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Modulation of ageing mice microglia functions during neuroinflammation using synthetic cannabinoids

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ABSTRACT

Ageing is marked by a gradual rise in systemic inflammation, with neuroinflammation being a key feature. Neuroinflammation, which refers to the immune response within the CNS, is primarily mediated by microglia. These resident macrophages in the CNS parenchyma are essential for maintaining homeostasis and initiating immune responses. Their function depends on the timely activation and deactivation of microglia to regulate these processes effectively. Excessive activation of microglia has been shown to disrupt brain functions and promote a proinflammatory response leading to dysfunctional microglia that are unable to carry out immune response due to various reasons, with major implications in neuroinflammation. Therefore, there is a need to monitor and modulate the functionality of microglia to elicit a healthy immune response. The endocannabinoid system is a negative feedback system that is activated to modulate various mechanisms related to inflammation. In our research, we therefore investigated the functionality of microglia in relation to phagocytosis and oxidative stress during neuroinflammation by stimulating the endocannabinoid receptors *Cnr1* and *Cnr2* with synthetic cannabinoid compounds in ageing mice. Our results show that the expression of the endocannabinoid system (ECS) increases with age. Activation of CB_1 and CB_2 receptors reduces reactive oxygen species (ROS) in both young and aged mice, with the effect being more pronounced effect in younger mice. In aged mice, the upregulation of these receptors indicates persistent inflammation, while microglial phagocytosis is modulated through CB_1 receptors in both age groups.

1. Introduction

Ageing is a complex process characterised by a number of interrelated factors such as genomic instability, mitochondrial dysfunction, cellular senescence, stem cell depletion, altered intercellular communication and progressive increase in systemic inflammation (Villalobos et al., 2022). In the CNS, cellular ageing and dysfunction are exacerbated by chronic neuroinflammation (Megur et al., 2020; Mou et al., 2022; Müller et al., 2019). Microglia, the resident macrophages, are identified as a distinct cell population in the CNS. According to current understanding, microglia are considered the quintessential tissue-resident macrophage-like innate immune cells of the CNS that possess memory-like functions and enable context-dependent responses (Belhocine et al., 2022; Bohlen et al., 2017). These multifunctional cells interact with various other CNS cell types, including neurons, astrocytes, and oligodendrocytes (Nutma et al., 2020). Activation of microglia promotes neuroinflammation, and subsequent deactivation is crucial for a healthy immune response and brain functionality. Ageing is characterised by the increase in inflammation and due to various reasons, chronic activation or long-term inflammation can cause microglia dysfunction which induces secondary neuronal damage and impairs global cognitive function (Fig. 1) (Vijaya et al., 2024; Zhao et al., 2019).

The endocannabinoid system (ECS) is a neuromodulatory system that plays a critical role in the regulation of multiple physiological processes, including memory, mood, anxiety etc., and is therefore an important target in the treatment of disease and neurodegeneration (Maldonado et al., 2020; Sharma et al., 2021). The ECS consists of the endocannabinoids anandamide and 2-arachidonoylglycerol, the enzymes that synthesize and degrade enzymes of endocannabinoids, and the homologous G protein-coupled receptors, namely cannabinoid type 1 (CB_1) and type 2 (CB_2) receptors (Kilaru and Chapma, 2020). CB_1 receptors are abundant in neurons; when activated, they often inhibit the

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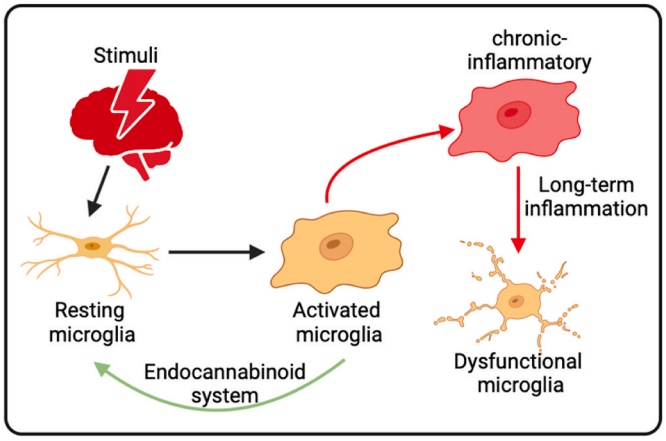

