



The Diminished Availability of 2-AG in Aged Synaptic Terminals is Ameliorated by a Full-Spectrum Cannabis Extract with a High THC Content

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Received: 10 February 2026 / Revised: 11 March 2026 / Accepted: 13 March 2026

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Abstract

We have previously demonstrated that the endocannabinoid system is dysregulated in the synaptic terminals of the cerebral cortex in aged rats. Specifically, the availability of the neuroprotective endocannabinoid 2-arachidonoylglycerol (2-AG) is reduced due to impairments in the enzymes involved in its metabolism, a deficit only partially compensated by the binding of cannabinoid receptor ligands. Given that Δ^9 -tetrahydrocannabinol (THC) acts as a ligand for cannabinoid receptors (CBR), we designed the present study to investigate the effects of a full-spectrum cannabis extract with a high THC content, the THC-free fraction of this cannabis extract, and pure THC on the previously mentioned aging model. Thus, 2-AG metabolic enzymes were assayed incubating synaptosomes from aged and adult rat cerebral cortex, with ethanolic cannabis extract, the THC-free fraction of this cannabis extract or pure THC, and the corresponding radiolabeled substrates. Our key findings indicate that the age-related decline in 2-AG bioavailability: (a) is exacerbated in the presence of either the THC-free fraction from the cannabis extract or pure THC, primarily due to a significant decrease in 2-AG synthesis; and (b) is partially mitigated by the inhibition of 2-AG hydrolysis when the extract contains THC. These results provide compelling evidence for the regulation of 2-AG metabolism by a full-spectrum cannabis extract with high THC content, supporting the theory of the entourage effect among cannabis phytochemicals. This highlights the potential of high THC content extracts as therapeutic agents for restoring the decreased 2-AG levels observed in the aging brain.

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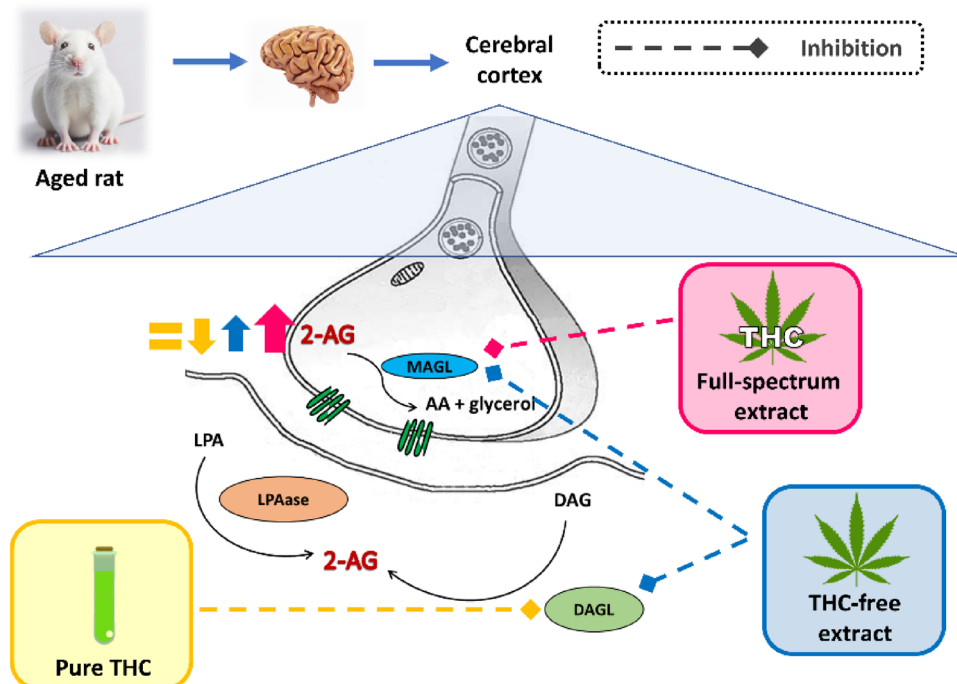
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Graphical Abstract



Keywords Aging · 2-arachidonoylglycerol metabolism · Cerebral cortex · Synaptosomes · THC

Abbreviations

2-AG	2-arachidonoylglycerol
AEA	Anandamide
BSA	Bovine serum albumin
CBR	Cannabinoid receptors
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CC	Cerebral cortex
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DTT	Dithiotreitol
ECS	Endocannabinoid system
LPA	Lysophosphatidic acid
LPAase	Lysophosphatidate phosphohydrolase
LPC	Lysophosphatidilcholine
MAG	Monoacylglycerol
MAGL	Monoacylglycerol lipase
ABHD6	Serine hydrolase alpha-beta-hydrolase domain 6
NEM	N-ethylmaleimide
LPA-PLA1	Lysophosphatidate phospholipase A1
SYN	Synaptosomes
THC	Δ^9 -tetrahydrocannabinol

Introduction

The endocannabinoid system (ECS) consists of the endogenous ligands, mainly anandamide (AEA) and 2-arachidonoylglycerol (2-AG) [1, 2], the CB1 and CB2 membrane receptors (CB1 and CB2) [3], and the enzymes involved in AEA and 2-AG metabolism [4–8]. Diacylglycerol lipase (DAGL) and lysophosphatidate phosphohydrolase (LPAase) are the primary enzymes responsible for 2-AG synthesis, while monoacylglycerol lipase (MAGL) and serine hydrolase alpha-beta-hydrolase domain 6 (ABHD6) are the main enzymes mediating its hydrolysis at pre-synaptic and post-synaptic level, respectively [6, 8, 9].

2-AG is a crucial endocannabinoid implicated in various physiological and pathological processes, notably in neuroprotection against neurodegenerative disorders via activation of cannabinoid receptors (CBR) in neuronal synapses [10–12]. This endocannabinoid acts as a retrograde messenger regulating the release of various neurotransmitters, which underscores the importance of preserving its tone to prevent neurodegenerative processes. Therefore, the availability of 2-AG at the synaptic level represents a key aspect in the study of mechanisms underlying neurodegeneration. Our previous research demonstrated age-related alterations in the expression of proteins (receptors and enzymes), as well as in the enzymatic activities involved in the synthesis

(DAGL) and hydrolysis of 2-AG (mainly MAGL), in synaptosomes (SYN) from aged rats. Aging was associated with a decrease in DAGL activity, a slight increase in LPAase activity, and an increase in MAGL-mediated hydrolysis at synaptic terminals, indicating an imbalance in the enzymatic pathways regulating 2-AG metabolism. These findings also revealed a reduction in 2-AG levels from the cerebral cortex of aged rats [13], with significant modulation of 2-AG metabolism by CBR ligands [14].

