close to the order of magnitude used for the treatment of the cells (0.6 mM). The latter is comparable to the concentration of the OH^- present, possibly leading to altered kinetics of the reaction. The ratio of the two peaks is significantly changed within 2 min. We have to add, however, that our experiments were carried out in pure physiological solution, free of cells or biological membranes, which helped us to significantly reduce the time frame of sample preparation and concomitant artifactual isomerization. This approach is different from those publications, in which 1-AG and 2-AG were isolated from biological samples requiring lengthy purification steps that provides possibility for post-isolation artifacts (Ferrer et al., 2003; Suplita et al., 2006). Importantly, acyl migration takes place in any protic solvent

(including water) and is actually catalyzed by OH^- present in all aqueous solutions at physiological pH (Rouzer et al., 2002). Therefore, we assume that 2-AG isomerizes to 1-AG on the minute timescale in any water-based media including the one used in the present study. In addition, rearrangement of 2-AG is accelerated in the presence of protein, that is likely to act as a catalytic surface that increases the reaction speed. Since actually all intracellular or extracellular fluids of the living organisms contain proteins, acyl migration is likely to be of significance in vivo as well.

Overexpression of CB_1 Receptor in COS7 Cells

First, we aimed to establish a simple in vitro model expressing CB_1 to study the cellular effects of 2-AG and 1-AG. Thus, we transiently transfected COS7 cells with pcDNA3 CB_1 by electroporation after 5–7 passages to achieve overexpression of CB_1 receptor, and verified the expression of CB_1 by immunocytochemistry. CB_1 immunostained puncta could be observed in high densities along the cell membrane and also in the cytoplasm of the transfected cells, identified by their RFP expression. We also quantified CB_1 protein levels 3 days after transfection. Western blot analysis with an anti-CB_1 antibody confirmed the expression of CB_1 in COS7 cells, and found an approximately 5.6-fold increase in CB_1 expression in the transfected cells.

Despite Its Conversion into 1-AG, the Biological Effects of 2-AG at Cannabinoid Receptors Are Barely Affected on the Minute Timescale

Since 2-AG gradually disappears in HBSS due to isomerization, acyl migration may strongly influence the potency of 2-AG. Thus, we examined the effects of decreasing effective 2-AG concentration on CB_1-induced Ca^{2+} transients by loading CB_1 transfected COS7 cells with the fluorescent Ca^{2+} indicator Fluo-8-AM and monitoring Ca^{2+} signals evoked by administration of 2-AG. We identified all CB_1-transfected cells in the visual field based on the RFP signal, and selected cells with sharp and distinct RFP-defined contour for analysis. To allow reliable comparison of the experimental data, we performed all Ca^{2+} imaging with identical settings and acquisition parameters. Changes in the fluorescence signal intensity over baseline ($\Delta F/F0$) were plotted against time to illustrate alterations in the intracellular Ca^{2+} concentration. In the end of all Ca^{2+} measurement experiments, we applied 180 mM ATP as final treatment to verify viability of cells and obtain maximum Ca^{2+} response. Cells showing no response to ATP

treatment were discarded from further analysis. Values of the AUC of Ca^{2+} transients evoked by ATP were considered as maximal responses for each cell. AUC values of cannabinoid-evoked Ca^{2+} signals were also calculated and normalized against responses to ATP by expressing CB_1-dependent responses as percentage of maximal response. Cells in each well were treated only once to avoid desensitization of CB_1 and allow registration of consistent responses. With these experimental settings, we treated the cells with 2-AG solution, that had a 1 mM starting concentration and was administered either immediately ("0-min experiment"), or incubated for 2.5, 5, and 10 min at room temperature before application. This amount of 2-AG is about 50% of the concentration that evokes maximal response, so any change in 2-AG levels will be approximately linearly reflected by changes is the Ca^{2+} transients. Freshly prepared and immediately applied 2-AG induced robust transient-like elevation of intracellular Ca^{2+} concentration in CB_1 transfected COS7 cells. Two and half minutes after preparation, 2-AG evoked Ca^{2+} signals with AUC values practically identical to that of 0-min experiment, and an additional incubation of 2-AG solution for 2.5 min caused only a marked diminution of Ca^{2+} responses. Application of 2-AG 10 min after preparation caused a weak and still not significant attenuation in cytosolic Ca^{2+} elevation. 2-AG is readily converted into 1- AG with a half-life of 8.8 min. However, the decrease in 2-AG concentration was not reflected in the evoked Ca^{2+} signals, as we detected only an insignificant minor decrease in biological responses.