Fig. 1. Summary of microglia activation during inflammation.

release of neurotransmitters (Rapaka et al., 2021). CB_1 receptors are predominantly found in neurons within the CNS, where their activation typically inhibits neurotransmitter release (Rapaka et al., 2021). In contrast, CB_2 receptors are commonly located on macrophages and other immune cells, including microglia, and play a key role in regulating inflammation (Ruiz de Martín Esteban et al., 2022). Since microglia express both CB_1 and CB_2 receptors, their activity can be modulated through the activation of these receptors (Young and Denovan-Wright, 2022a). However, the pharmacological effects of CB_1 and CB_2 receptors on the proinflammatory activity of microglia have been sparsely characterized using agonists and antagonists, particularly in ageing animal models. Nevertheless, the biological significance of these receptors in relation to neurones has been extensively studied (Barrie and Manolios, 2017) by using various synthetic cannabinoid compounds like rimonabant, JWH-133, ACEA and SR144528 (Aso et al., 2012; Cabañero et al., 2020; Martín-García et al., 2010; Rinaldi--Carmona et al., 1998).

In the current era of multi-omics research, microglia have become the most important point in neuroimmunological studies due to significant advances in sequencing technologies. Microglia may not only act as protector of the CNS in various pathologies, but also promote disease progression through their chronic activation (Hanisch and Kettenmann, 2007; Subhramanyam et al., 2019). Genome-wide association studies have identified several risk factors associated with CNS diseases such as Alzheimer's, Parkinson's, schizophrenia, autism and multiple sclerosis (Chen et al., 2023). Therefore, microglia have emerged as a novel and promising therapeutic target for various neuropsychiatric disorders (Zhu et al., 2023).

In this work, we aimed to investigate the modulation of microglia functionality by using synthetic cannabinoids in ageing mice. We demonstrated that each class of cannabinoid receptor agonist attenuates inflammatory activity and oxidative stress levels in microglia and worsens in the presence of an antagonist. Furthermore, it was important to evaluate these effects in relation to old animals, as ageing promotes inflammation and microglial dysfunctionality. Taken together, the data suggest that the interaction between microglia and endocannabinoid systems is a promising tool to attenuate microglia-mediated neuroinflammation and subsequently prevent neurodegenerative diseases.

2. Materials and methods

2.1. Animals

For this study, 8 young (2-month-old) and 8 adult (12-month-old) male C57BL/6J mice (Janvier Laboratories, France) were used. The permission to perform the experiments was given by the Lithuanian State Food and Veterinary Service (No. G2–104), and the maintenance and experiments complied with the requirements of 2010/63/EU Directive. The animals were housed under controlled conditions (21 °C ± 1 °C, humidity 55 % ± 10, food and water were supplied *ad libitum*) and under veterinary supervision. The animals were culled using cervical dislocation.

2.2. Isolation and maintenance of microglial cells

Brains were removed from the animals as quickly as possible and kept cold (+4 °C) in a medium containing antibiotic. Isolation of microglia was performed according to the protocol described in (Vijaya et al., 2023). Cells were seeded in a T25 culture flask in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; ThermoFisher Scientific, USA) with GlutaMAX™ supplement (ThermoFisher Scientific; USA) containing 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin (10,000 units). The next day, the medium was supplemented with macrophage colony-stimulating factor (MCSF; 100 ng/mL; R&D Systems, UK) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 100 ng/mL; R&D Systems, UK).

2.3. Treatment of microglia cells

For experiments, 100,000 cells/well were seeded in a 48-well plate. The cells were treated with LPS (10 ng/mL) 24h after seeding and incubated for 12h. Then cannabinoid compounds were added at different concentrations for 24h (Fig. 2A). Positive (with LPS, no cannabinoids) and negative (no LPS, no cannabinoids) controls were also prepared. After the treatment, phagocytosis and ROS assays were performed (Fig. 2B). CB_1 receptor agonist (ACEA: 0.5 μM, 1 μM and 2 μM), CB_1 receptor antagonist (Rimonabant: 0.5 μM, 1 μM, 10 μM). CB_2 receptor agonist (JWH-133: 0.5 μM, 1 μM, 10 μM), CB_2 receptor antagonist (SR144528: 50 nM, 100 nM, 1000 nM).