Cannabis sp. (cannabis) contains a variety of metabolites, including terpenoids, sterols, and flavonoids [15], in addition to phytocannabinoids [16, 17], as well as other compounds. This diversity suggests that the therapeutic effects of this plant may arise from the synergistic action of multiple compounds rather than a single active constituent [18–20]. In neurodegenerative conditions, cannabis-derived compounds have been shown to exert neuroprotective effects and are currently being evaluated in several clinical studies [21]. In the context of aging, the CBR agonist THC has been reported to improve spatial learning and enhance cognitive performance in aged mice [22]. Based on these findings, together with our previous results demonstrating the modulation of 2-AG metabolism by CB receptor ligands [14], we hypothesize that THC-containing cannabis extracts may enhance the metabolism of the neuroprotective endocannabinoid 2-AG, potentially contributing to the mechanism that underlie the cognitive improvements reported for THC. To corroborate this hypothesis, we conducted experiments to assess the enzymatic activities of DAGL, LPAase, and MAGL in SYN isolated from the CC of both adult and aged rats. The study involved treatment with a full-spectrum cannabis extract containing THC (full-spectrum extract), the THC-free fraction from this extract (THC-free extract), and pure THC (THC). By comparing the effects of these treatments on 2-AG metabolism, we aimed to determine whether THC alone or in combination with other cannabis metabolites could influence the enzymatic regulation of 2-AG, thereby contributing to its neuroprotective potential in the context of aging. The outcomes on 2-AG metabolism in the presence of THC alone or within the full-spectrum extract would help elucidate whether THC exerts a specific effect or if a synergistic interaction with other metabolites exists, potentially counteracting the age-related decline in 2-AG levels [13].

Materials and Methods

Materials

Cannabis sp. flowers were provided by the non-profit organization Asociación Civil Cultivo mi Medicina, in

Bahía Blanca, Buenos Aires, Argentina. Standard solutions of cannabinal (CBN), cannabidiol (CBD), and (-)- Δ^9 -tetrahydrocannabinol ((-)- Δ^9 -THC) (1 mg/mL in methanol) were obtained from Cerilliant Corporation (Round Rock, TX, USA). [2 - 3 H]Glycerol (2 Ci/mmol) was sourced from Perkin Elmer (Boston, MA, USA). Lysophosphatidic acid, 1-oleoyl [oleoyl-9,10- 3 H(N)] (54 Ci/mmol) was acquired from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). Additional reagents, including oleoyl-L- α -lysophosphatidic acid, *N*-ethylmaleimide (NEM), bovine serum albumin (BSA), 2,5-diphenyloxazole (PPO), and 1,4-bis(5-phenyl-2-oxazolyl) benzene (bis-MSD), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals utilized in this study were of the highest available purity.

Preparation of the Cannabis Extract

Female inflorescences of cannabis were kept at -20 °C in light-protected, nitrogen-filled tubes. Before extraction, acidic cannabinoids decarboxylation was achieved by pre-heating the inflorescences in an oven at 115 °C for 40 min [23]. Cannabis extraction was subsequently performed employing absolute ethanol (EtOH) (5% w/v) as extraction solvent, as described by Romano and Hazekamp [24], with minor modifications.

Preparation of the THC-Free Cannabis Extract

EtOH was evaporated from the cannabis extract under a stream of nitrogen to obtain the resin. This resin (1 mg) was separated by preparative thin-layer chromatography (TLC) using a plastic-backed plate coated with silica gel 60 (0.2 mm thickness, Merck), with dimensions of 12.5 cm by 20 cm. The mobile phase consisted of a hexane: ethyl acetate mixture (90:10). A THC standard was spotted along both edges of the plate. One side was developed using iodine vapors, while the other side was developed with a *p*-anisaldehyde solution (5 mL *p*-anisaldehyde, 5 mL concentrated H_2SO_4 , 1 mL glacial acetic acid, and 90 mL EtOH) followed by heating with a heat gun. The TLC plate was fractionated to isolate THC from other compounds. The THC fraction and the THC-free fractions were scraped separately, and then washed with EtOH, filtered, and evaporated under a nitrogen stream. The THC-free fractions were subsequently combined to obtain a THC-free extract.

Identification and Quantification of Phytocannabinoids

Ethanol extract was analyzed using High-Performance Liquid Chromatography with Diode-Array Detection

(HPLC-DAD). Prior to quantification, the resin was re-suspended in methanol.

The HPLC system used was a Thermo Scientific Dionex Ultimate 3000 equipped with a Diode-Array Detector. Samples were detected at a wavelength of 220 nm following separation on a Phenomenex Luna C8 column (250 × 4.6 mm, 5 μm). The mobile phase consisted of acetonitrile and water in an 80:20 ratio, with a flow rate of 1 mL/min. The HPLC method was adapted from the protocol described by Raharjo and Verpoort [25].

To identify and/or quantify phytocannabinoid peaks in the chromatograms, CBD, CBN, and (-)-Δ⁹-THC standard solutions were used. THC fraction and THC-free fractions were analyzed by both HPLC (as described above) and gas chromatography mass spectrometry (GC-MS). For GC-MS analysis, a Hewlett Packard CG-6890 with an MS-5972 A mass selective detector was employed, using an HP5-MS capillary column (30 m × 250 μm × 0.25 μm). Mass spectra were obtained at 70 eV in the m/z range of 35 to 700 a.m.u, with helium as the carrier gas at a flow rate of 1.0 mL/min. Identification of chromatographic peaks was based on comparison with the MS library, with global match score for THC exceeding 900. Results from these analyses are shown in Supplementary Material.

Preparation of Synaptosomes

Adult (4 months old, 250–300 g) and aged (28 months old, 450–500 g) Wistar rats (50% male and 50% female) were used in this study. Animals were maintained under controlled environmental conditions and housed two per cage in standard polycarbonate cages, with *ad libitum* access to chow and water. Rats were obtained from the Laboratory of Experimental Animals (Laboratorio de Animales de Experimentación, LAE), Faculty of Veterinary Sciences, National University of La Plata (La Plata, Argentina), and had been maintained for several years in the institutional animal facility.