1-AG Concentration-Dependently Increases Intracellular Ca^{2+} Concentration by Activating Cannabinoid Receptors

As the arachidonoyl moiety moves from the 2-position to the 1-position of glycerol, not only the effective concentration of 2-AG decreases continuously, but also the amount of 1-AG increases gradually in aqueous solutions until the equilibrium between 2-AG and 1-AG is reached at approximately 1:9 ratio. Here we investigated if the 1-isomer interacts with CB_1 in our experimental conditions, and compared its pharmacological properties to those of 2-AG. We administered 1-AG solution of various concentration spanning from 10 nM to 100 mM to CB_1 transfected COS7 cells, and examined if the treatment induced any change in the intracellular Ca^{2+} concentration in our experimental conditions. Starting at low micromolar concentration, 1-AG evoked Ca^{2+} transients with strictly concentration-dependent amplitude and AUC values. To compare the potency and efficacy of the two isomers, we repeated the above experiment, now by applying various concentration of 2-AG spanning from 10 nM to

100 mM. In agreement with earlier observations (Sugiura et al., 1999), 2-AG also induced Ca^{2+} transients even in nanomolar concentration. Concentration-response curve of 1- and 2-AG clearly demonstrates that potency of 2-AG (EC50 D 0.6 mM) is threefold higher than that of 1-AG (EC50 D 1.9 mM), and 2-AG was also found to be more efficacious. To demonstrate that 1- and 2-AG induce Ca^{2+} signals through a CB_1 receptor mediated pathway, we applied the selective CB_1 antagonist AM251. Effects of 1-AG were exclusively mediated by CB_1 receptors, since application of AM251 prevented the raise in intracellular Ca_{2+} in response to 10 mM 1-AG in 99.37 0.51% of the cells. Application of AM251 completely abolished 2-AG evoked Ca^{2+} transients also in the majority of the cells, however, 2.33 0.89% of COS7 cells still showed responses to 1 mM 2-AG. Preincubation with AM251 caused a pronounced decrease in, but could not fully prevent the 2-AG induced raise of, intracellular Ca^{2+} concentration in these cells (data not shown). Preincubation of the cells with AM251 at 5 mM caused an apparent drop of the baseline in case of 6.16 ± 0.91 of the cells. This may most probably be the result of the inhibition of a basal endocannabinoid tone by AM251, but, as an inverse agonist, it may also decrease constitutive CB_1 receptor activity. Thus, it is very likely that the AM251-induced drop of the baseline is CB_1-dependent, but we cannot exclude other mechanisms in the background of this phenomenon, such as the inhibition of adenosine A1 receptors by AM251.

Accumulation of 1-AG Compensates the Decreasing 2-AG Concentration and Consequent Drop in Biological Responses

Isomerization of 2-AG creates an interesting situation, since both 1- and 2-isomers of arachidonoylglycerol target CB_1 and it is very likely that they will compete for the ligand binding sites of CB_1. However, depending on the relative concentration of the two isomers with different potency and ligand binding sites available in the biological environment, combined effects of 1-AG and 2-AG may be either additive or antagonistic. We hypothesized that, as isomerization proceeds after the moment of 2-AG mobilization, the antagonistic effect of accumulating 1-AG becomes gradually more pronounced, resulting in the attenuation of 2-AG evoked biological responses. To investigate this possibility, we first calculated the change in ratio of the two isomers over 10 min. Based on our measurements, the amounts of 2-AG and 1-AG change so that, 0.5, 2.6, 4.2, and 9.8 min after dissolving 2-AG in HBSS, their ratio is approximately 9:1, 8:2, 6:4, and 5:5, respectively. Thus, in this experiment we treated transfected COS7 either with 2-AG alone in a descending serial dilution (0.9, 0.8, 0.6, and 0.5