2.4. Evaluation of the phagocytic properties of microglia

The phagocytic capacity of the microglia was determined by the uptake of 1 μm fluorescent latex beads (Sigma Chemical Co., USA). Cells were incubated with 0.025 % (w/w) fluorescent latex beads for 4 h at 37 °C and 5 % CO_2, then washed twice with PBS and fixed with freshly prepared 4 % (w/v) paraformaldehyde in PBS. Cells were permeabilized for 15 min (PBS + 1 % Triton X-100) was done for 15 min and then blocked for 30 min in PBS containing bovine serum albumin (3 % BSA) and foetal bovine serum (10 % FBS) and incubated for 1 h at room temperature. Primary conjugated antibodies (anti-CD11b; 1:150; PE, ThermoFisher Scientific; USA) and DAPI dye (5 mg/mL) were used, and fluorescence was visualized using a fluorescence microscope (Fig. S1A). Analysis was performed using ImageJ software and GraphPad software. The average percentage of CD11b + cells with >10 ingested latex beads was calculated from 3 technical repeats in each treatment group.

2.5. Assessment of microglial oxidative stress (ROS)

ROS generation was assessed by staining with CellRox Deep Red (ThermoFisher, USA) according to the manufacturer's instructions. Briefly, CellRox Deep Red solution was added to the media following treatment and incubated for 30 min at 37 °C and 5 % CO_2 in the dark. Cells were then washed and fixed with 4 % paraformaldehyde for 15 min. The nuclei were counterstained with DAPI dye (5 mg/mL), and fluorescence was visualized using a fluorescence microscope (Fig. S1B) (Olympus IX51, Japan). Analysis was performed using ImageJ software. The averages of Log10 transformed fluorescence intensity data from 3 technical repeats in each treatment group was calculated.