Sample size was calculated a priori using the Snedecor and Cochran (1989) [26] formula, based on preliminary data [13, 14] to estimate the pooled standard deviation (s) and the expected difference between groups (d). Calculations assumed a significance level of $\alpha=0.05$ and statistical power of $1-\beta=0.8$. Estimated sample sizes ranged between 1.08 and 1.7 animals per group, depending on the enzyme analyzed. Accordingly, experiments were performed with a minimum of three biological replicates per group, each consisting of a pool of two animals. Due to the unpredictable mortality rates of aged animals, extra animals were included beyond the minimum required. When these animals survived, they were used for additional repetitions to confirm observed trends, within the constraints of variable animal

availability. Both adult rats and aged rats were first sedated with carbon dioxide gas and subsequently euthanized by decapitation. Immediately following euthanasia, the cerebral cortices were dissected and used to isolate SYN as previously described [13, 27]. The isolated SYN were stored at -80 °C until further use. All experimental procedures were conducted following the approval of the Institutional Animal Care and Use Committee of the Universidad Nacional del Sur (IACUC, protocol number 228–2023-CDBByF Res: 609/23) and in conformity with the guidelines outlined in the Care and Use of Laboratory Animals (National Institutes of Health).

Preparation of Radiolabeled Substrates

[2-³ H]Glycerolphosphatidylcholine (PC) and [2-³ H]Triacylglycerol (TAG) were synthesized from bovine retinas that were incubated with [2-³ H]glycerol, as described by Pasquaré de Garcia and Giusto [28]. 1,2-Diacyl-sn-glycerol (DAG) and monoacyl-sn-glycerol (MAG) were generated by the action of phospholipase C from *Clostridium welchii* (Grade B, Calbiochem, Los Angeles, CA) on [2-³ H]PC [29], and through the hydrolysis of [2-³ H]TAG with pancreatic lipase, respectively [13].

Lipids were extracted according to the method outlined by Folch et al. [30] and then separated by one-dimensional TLC, following the procedures detailed in prior publications [13, 29, 31]. The specific activities of the substrates employed in the enzymatic assays, as determined using Gas Liquid Chromatography, were 0.17 μCi/μmol for [³H]DAG and 1.15 μCi/μmol for [³H]MAG.

DAGL Activity Assay

The enzymatic assay was carried out using [³H]DAG, which was prepared by sonicating [³H]DAG plus unlabeled DAG, with equal molar amounts of lysoPtdCho in a 50 mM MOPS buffer (pH 7.4) containing 0.25% fatty acid-free bovine serum albumin (BSA), resulting in a final volume of 100 μL [31]. The assay for DAGL was started by adding DAG suspensions (600 μM, 11,500 DPM) to SYN (100 μg of protein).

LPAase Activity Assay

The incubation mixtures consisted of unlabeled 1-oleoyl LysoPtdOH and 1-oleoyl [oleoyl-9,10-³H(N)]-LysoPtdOH (20 μM, 6,000 DPM), 100 mM Tris-HCl (pH 7.4), 1.2 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), and SYN (50 μg protein) in a total volume of 100 μL [32]. Prior to substrate addition, SYN were pre-incubated with 2 μM KML29, a selective MAGL inhibitor

[33], for 30 min; and *N*-ethylmaleimide (NEM) for 10 min to specifically assess NEM-insensitive LAPase activity.

MAGL Activity Assay

This activity was evaluated by incubating SYN (50 µg protein) with [³H]-MAG (20 µM, 5,000 DPM) in a Tris-HCl buffer (50 mM, pH 7.5) containing 1 mM EDTA, in a total volume of 100 µL, as described by Baker and Chang [34] and Blankman et al. [35].

For each enzymatic assay, SYN were co-incubated for 20 min with the respective substrate and either the full-spectrum extract, the THC-free extract, THC, or their vehicle (ethanol), which served as the control. All assays were conducted at 37 °C and the reactions were stopped by the addition of chloroform: methanol (2:1, v/v) or chloroform: methanol (1:1, v/v). The conditions for the enzymatic assays were previously optimized and reported in earlier publications [13, 14, 36]. Lipid products were then extracted following the method of Folch et al. [30] and separated using previously described techniques with minor modifications, and glycerol was recovered from the aqueous phase [13, 37]. The radiolabeled products were quantified after adding 0.25 mL of water and 5 mL of a 0.5% mixture of 98% PPO and 2% bis-MSD in toluene/Arkopal N-100 (4:1, v/v).

Blank controls were prepared in the same manner as the enzymatic assays, except that the SYN proteins were previously denatured by adding chloroform: methanol (2:1, v/v).

Protein concentration was measured using the DC Protein Assay (#500–0116, BIO-RAD, Hercules, CA, USA), following the method established by Lowry et al. [38].

Statistical Analysis

A minimum of three experiments were conducted, utilizing a pool of two animals per experiment. Each experiment included at least three replicates per condition, with each experiment treated as an individual sample (*n*). Potential outliers were visually inspected using Q–Q plots. Prior to each statistical analysis, the assumption of normality was verified using the Shapiro–Wilk ($p > 0.05$) test. Data were analyzed using two-way ANOVA, when no significant interaction between age and THC treatment was found, a one-way ANOVA was applied for each age group. In all cases, Fisher's LSD post hoc test was used, as indicated in the respective figures. For all column figures, bars represent mean ± standard error of the mean (SEM), with individual data points shown (*n*). Statistical analysis was performed with Infostat version 2018 program (<http://www.infostat.com.ar>).

Results

Analysis of the Full-Spectrum Extract and the THC-Free Extract

Phytocannabinoid quantification of the full-spectrum extract by HPLC revealed a THC content of 37.73 mg/g inflorescence, with a minimum content of acidic THC (THCA) and CBN. No detectable amounts of CBD were observed (Limit of detection for CBD = 0.73 µg/mL, corresponding to 0.6 mg CBD/g inflorescence) (data not shown).

When the full-spectrum extract was chromatographed by preparative TLC, analysis of the developed plate allowed to identify a fraction that matched the pure THC standard ($R_f = 0.65$). This fraction (fraction 3) and the THC-free fractions (fraction 1 corresponding to the origin of the TLC plate; fraction 2 corresponding to the compounds that migrate above the origin but remained below the THC-enriched band and fraction 4 corresponding to the compounds that migrated beyond the THC-containing band) were then separately removed from the plate and analyzed by HPLC and GC-MS. These analyses confirmed the non-detectable levels of THC in the THC-free fractions, while the fraction that matched the pure THC standard contained mainly THC with a purity of 92% (shown in Supplementary Material). As mentioned in Material and Methods section, THC-free fractions were combined for subsequent assays to investigate the effects of the THC-free extract.

To examine the impact of the THC-free extract, the full-spectrum extract, and pure THC on 2-AG metabolism, we assessed the activities of MAGL (Figs. 1 and 2), DAGL (Fig. 3), and LPAase (Fig. 5) in both adult and aged SYN.