mM), or with a mixture containing increasing quantities of 1-AG (0.1, 0.2, 0.4, and 0.5 mM) complementing the decreasing concentration of 2- AG to mimic the progress of isomerization. As expected, the drop in effective 2-AG concentration was paralleled by a pronounced diminution of evoked Ca^{2+} transients. Surprisingly, however, effects of 1-AG on 2-AG evoked Ca^{2+} of the two isomers. Although we observed a weak antagonistic effect of 0.1 mM 1-AG against 0.9 mM 2-AG, gradually increasing concentration of 1-AG proved to be additive and increased Ca^{2+} signals evoked by declining quantities of 2-AG. The slope of concentration-response curve of decreasing [2-AG] – increasing [1-AG] pairs mimicked surprisingly well the time-dependent minor and not significant changes of 2-AG induced Ca^{2+} responses. Our measurements showed also that the effect of 1-AG on 2-AG dependent cellular responses is neutral at approximately at 0.8 mM 2-AG to 0.2 mM 1-AG ratio, that is reached in 2.3 min from the moment when 2-AG meets the aqueous medium.

DISCUSSION

Endogenous cannabinoids are ubiquitous intercellular messengers playing essential role in a variety of physiological and pathological processes. Although the endocannabinoid system has been extensively investigated in practically every type of mammalian tissue (Kano et al., 2009; Maccarrone et al., 2015), several questions and controversies remain to be solved (Piomelli, 2014). One of these issues involve the mechanisms that decreases the concentration and terminates the effects of the endocannabinoid 2-AG. MGL which accounts for the majority of 2-AG breakdown in the brain (Beltramo and Piomelli 2000; Cravatt et al. 2001; Blankman et al. 2007; Hashimotodani et al. 2007; Vandevoorde and Lambert 2007; Pan et al. 2009; Murataeva et al. 2014), has been found in the cytoplasm of presynaptic terminals (Straiker et al. 2009; Dinh et al. 2002; Gulya´s et al. 2004). Thus, it is optimally positioned to break down 2-AG that has engaged presynaptic CB_1s. Besides the enzymatic degradative pathways, 2-AG, as member of 2-monoglycerol family, is chemically unstable and prone to acyl migration which results in the formation of 1-AG (Martin, 1953). This molecular rearrangement generates difficulties when quantifying amounts of 2-AG from biological samples (Vogeser and Schelling, 2007; Astarita and Piomelli, 2009; Pastor et al., 2014) and interpreting the effects of 2-AG at cannabinoid and TRP receptors (Zygmunt et al., 2013).

MGL in axon terminals and glial cells

A heterogenous pattern of expression of MGL can be observed in many regions of the central nervous system. MGL expression was highly heterogeneous in the cerebellum, being rich within parallel fiber terminals but absent in axon terminals of climbing fibers as well as of basket cells and stellate neurons. These axon terminals were found to be negative for MGL nonetheless they were rich in CB_1s (Ohno-Shosaku et al. 2012). Heterogeneous expression of MGL has also been found in the dentate gyrus, where MGL is expressed in some GABAergic inhibitory terminals of both CB_1 positive and negative interneurons (Uchigasima et al. 2011). Our present results provide additional proof in support of the partial dissociation of CB_1 and MGL. In one of our earlier paper we reported that nearly half and more than 20 % of the axon terminals of peptidergic and non-peptidergic nociceptive primary afferents, more than one-third and approximately 20 % of the axon terminals of putative glutamatergic and GABAergic spinal interneurons, respectively, were positively stained for CB_1 (Hegyi et al. 2009). Here we showed that only 17 % of the axon terminals of peptidergic nociceptive primary afferents and