2.6. Microglia endocannabinoid gene expression

To assess gene expressions of CB_1 and CB_2 cannabinoid receptors in

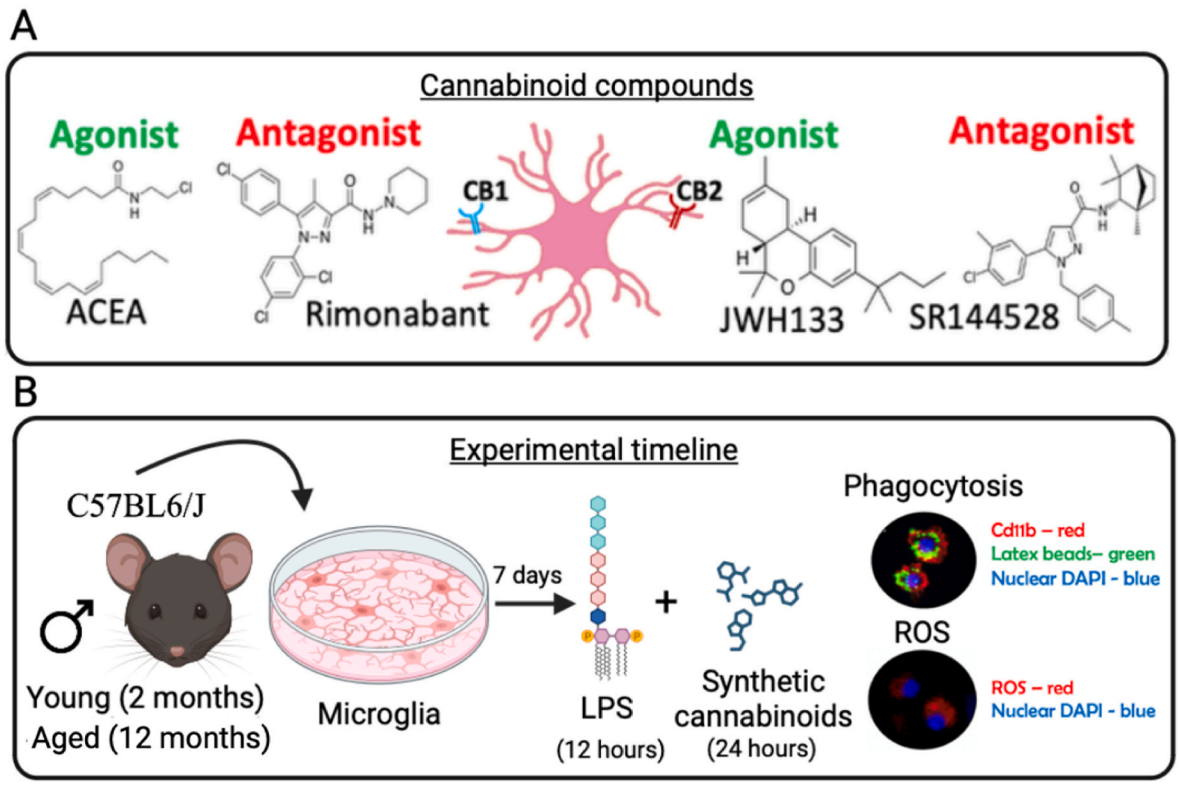

Fig. 2. Design of the experiments. (A). CB_1 and CB_2 receptor agonists and antagonists used in the experiments. (B). Timeline of the treatments and the assays performed.

microglia cells, we performed qRT-PCR using QuantStudioTM 5 Real-Time PCR System (Thermo Fisher Scientific, USA). Total RNA was extracted using a High Pure RNA Isolation kit (Macherey-Nagel, Germany) according to the manufacturer's protocol, and subjected to cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, USA). CB_1 receptor primer sequence: F: 5′-TATGTGTCATCCTTCACTCCCG, R: 3′- CTATCTTTGCGGTGGAACACG and CB_2 receptor primer sequence: F: 5′- GTGTTACCCGCCTACCTACA, R: 3′- CAATGCTGAGAGGACCCACAT. Alpha tubulin was used as a housekeeping control gene, F: 5′-TGTGGATTCTGTGGAAGGC- 3′, R: 5′-ATGAAAGCACACATTGCCAC-3′. qRT-PCR data was analyzed using ΔΔCt method with each sample input amount normalized by comparing it to Alpha tubulin expression. To standardize $2^{\Delta\Delta Ct}$ values, each value was divided by the average of young control group, which was assigned the value of 1 (arbitrary unit).

2.7. Microglia endocannabinoid protein expression analysis

Microglial cells were extracted, cultured, treated with LPS and then lysed for proteins using trizol extraction technique. SDS PAGE was performed to separate the proteins according to their molecular weight in the 12 % acrylamide gel. The proteins from the gel were transferred to a nitrocellulose membrane using semi-dry transfer (25 V for 35 min). The membranes were blocked with 3 % bovine serum albumin (BSA; Sigma) in TBST (Tris buffer saline tween) (50 mmol/L Tris, pH 7.6; 0.9 % NaCl; and 0.1 % Tween-20) for 1 h at room temperature (RT). The membranes were incubated overnight at 4 °C with the primary antibodies against CB1 (1:1000; ThermoFisher Scientific; USA), CB2 (1:1000; ThermoFisher Scientific; USA). After washing three timed with TBST, the membranes were incubated with the corresponding secondary antibodies (1:10000; ThermoFisher Scientific; USA) for 1 h at RT. After incubation with the secondary antibodies, the membranes were incubated for (5 min) with Pierce Enhanced Chemiluminescence Substrate (Thermo Fisher Scientific; Lithuania) for the detection of HRP (Thermo Fisher Scientific; Lithuania). Imaging and quantification of the bands were performed with the Alliance Q9 software (UK). Proteins were standardized using house-keeping protein Alpha tubulin (1:1000; ThermoFisher Scientific; USA) and normalized against young microglia cells for plotting the data.