Effect of the THC-Free Extract, the Full-Spectrum Extract, and THC on MAGL Activity

To determine the optimal concentration of THC in the full-spectrum extract that can affect 2-AG metabolism, we selected the measurement of MAGL activity, as it is the simplest assay involved in the availability of this endocannabinoid. The results demonstrated a 7% inhibition of this activity only in aged SYN and with the amount of full-spectrum extract containing a THC concentration of 1 µM (Fig. 1b). Accordingly, 1 µM THC was selected for the subsequent enzyme assays in both, the full-spectrum extract and the pure phytocannabinoid conditions. Additionally, when the effect of the THC-free extract was assayed, it was added in the same amount as the full-spectrum extract containing 1 µM THC; namely, the combined THC-free fractions derived from chromatographing an extract amount equivalent to that containing 1 µM THC were used. Thus, the THC-free extract conditions contained the same components in the

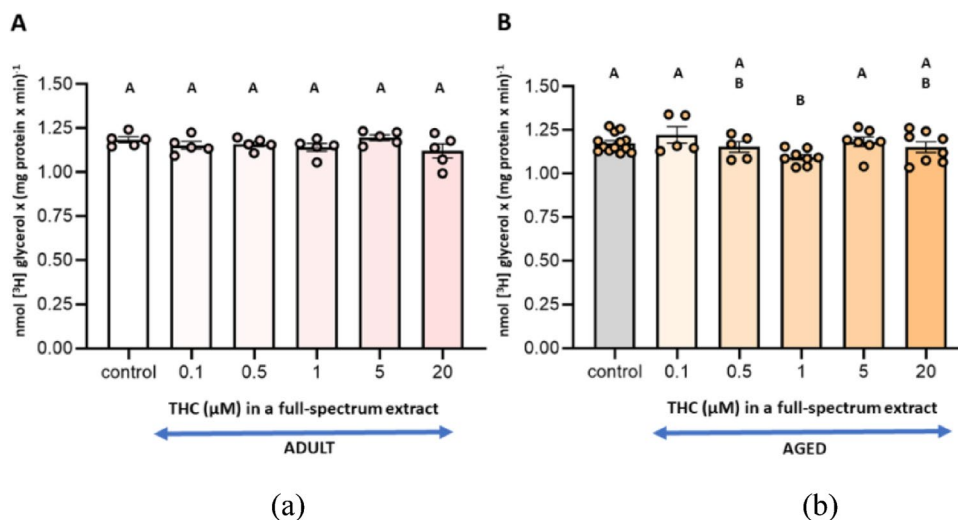


Fig. 1 a Effect of a full-spectrum extract in different THC concentrations on monoacylglycerol lipase (MAGL) activity in synaptosomes from adult rat cerebral cortex and; **(b)** aged rat cerebral cortex. The substrate [³H] MAG was added exogenously under the assay conditions detailed in the Materials and Methods section. Incubation conditions were optimized for MAGL activity. Results are expressed as nmol of glycerol per mg of protein per minute and are shown as mean ± standard error of the mean (SEM), with individual data points

representing the sample size of each group (*n* varies across columns). Data were analyzed by one-way ANOVA (**a** (overall model): $F=1.34$, $p=0.2802$; **b** (overall model): $F=2.49$, $p=0.0473$), followed by Fisher's LSD post-test in **b**. Identical letters in A and B indicate no statistically significant differences ($p \geq 0.05$), whereas different letters in **b** signify statistically significant differences ($p < 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<http://www.graphpad.com>)

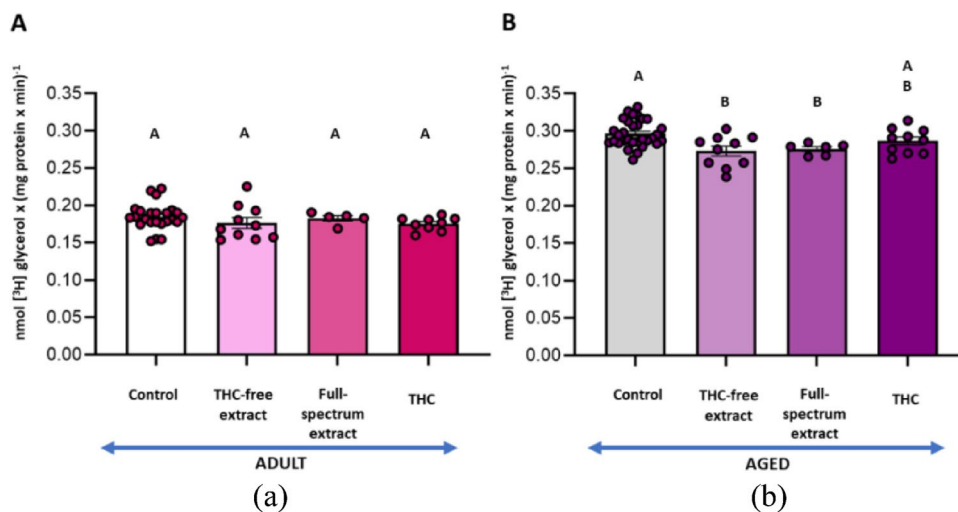


Fig. 2 a Effect of the THC-free extract, the full-spectrum extract, and THC on monoacylglycerol lipase (MAGL) activity in synaptosomes from adult rat cerebral cortex; and **(b)** aged rat cerebral cortex. The assay conditions are outlined in Fig. 1 and detailed in the Materials and Methods section. Results are expressed as nmol of glycerol per mg of protein per minute and are shown as mean ± standard error of the mean (SEM), with individual data points representing the sample

size of each group (*n* varies across columns). Data were analyzed by one-way ANOVA (**a** (overall model): $F=0.95$, $p=0.4242$; **B** (overall model): $F=5.90$, $p=0.0015$), followed by Fisher's LSD post-test in **b**. Identical letters in A and B indicate no statistically significant differences ($p \geq 0.05$), whereas different letters in **b** denote statistically significant differences between conditions ($p < 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<https://www.graphpad.com>)

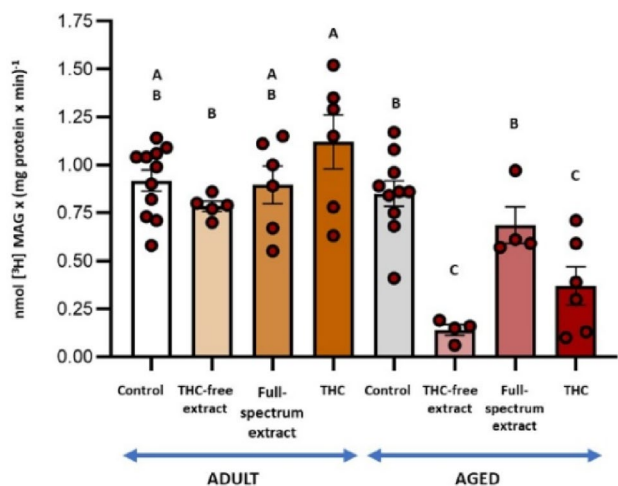


Fig. 3 Effect of the THC-free extract, the full-spectrum extract, and THC on diacylglycerol lipase (DAGL) activity in synaptosomes from Adult and Aged rat cerebral cortex. The substrate [³H]DAG was added exogenously and the assay was conducted according to the conditions specified for DAGL activity as was described in the Materials and Methods section. Results are expressed as nmol of MAG per mg of protein per minute and are shown as mean ± standard error of the mean (SEM), with individual data points representing the sample size of each group (n varies across columns). Data were analyzed by two-way ANOVA (overall model: $F = 11.32, p = 0.0001$), followed by Fisher's LSD post-test. Statistical differences between conditions are denoted by different letters ($p < 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<https://www.graphpad.com>)

same amounts as the 1 μM THC full-spectrum conditions, except for THC.