10 % of the axon terminals of putative excitatory neurons are immunoreactive for MGL, whereas axon terminals of non-peptidergic primary afferents and inhibitory neurons showed only a minor degree, if any, immunolabeling for MGL. The remarkably different distribution patterns indicate that MGL can be expressed only in a proportion of CB_1 positive axon terminals in the superficial spinal dorsal horn. Moreover, MGL is almost completely absent in CB_1 expressing axon terminals of non-peptidergic primary afferents and inhibitory neurons. Accepting that MGL-mediated hydrolysis is the primary mechanism for 2-AG inactivation in intact neurons (Beltramo and Piomelli 2000; Cravatt et al. 2001; Dinh et al. 2002; Vandevoorde and Lambert 2007; Murataeva et al. 2014), we may assume that the magnitude and time course of 2-AG mediated retrograde signaling on CB_1 positive axon terminals in the superficial spinal dorsal horn may vary in a wide range depending on the presence or absence of MGL in the axon terminals. According to our finding, 2-AG release may mediate rapidly developing and short lasting DSE only in a population of synapses formed by axon terminals of peptidergic nociceptive primary afferents and excitatory neurons, whereas 2-AG mediated DSI which may appear only in approximately 20 % of synapses established by axon terminals of inhibitory neurons (Hegyi et al. 2009) can be a slowly developing and long lasting event in the superficial spinal dorsal horn. As another plausible option, MGL positive astrocytes located adjacent to synaptic contacts may also substantially participate in the degradation of 2-AG at axon terminals that express CB_1 but lack MGL.

A great deal of experimental data indicates that, in addition to neurons, 2-AG is produced and inactivated by glial cells (Walter and Stella 2003; Walter et al. 2004; Stella 2009). Bergman glial cells in the cerebellum (Tanimura et al. 2012), astrocytes in the dentate gyrus (Uchigasima et al. 2011) and hypothalamus (Di et al. 2013), as well as microglial cells in culture (Witting et al. 2004) have been shown to express MGL. Due to their close association with synaptic contacts, astrocytes can control extracellular neurotransmitter levels by forming a physical diffusion barrier and by mediating transmitter uptake (Piet et al. 2004;Gordonet al. 2009; Tasker etal. 2012; Di et al. 2013). As we presented here, similarly to the dentate gyrus and hypothalamus (Uchigasima et al. 2011; Tanimura et al. 2012), a population of astrocytes, but not microglial cells, express MGL also in the superficial spinal dorsal horn. Astrocytes, therefore, may provide dynamic control over the extracellular spatial distribution of stimulus-evoked 2-AG under different physiological conditions (Di et al. 2013). The perisynaptic buffering of 2-AG by astrocytes, can restrict 2-AG action to those CB_1 expressing axon terminals of nociceptive primary afferents and excitatory spinal neurons, the activation of which evoke postsynaptic 2-AG release (Di et al. 2013). However, in case of changing

functional conditions, which results in the attenuation of astrocytic buffering, 2-AG can diffuse out from its site of release and can act heterosynaptically (Kano et al. 2009; Uchigasima et al. 2011; Tanimura et al. 2012; Di et al. 2013). It is important to note that microglial cells are essential constituents of the spinal endocannabinoid system, since they express CB_1 (Hegyi et al. 2009) and may also release 2-AG and anandamide (Hegyi et al. 2012). Surprisingly, however, we were unable to confirm the presence of MGL on these cells. Although our group has not investigated alternative enzymatic routes of 2-AG degradation, we assume, that in spinal microglial cells - similarly to BV-2 cells - the ABHD6 is responsible for this process (Marrs et al. 2010; Muccioli et al. 2007). Our findings verifying the differential expression of MGL in astrocytes and microglial cells suggest that these cells may play different roles in the degradation of endocannabinoids. Therefore, their roles in the endocannabinoid-mediated modulation of spinal pain processing, and the induction and maintenance of pathological pain can also be very different.