2.8. Statistical analysis

Data was analyzed with ANOVA using Tukey's post hoc test. Phagocytosis and gene expression data are represented as bar graphs with ±SEM (standard error of mean), ROS and protein expression data is represented as boxplots. The significance level was set at $p < 0.05$. GraphPad Prism was used for statistical analysis (GraphPad Prism version 9.3.1, USA).

3. Results

3.1. Evaluation of microglia properties

3.1.1. CB_1 receptor agonist arachidonyl-2′-chloroacetamide (ACEA): young mice

- The phagocytic properties of microglia

To investigate the phagocytic capability of microglia, the efficacy of the uptake of fluorescent latex beads was evaluated. For statistical analysis, one-way ANOVA was used [$F_{(4,20)} = 15.37$; $p = 0.0001$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control ($p = 0.001$). However, when CB_1 receptor agonist was added, the cells showed a decrease in phagocytic activity compared to the control. In the LPS groups, a significant increase in phagocytosis was observed at 1 μM ($p = 0.001$) and 2 μM ($p = 0.001$) compared to LPS. In addition, a significant

difference was observed between the LPS groups 0.5 μM and 1 μM (p = 0.0155), and 0.5 μM and 2 μM (p = 0.0177) (Fig. 3A and Fig. S2A).

- Microglia oxidative stress

Reactive oxygen species play a key role as cellular defence mechanisms during inflammation, therefore, it is crucial to determine ROS production. A one-way ANOVA was used for statistical analysis [$F_{(4,20)} = 16.00$; $p < 0.001$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS only in LPS-treated microglia compared to control ($p = 0.00$). Interestingly, among the LPS groups, all concentrations 0.5 μM (p = 0.001), 1 μM (p = 0.001), and 2 μM (p = 0.001) showed a significant reduction in ROS production compared to the LPS-treated microglia (Fig. 3C and Fig. S2B).

3.1.2. CB_1 receptor agonist arachidonyl-2′-chloroacetamide (ACEA): aged mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,20)} = 8.621$; $p = 0.0003$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control (p = 0.0056). However, when CB_1 receptor agonist was added, the cells showed an increase in phagocytic activity only at 2 μM (p = 0.0016) compared to the control. Among the LPS groups, a significant increase in phagocytosis was observed only at 0.5 μM ($p = 0.0109$) compared to LPS. We also saw a significant difference between 0.5 μM and 2 μM LPS groups (p = 0.0032) (Fig. 3B and Fig. S3A).

- Microglia oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4,20)} = 4.487$; $p <$ 0.0095], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS only in LPS-treated microglia compared to the control (p = 0.0158). Among the LPS groups, only 2 μM (p = 0.0108) showed a significant reduction in ROS production compared to LPS-treated microglia (Fig. 3D and Fig. S3B).

3.1.3. CB_1 receptor antagonist rimonabant: young mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,20)} = 10.23$; $p = 0.001$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control (p = 0.001).