In adult SYN, no significant changes in MAGL activity were observed under any of the conditions assayed (Fig. 2a). Conversely, in aged SYN, both the full-spectrum extract and the THC-free extract led to a similar reduction in MAGL activity, approximately 7.4%. THC did not result in significant differences in MAGL activity compared to the control or to the different extracts (Fig. 2b).

Effect of the THC-Free Extract, the Full-Spectrum Extract, and THC on DAGL Activity

Assessment of DAGL activity, an enzyme involved in 2-AG synthesis, revealed no significant effects of the treatments in adult SYN. In contrast, in aged SYN, the THC-free extract and the THC resulted in inhibition of DAGL activity by 84% and 57%, respectively, with no statistical differences between conditions (Fig. 3). DAGL activity assays also revealed glycerol formation, demonstrating the coupling of MAGL activity with DAGL activity (Fig. 4). In adult SYN, none of the treatments resulted in significant changes (Fig. 4a). In contrast, in aged SYN, both the THC-free extract and THC decreased MAGL activity coupled to DAGL by 22% and 33%, respectively, although the difference between these two treatments was not statistically significant (Fig. 4b).

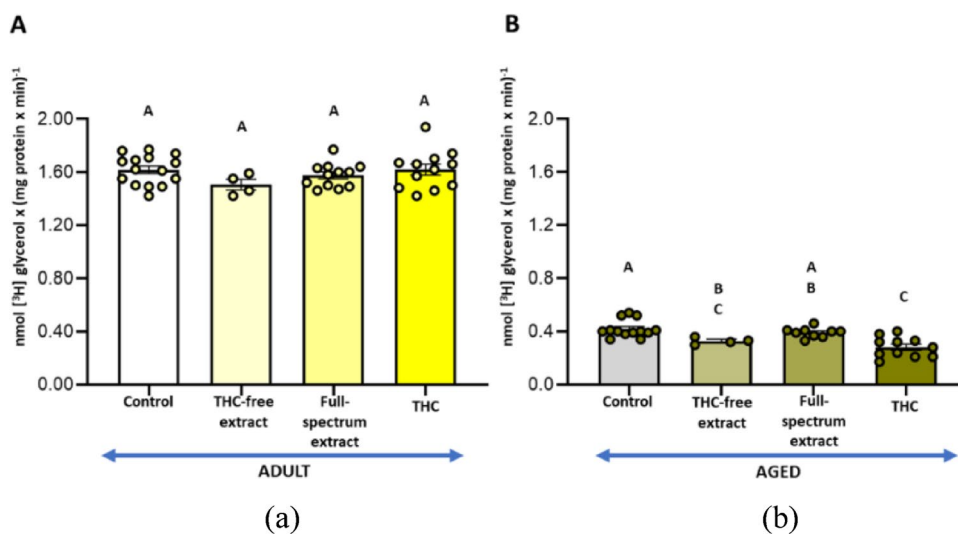


Fig. 4 a Effect of the THC-free extract, the full-spectrum extract, and THC on monoacylglycerol lipase (MAGL) activity coupled to diacylglycerol lipase (DAGL) in synaptosomes from adult rat cerebral cortex and; **b** aged rat cerebral cortex. MAGL activity was measured in conjunction with DAGL activity. The substrate [³H]MAG was endogenously produced from the hydrolysis of [³H]DAG by DAGL. Incubation conditions were as detailed for DAGL activity in the Materials and Methods section. Results are reported as nmol of glycerol per mg of protein per minute and are shown as mean ± standard error of

the mean (SEM), with individual data points representing the sample size of each group (n varies across columns). Data were analyzed by one-way ANOVA (**a** (overall model): $F = 1.28, p = 0.2932$; **b** (overall model): $F = 11.55, p = 0.0001$), followed by Fisher's LSD post-test in **b**. Similar letters in A and B denote no statistically significant differences ($p \geq 0.05$), whereas different letters in **b** indicate statistically significant differences between conditions ($p < 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<https://www.graphpad.com>)

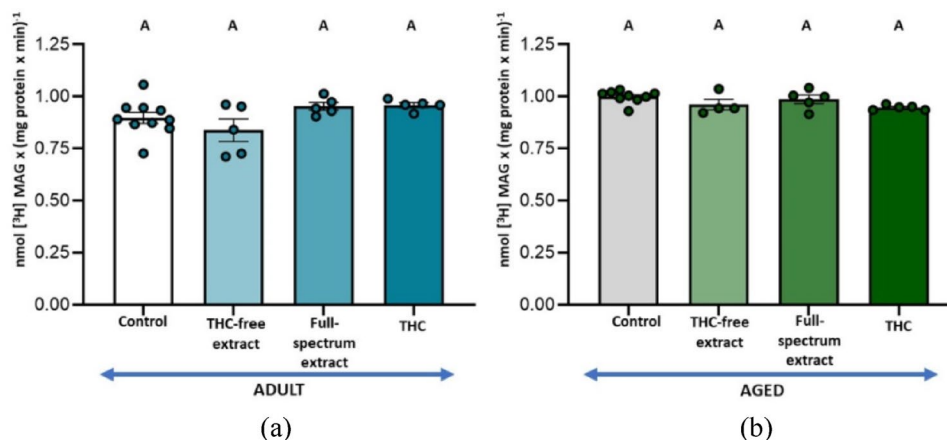


Fig. 5 **a** Effect of the THC-free extract, the full-spectrum extract, and THC on lysophosphatidate phosphohydrolase (LPAase) activity in synaptosomes from adult rat cerebral cortex and; **b** aged rat cerebral cortex. The substrate [^3H]LPA was added exogenously and the assay was conducted according to the conditions specified for LPAase activity as was described in the Materials and Methods section. Results are reported as nmol of MAG per mg of protein per minute and are shown

as mean \pm standard error of the mean (SEM), with individual data points representing the sample size of each group (n varies across columns). Data were analyzed by one-way ANOVA (**a** (overall model): $F=2.58$, $p=0.0804$; **b** (overall model): $F=2.67$, $p=0.0769$). Similar letters indicate no statistically significant differences between conditions ($p \geq 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<https://www.graphpad.com>)