Based on our data, the number of MGL positive synapses is much lower than those positive for CB_1 suggesting that in synapses carrying CB1 but lacking MGL, either a different mechanism is responsible for the degradation of 2-AG, or the effects induced by 2-AG are prolonged.

We have to mention however, that nearly at the same time we published our paper about the spinal expression of MGL, an other hungarian scientific group also demonstrated the presence of MGL in the superficial spinal dorsal horn, and the results of their experiments, that overlap with our studies confirmed our observations (Horváth et al. 2014).

The role of acylmigration in the degradation of 2-AG

Here we investigated the kinetics of 2-AG isomerization into 1-AG and found that acyl migration is a rapid molecular rearrangement with a half-time of 16.16 min in HBSS and 8.8 min in HBSS containing 10% serum at 37C. Although our measurements were carried out in cell-free physiological solutions, these results may indicate the biological relevance of 2-AG isomerization in certain cellular environments, since 2-AG degrading hydrolases decrease the amounts of 2-AG with a halflife of 19–28 min depending on the cell density (Di Marzo et al., 1999). In that case, acyl migration and 1-AG formation seems to be approximately two times faster than enzymatic inactivation of 2-AG. Importantly, isomerization does not have an impact on the findings of the cited paper, since 1- and 2-monoacyglycerols are equally accepted by the investigated hydrolases, and their catabolism results in the formation of the same end-products (Tornqvist and Belfrage, 1976; Di Marzo et al., 1999). In many other physiological conditions,

however, where the half-life of 2-AG signaling is shorter than the rate of isomerization, formation of 1-AG is unlikely to play physiologically relevant role. This may be the case in 2-AG mediated retrograde synaptic transmission, where the half-lives of depolarization induced suppression of excitation of inhibition has been shown to fall in the 15–40 s range (Kano et al., 2009). Earlier studies demonstrated, that isomerization of 2-AG depends primarily on the pH and partly on the ionic strength of the milieu, and found faster acyl migration with a half-life of 10 min in serum-free and 2.3 min in serum-supplemented RPMI medium at 37°C (Rouzer et al., 2002). Although we cannot fully explain this difference, we assume that the faster isomerization may be the result of the richer and well supplemented RPMI medium used in the cited study. The somewhat slower chemical transformation which we found still indicates that, regardless of the presence of monoacylglycerol degrading enzymes, release of 2-AG into aqueous extracellular space leads to the formation and possibly to the temporary accumulation of 1-AG, which gives special importance to bioactivity or inactivity of 1-AG at cannabinoid receptors. Therefore, we next investigated if 1-AG has any CB1 mediated biological activity, and found that 1-AG transiently increases intracellular Ca^{2+} concentration in a dose dependent manner. This finding, in good agreement with earlier and frequently overlooked studies (Sugiura et al., 1999), demonstrates that 1-AG is indeed a bioactive molecule that activates CB_1. EC50 of 1-AG was found to be one order of magnitude higher than that of 2-AG, and 2-AG was more efficacious in producing biological responses in CB_1 transfected COS7 cells. However, 1-AG can also be considered as a high efficacy agonist at CB_1 receptor, and isomerization of 2-AG, therefore, does not represent a non-enzymatic inactivation mechanism, but yields another bioactive molecule activating the same receptors as its precursor. We have to add, however, that during physiological conditions, a cellular environment may never reach such a high concentration of 1-AG that evokes maximal response. Thus, 1-AG is likely to incompletely activate CB_1 receptors. Acyl migration following 2-AG release creates a rather unique situation, when the monoacylglycerol concentration is relatively constant, but the ratio of the two isomers changes rapidly as 2-AG is gradually replaced by 1-AG, and the two isomers may compete for ligand binding sites of CB_1. Therefore, we next studied the biological consequences of this process by preparing mixtures of the two isomers in a ratio that simulates the time-dependent change in their relative quantities. The moderate and not significant diminution of effects of 2-AG on Ca^{2+} signaling over time was surprisingly similar to that of the appropriate artificial mixtures of the two isomers representing given time point of isomerization. Importantly, results of both experiments were significantly different from the more pronounced decline evoked by decreasing quantities of 2-AG alone indicating, that