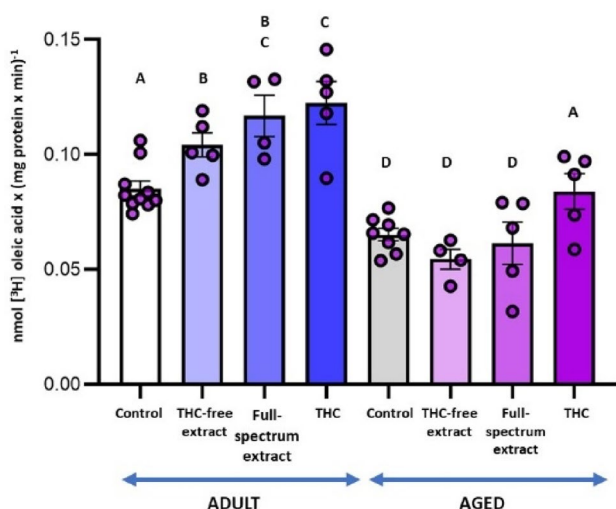


Fig. 6 Effect of the THC-free extract, the full-spectrum extract, and THC on Lysophosphatidate phospholipase A1 activity in synaptosomes from Adult and Aged rat cerebral cortex. Incubation conditions were consistent with those outlined for LPAase activity and specified in the Materials and Methods section. Results are presented as nmol of oleic acid per mg of protein per minute and are shown as mean \pm standard error of the mean (SEM), with individual data points representing the sample size of each group (n varies across columns). Data were analyzed by two-way ANOVA (overall model: $F=15.94$, $p=0.0001$), followed by Fisher's LSD post-test. Different letters denote statistically significant differences between conditions ($p < 0.05$). Graphic was performed using GraphPad Prism 9.5.1 (<https://www.graphpad.com>)

Effect of the THC-Free Extract, the Full-Spectrum Extract, and THC on LPAase Activity

LPAase, another enzyme involved in 2-AG synthesis, was also evaluated (Fig. 5). In this context, LPAase activity was unaffected by any of the treatments, both in adult (Fig. 5a) and aged (Fig. 5b) SYN.

Effect of the THC-Free Extract, the Full-Spectrum Extract, and THC on LPA-PLA1 Activity

Given that the LPAase substrate used in the assay was labeled with [^3H]-oleic acid at the 1-position, and that the assay was conducted in the presence of a MAGL inhibitor, the release of [^3H]-oleic acid specifically reflects the activity of the LPA-PLA1 enzyme (Fig. 6). Notably, in adult SYN, there was a significant increase in enzymatic activity induced by the THC-free extract (22%), the full-spectrum extract (37%), and THC (44%), with the difference between the first and last treatments being statistically significant. In aged SYN, only THC elicited a significant increase in enzymatic activity (29%), restoring it to levels observed in the adult control.

Discussion

Aging is a complex and multifactorial biological process that leads to a progressive decline in the physiological functions of all organ systems, with the nervous system being particularly susceptible. At the cerebral level, this process is

characterized by synaptic dysfunction, a reduction in neurogenesis, and a diminished responsiveness of glial cells [39–41]. In some cases, this physiological aging may constitute an antecedent stage to the pathological aging that is characteristic of neurodegenerative disorders. Extensive research has elucidated the integral roles of the ECS in modulating crucial physiological processes, among them the cognitive decline, a hallmark of normal aging. 2-AG is a bioactive lipid whose function is precisely controlled by its tissue levels, which are critically dependent on the finely tuned balance between its synthesis and degradation. Disruption of this balance can lead to dysregulation of 2-AG activity and affect its physiological functions. The synaptic terminal is a vulnerable neuronal structure where aging disrupts the ECS, particularly 2-AG metabolism [13]. With respect to synaptic dysfunction occurring in aging, recent studies have demonstrated that low doses of THC (1–3 mg/kg/day) significantly improve spatial learning and enhance cognitive performance in aged mice. However, a 1:1 combination of THC and CBD does not produce these effects [22]. Although Δ^9 -THC is widely recognized as the principal phytocannabinoid responsible for the undesired psychoactive effects associated with cannabis exposure, including alterations in cognition, perception, and mood, increasing evidence indicates that its neurobiological actions are complex and strongly dependent on dose, exposure conditions, and the phytochemical composition of the preparation. Clinical and experimental studies have documented that THC can induce transient psychotropic and somatic effects in humans, highlighting the importance of carefully considering these properties when discussing its potential therapeutic applications [42, 43]. At the same time, accumulating evidence indicates that cannabinoids modulate several signaling pathways involved in synaptic function, neuroinflammation, and neurodegeneration. In particular, the endocannabinoid system plays a key regulatory role in brain homeostasis, and age-related alterations in this system — including reductions in endocannabinoid levels such as 2-AG — have been associated with cognitive decline and neurodegenerative processes. Recent reports have therefore proposed that modulation of the endocannabinoid system by phytocannabinoids could represent a potential strategy to counteract aging-related neuronal dysfunction [44]. In this context, the present findings indicate that a THC-rich full-spectrum cannabis extract can modulate key enzymatic steps of 2-AG metabolism in aged synaptic terminals. Importantly, the pharmacological effects of complex cannabis extracts may differ from those of isolated THC due to potential interactions among multiple phytocannabinoids and other bioactive constituents. Therefore, while the psychotropic properties of THC must be considered when evaluating therapeutic strategies, our results support the notion that THC-containing cannabis

preparations may influence endocannabinoid signaling at the synaptic level, providing a mechanistic framework for further investigation in aging and neurodegenerative conditions. Based on this, and on our previous findings, which demonstrated a reduction in the availability of the endocannabinoid 2-AG in synaptic terminals of the CC from aged rats, and its partial reversal by CBR ligands [13, 14], we hypothesized that cannabis extracts with a specific phytocannabinoid composition could modulate the endogenous metabolism of this neuroprotective endocannabinoid.

To address this hypothesis, we commenced our study with an extract obtained from a plant containing 37.72 mg of THC/g inflorescence. The effects of the THC-free extract, the full-spectrum extract, and THC were assessed on the synthesis (via DAGL and LPAase) and hydrolysis (via MAGL) of 2-AG. Through a dose-dependent analysis of MAGL activity, we identified 1 μ M THC as an appropriate concentration for subsequent experiments. The THC concentration range assayed in this analysis was based on studies on synaptic terminals that assess the effects of this phytocannabinoid on catecholamine uptake, release [45] and synthesis [46]; calcium uptake [47]; and cAMP levels [48], among other parameters. Although the magnitude of MAGL inhibition at 1 μ M THC was modest (7%), this value was deemed biologically relevant because 2-AG is a retrograde signaling molecule present at low basal levels, and even small changes in its enzymatic regulation can significantly affect synaptic endocannabinoid tone, where local signaling is tightly controlled. The selected THC concentration also modulated DAGL activity but did not alter LPAase activity in aged SYN. However, the possibility that different THC concentrations could impact LPAase activity cannot be excluded. As only full-spectrum and THC-free extracts —but not pure THC— diminished MAGL activity, our findings in aged SYN, indicate that this effect of cannabis is independent of THC (Fig. 2b). This suggests that one or more metabolites present in the extract, other than THC, may mediate the observed inhibition.

Reports about MAGL in wild-type COS cells demonstrated that certain herbal extracts with diverse phytocannabinoid compositions —unlike the pure cannabinoids— could inhibit MAGL activity [49]. As mentioned above, cannabis produces a diverse varied spectrum of bioactive compounds, including cannabinoids and terpenoids. β -caryophyllene, a sesquiterpene with pharmacological properties, including antioxidant and anti-inflammatory activities [50], has been shown to inhibit MAGL in an analgesia model [51]. This terpene has often been described as a ligand for CB2 [52]. In this concern, MAGL activity has been previously shown to be modified by CBR ligands [14]. Although this sesquiterpene was not detected among the compounds identified in the THC-free fraction used in our assays, the caryophyllene

oxide present in the fraction could potentially be a metabolite derived from β -caryophyllene oxidation during GC analysis (as shown in the Supplementary Material). Additional biomolecules identified in the THC-free extract included amyrins (shown in Supplementary Material). The triterpenes α - and β -amyryns have also been documented to inhibit the hydrolysis of 2-AG [53]. These compounds may contribute to the inhibition of MAGL, thereby enhancing the availability of the neuroprotective 2-AG.

With respect to 2-AG synthesis, the co-presence of THC with other cannabis metabolites nullifies the inhibitory effect that each exerts individually on DAGL activity in aged SYN, (Fig. 3). Given that DAGL and MAGL operate via a shared mechanism of action—both enzymes catalyze the hydrolysis of a fatty acid esterified to glycerol—and both enzymes are members of the lipase-3 family of serine hydrolases [49], it can be hypothesized that the inhibitory effect attributed either to β -caryophyllene or amyrins on MAGL activity may also extend to DAGL activity. Although non-THC compounds present in the THC-free extract, inhibit both MAGL and DAGL activity, THC only inhibit DAGL. This could reflect a different mechanism of action of this THC and the non-THC compounds (e.g. amyrins) on DAGL inhibition.

The fact that THC and non-THC compounds inhibition of DAGL is abolished in the presence of the cannabis extract, may appear contradictory. However, this apparent inconsistency highlights the complexity of the enzymatic network and the possibility of interactions among phytochemicals within the extract. The ‘entourage effect’ caused by the interactions among the different constituents from cannabis plant have been documented in cell-based and animal models. This concept proposes that terpenoids and phytocannabinoids as well as other constituents can individually modulate the activity of one another, thereby enhancing or modifying their overall effects and ultimately leading to increased or novel therapeutic outcomes that are not achieved with their isolated components [18–20]. In this concern, the effect of THC on CB1 and CB2 receptors have been reported to range from antagonistic to agonistic, depending on factors such as the presence of other ligands that interact with the same targets [54]. Amyryns has also been shown to exhibit potent biological activity by binding to the CB1 with an affinity 200–300 times greater than that of THC [55]. Thus, it could be hypothesized that terpenes such as amyrins, by binding to the CB1 receptor, may modulate the effect of THC on this receptor, and possibly explain why the presence of both compounds in the full-spectrum cannabis extract leads to different effects compared to those exerted by each compound separately. In this regard, DAGL activity has been previously shown to be modified by CBR ligands [14].

It is noteworthy that, although two DAGL isoforms have been identified, we have previously shown that, in synaptosomal CC fractions, DAGL- β is present in both adult and aged rats, whereas DAGL- α is confined to membrane fractions and is undetectable in synaptosomal CC fractions from either age group [13].

MAGL activity was assessed not only with an exogenous substrate but also with MAG produced during DAGL assay, exhibiting different patterns in aged SYN: (1) the inhibition exerted by the THC-free extract in MAGL coupled to DAGL was four times higher than those observed with the exogenous substrate; (2) while in the MAGL coupled to DAGL assay THC inhibits glycerol production, in MAGL assay this effect was not observed, and (3) in MAGL coupled to DAGL, the presence of THC in the extract restored its own inhibitory effect. This suggests that the observed inhibition in MAGL coupled to DAGL activity is, at least in part, due to the experimental conditions affecting DAGL, as the inhibition of this activity by both the THC-free extract and THC reduces the availability of the substrate for MAGL.

As mentioned previously, LPAase activity was unaffected under the experimental conditions assayed. Previous investigations by our group in 2-AG metabolism in various models—such as amyloidosis induced in synaptic terminals by the oligomeric form of beta-amyloid peptide [36] and the effect of light on retinal photoreceptor cells [56] demonstrated that, whereas DAGL and MAGL activities were significantly altered, LPAase activity remained unchanged.

Upon analyzing the results related to the enzymes involved in 2-AG metabolism under the different experimental conditions, it becomes evident that the metabolic processing of 2-AG is not significantly altered in SYN derived from adult rats. This observation implies that the endogenous tone of 2-AG is adequately maintained to support synaptic function under physiological conditions, while in aged organisms this tone is reported to be mainly reduced [13, 57]. This could explain the fact that THC-containing cannabis extracts effects are shown to be positive in aged organisms and not in younger ones [58], and these results are consistent with that observed in the present study.