accumulating 1-AG stabilizes the cannabinoid signal and masks the biological consequences of isomerization-related drop in 2-AG concentration. This may explain why chemical instability of 2-AG did not have any obvious impact on experimental settings with 5–10 min or even longer time frames (Szabo et al.,2006; De Luca et al., 2014; Stanley and O'Sullivan, 2014; Griebel et al., 2015), and it is also very likely that such measurements result in data mirroring at least partly or mostly effects of 1-AG. Bioactivity of 1-AG at CB_1 and its ability to effectively compensate the rapid elimination of 2-AG may represent an essential mechanism in maintaining long-lasting effects of 2-AG and also basal endocannabinoid tone. However, our results and interpretation have certain limitations. We carried out experiments on transfected cell cultures, which may be different from most in vivo conditions in terms of cell physiology and metabolism, intercellular connections and extracellular environment. For instance, various enzymes degrading monoacylglycerols may effectively modify the ratio of the two isomers. MGL equally accepts both 1-AG and 2-AG, whereas ABHD6 and ABHD12 show preference to 1-AG over the 2-isomer (Navia-Paldanius et al., 2012). Thus, differential expression of these hydrolases in various types of cells and tissues may result in highly different 1-AG/2-AG ratios. Moreover, 2-AG release into aqueous extracellular space is frequently mentioned as an important step of cannabinoid signaling, but its proper mechanism is poorly understood (Bisogno et al., 1997; Sugiura and Waku, 2000; Di Marzo et al., 2005), and is further complicated by lateral diffusion, i.e., 2-AG is dissolved in, and travels along cell membranes, which may delay or prohibit acyl migration (Makriyannis et al., 2005; Hurst et al., 2010). Thus, biological processes associated with 2-AG release may represent composite effects of various mixtures of the 1- and 2- isomers. Fast endocannabinoid signaling, such as homosynaptic retrograde neurotransmission is most likely dominated by 2-AG, whereas in case of prolonged effects of cannabinoids, like tonic cannabinoid receptor activation (Sagar et al., 2010), participation of 1-AG in stabilizing the net cannabinoid signal and maintaining CB_1 activation may be more prominent. This latter possibility opens the question if prolonged incubation with the two isomers induce differential degrees of CB_1 desensitization which may further diversify the outcome of isomerization. Importantly, commercially available 2-AG preparations already contain approximately 10% 1-AG, therefore the isomerization process starts at 9:1 ratio. Although 1-AG masks the isomerization-associated drop in 2-AG concentration, the strength of the evoked biological responses will necessarily reflect the effects of various mixtures of the two isomers in only several minutes. Investigators should be aware of acyl migration in experiments employing 2-AG, and choose carefully a time frame

short enough to prevent formation of 1-AG so that the results can reliably be associated with 2-AG.

SUMMARY

The proper functioning of the nociceptive pain processing pathway is regulated by certain mechanisms, among others by the endocannabinoid system. This is supported by the presence of the components of the endocannabinoid system in the regions of the central nervous system responsible for pain processing, also in the dorsal horn of the spinal cord, the first relay station of the pain pathway. Our earlier studies demonstrated the presence of the cannabinoid receptor type-1 (CB1) and the synthesising enzyme of 2-AG, the diacilglycerol-lipase α (DGLα) in the superficial laminae of the spinal dorsal horn, however, less is known about the spinal expression of monoacilglycerol-lipase (MGL), the enzyme responsible for the degradation of 2-AG. Therefore our aim was first to study the cellular and ultrastructural distribution of the MGL in the superficial laminae of spinal dorsal horn in rodents by immunohistochemical studies at the light- and electron microscopic level.