2-AG not only functions as a bioactive lipid but also serves as a precursor for the synthesis of other lipids, such as arachidonic acid. The release of arachidonic acid could be regulated by the enzymatic activities of MAGL and LPA-phospholipase A (LPA-PLA). In our study, the production of fatty acid from LPA, when LPAase activity was assessed in the presence of a MAGL inhibitor, further indicated the hydrolysis of LPA by a LPA-PLA. This enzymatic activity, which was diminished in aged SYN compared to adult ones, was enhanced by THC in both age groups, restoring the activity in aged SYN to levels observed in control adults. These findings, along with the observation that LPAase

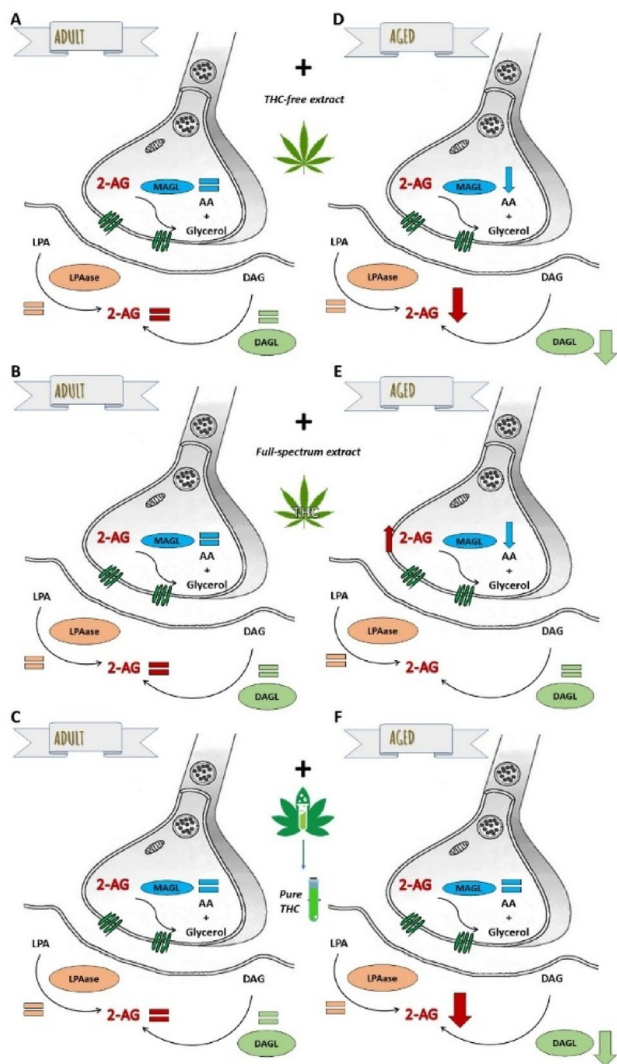


Fig. 7 Differential modulation of 2-AG metabolism in synaptic terminals by THC-free extract, full-spectrum extract, and THC. The relative size of the arrows reflects the magnitude of changes in enzymatic activities related to the synthesis (LPAase and DAGL) and hydrolysis (MAGL) of 2-AG, as well as its synaptic availability in aged synaptic terminals under the experimental conditions, relative to untreated aged synaptic terminals. AA, arachidonic acid. Graphic was performed using Corel draw 2021

activity remained unaltered under these conditions, suggest a reduced availability of the substrate (LPA) for the synthesis of the endocannabinoid 2-AG when THC was present. While our experimental conditions focus on evaluating LPA-PLA1, Yaginuma et al. (2022) demonstrated that LPA-PLA activity hydrolyzes esterified fatty acids at both the sn-1 and sn-2 positions of lysolipids with comparable efficiency [59]. Thus, the increased LPA-PLA activity induced by THC may indicate that this phytocannabinoid facilitates the release of arachidonic acid, which then serves as a substrate for the production of pro-inflammatory molecules. In this respect, it has been reported that THC-induced arachidonic acid

release occurs through a receptor-mediated process involving the activation of one or more phospholipases [60] and that the enzymes implicated in eicosanoid-mediated cannabinoid actions include MAGL, phospholipase A2, cyclooxygenases, lipoxygenases, and additional related enzymes [61].

The principal findings of this study, summarized in Fig. 7, reveal the following about 2-AG availability: (1) in adult SYN, remains unaffected by the THC-free extract, the full-spectrum extract, or THC (Fig. 7A-C); (2) in aged SYN, is decreased by both the THC-free extract and THC, predominantly via the suppression of DAGL activity (Fig. 7D and F); however, it is increased in the presence of the full-spectrum extract with high THC content by a decrease in MAGL activity (Fig. 7E). The reduction in 2-AG bioavailability induced by the THC-free extract and THC, which is reversed and also increased by the full-spectrum extract, suggests a beneficial interaction between the non-cannabinoid components of the extract and THC. This observation indicates that 2-AG availability could be enhanced by modulating its metabolism through a full-spectrum extract containing, at least, THC as phytocannabinoid. The enzymes involved in 2-AG metabolism represent promising therapeutic targets for mitigating synaptic dysfunction and protecting the nervous system from damage associated with the aging process, however if the observed effects of cannabis extracts are mediated by receptors, or if they result directly from modulation of enzymatic activities, are objectives to be elucidated in future studies.

In this study, we employed in vitro assays in isolated synaptosomes, providing a controlled and well-defined model to explore endocannabinoid metabolism at the synaptic level. This approach offers a solid framework that can be further expanded in future studies to determine whether cannabis extracts also modulate stimulus-evoked 2-AG release and to validate these findings in vivo, thereby extending their physiological relevance.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11064-026-04739-1>.

Acknowledgements The authors would like to acknowledge to the non-profit organization Asociación Civil Cultivo mi Medicina, Bahía Blanca, Buenos Aires, Argentina for providing the Cannabis sp. flowers used in this study.

Author Contributions Conceptualization, S.R.S., A.C.P. and S.J.P.; Methodology, S.R.S., A.C.P. and S.J.P.; Investigation, S.R.S. and A.C.P.; Resources (THC-free extract preparation and GC/MS analysis), F.A.M. and A.P.M.; Data curation (HPLC quantification), P.G.M.; Formal analysis, S.R.S., A.C.P. and S.J.P.; Writing—original draft preparation, S.J.P.; Writing—review and editing, all authors; Supervision, S.J.P. All authors have read and agreed to the published version of the manuscript.

Funding This work was supported by funds granted by the Ministerio Nacional de Ciencia, Tecnología e Innovación (Proyectos de Investigación y Desarrollo en Cannabis 3 A), the Agencia Nacional de Promoción Científica y Tecnológica (2017–1089), the Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 11220200100707), and the Secretaría General de Ciencia y Tecnología, Universidad Nacional del Sur (24/B250), Argentina, to Prof. S.J. Pasquaré.

Data Availability The datasets generated during and/or analysed during the current study can be available from the corresponding author on request.

Declarations

Competing Interests The authors declare no competing interests.

Ethical Approval The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Universidad Nacional del Sur, Argentina (protocol number 228-2023-CDBByF, Res. 609/23), and adhered to the standards set forth in the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (ILAR) of the National Academy of Sciences (Bethesda, MD, USA).

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