Our results suggest, that nearly 20% of the peptidergic primary afferent axon terminals, 10% of the axon terminals of excitatory interneurons and 20% of astrocytes are positive for MGL in laminae I. and II. However, the presence of MGL can be demonstrated in less than 3% of the axon terminals of non-peptidergic primary afferents and inhibitory interneurons and also microglial cells. This is a surprising finding if we consider the presence of CB1 in the majority of these structures which suggests, that they are under cannabinoid control. In this case, for the termination of the action of 2-AG has to be mediated by a different mechanism than the enzymatic route via MGL.

In the elimination of 2-AG we have to consider the termodinamical instability of 2-AG, by whichit is prone to spontaneous molecular rearrangement, during which the arachidonoil moiety of the molecule moves from the 2. carbon atom to the 1. carbon atom. This process is known as acylmigration and catalyses the formation of a termodinamically more stable molecule, 1-AG. In this way acylmigration can be a modulator of endocannabinoid signalling, since it can support the elimination of 2-AG signalling if it doesn't have biological activity. However, due to the structural similarity of 1-AG to 2-AG it can activate CB1, in this way the isomerization of 2-AG can be a fine modulator of the endocannabinoid signalling, where the strength and duration of 2-AG induced responses can be influenced by the forming 1-AG.

Therefore in our further experiments we studied the biological activity of 1-AG in COS-7 cell line, transfected with CB1 and also examined if the continuously accumulating 1-AG can affect the 2-AG signaling. Our results suggest, that 1-AG generates Ca2+ transients through a CB1-dependent manner, therefore it can be considered as a bioactive metabolite and the agonist of CB1. It is important to note however, that one order of magnitude higher concentration of 1-AG is necessary to evoke the same response as 2-AG does, so 1-AG is a weaker agonist of the CB1 receptor. Our results further demonstrate, that in low concentration 1-AG is a competitive antagonist of 2-AG, but the continuously increasing concentration of 1-AG seems to have an additive effect, since besides the decreasing concentration of 2-AG the forming 1-AG effectively compensated the weakening effects of 2-AG.

Our results indicate, that 1-AG, that is formed during the isomerisation of 2-AG is a bioactive ligand and is primarily responsible for the mediation of long lasting endocannabinoid-mediated processes. Because the spinal expression of MGL is lower than the expected, and the acylmigration is not a true inactivation process of 2-AG signalling we have to assume, that in the superficial laminae of the spinal dorsal horn other degradative routes serve for the termination of 2-AG mediated endocannabinoid signalling.

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Subject: PhD Publikációs Lista

Candidate: Klaudia Dócs
Neptun ID: DFAZTG
Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Dócs, K.**, Mészár, Z. M., Gonda, S., Kiss-Szikszai, A., Holló, K., Antal, M., Hegyi, Z.: The Ratio of 2-AG to Its Isomer 1-AG as an Intrinsic Fine Tuning Mechanism of CB1 Receptor Activation. *Front. Cell. Neurosci. 11*, 1-13, 2017.
   DOI: http://dx.doi.org/10.3389/fncel.2017.00039
   IF: 4.555 (2016)
2. **Dócs, K.**, Hegyi, Z., Holló, K., Kis, G., Hegedűs, K., Antal, M.: Selective axonal and glial distribution of monoacylglycerol lipase immunoreactivity in the superficial spinal dorsal horn of rodents.
   Brain Struct. Funct. 220 (5), 2625-2637, 2014.
   DOI: http://dx.doi.org/10.1007/s00429-014-0813-x
   IF: 5.618

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List of other publications

3. Balázs, A., Mészár, Z. M., Hegedűs, K., Kenyeres, A., Hegyi, Z., **Dócs, K.**, Antal, M.: Development of putative inhibitory neurons in the embryonic and postnatal mouse superficial spinal dorsal horn.
   Brain Struct. Funct. 222 (5), 2157-2171, 2017.
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Total IF of journals (all publications): 23,304
Total IF of journals (publications related to the dissertation): 10,173

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

25 January, 2018