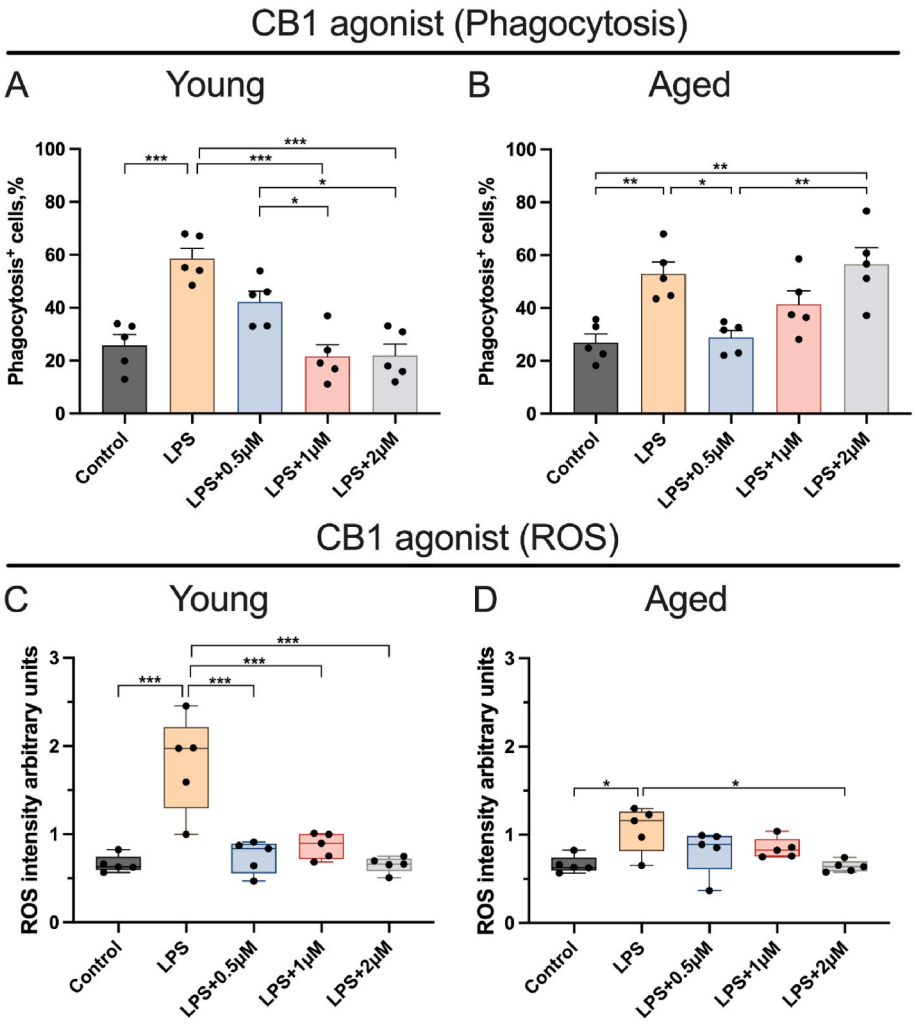

Fig. 3. Evaluation of the functionality of microglia. (A). The average percentage of young mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells ***p < 0.001; *p < 0.05. (B). The average percentage of aged mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells ***p < 0.001; *p < 0.05. (C). Average Log10 transformed ROS fluorescence intensity in young mice microglia. Data are presented as boxplots ***p < 0.001. (D). Average Log10 transformed ROS fluorescence intensity in aged mice microglia. Data are presented as boxplots ***p < 0.001.

However, when CB_1 receptor agonist was added, the cells showed an increase in phagocytic activity only at 10 μM ($p = 0.001$) compared to the control and no significant increase in phagocytosis was observed compared to LPS. Also, we saw a significant difference between LPS groups 0.5 μM and 10 μM ($p = 0.0383$), and 1 μM and 10 μM ($p = 0.0435$) (Fig. 4A and Fig. S4A).

- Microglia oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4,20)} = 5.732$; $p < 0.0031$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS only in the LPS-treated microglia compared to control ($p = 0.014$). Among the LPS groups, only 10 μM ($p = 0.0077$) showed a significant reduction in ROS production compared to control. Also, cells showed significant increase between LPS groups 1 μM and 10 μM ($p = 0.0427$) (Fig. 4C and Fig. S4B).

3.1.4. CB_1 receptor antagonist rimonabant: aged mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,20)} = 6.895$; $p = 0.0012$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control ($p = 0.0053$). Among the LPS groups significant decrease in phagocytosis was observed in 0.5 μM ($p = 0.0285$) and 10 μM ($p = 0.017$) compared to LPS (Fig. 4B and Fig. S5A).

- Microglia oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4,20)} = 7.166$; $p < 0.0009$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS in LPS treated microglia ($p = 0.0125$) and 10 μM ($p = 0.0259$) compared to control. Among the LPS groups, 0.5 μM ($p = 0.018$) and 1 μM ($p = 0.0105$) showed a significant reduction in ROS production compared to LPS-treated microglia. Also, cells showed significant difference between the LPS groups 0.5 μM and 10 μM ($p = 0.0367$), and 1 μM and 10 μM ($p = 0.0219$) (Fig. 4D and Fig. S5B).

3.1.5. CB_2 receptor agonist JWH-133: young mice

- The phagocytic properties of microglia

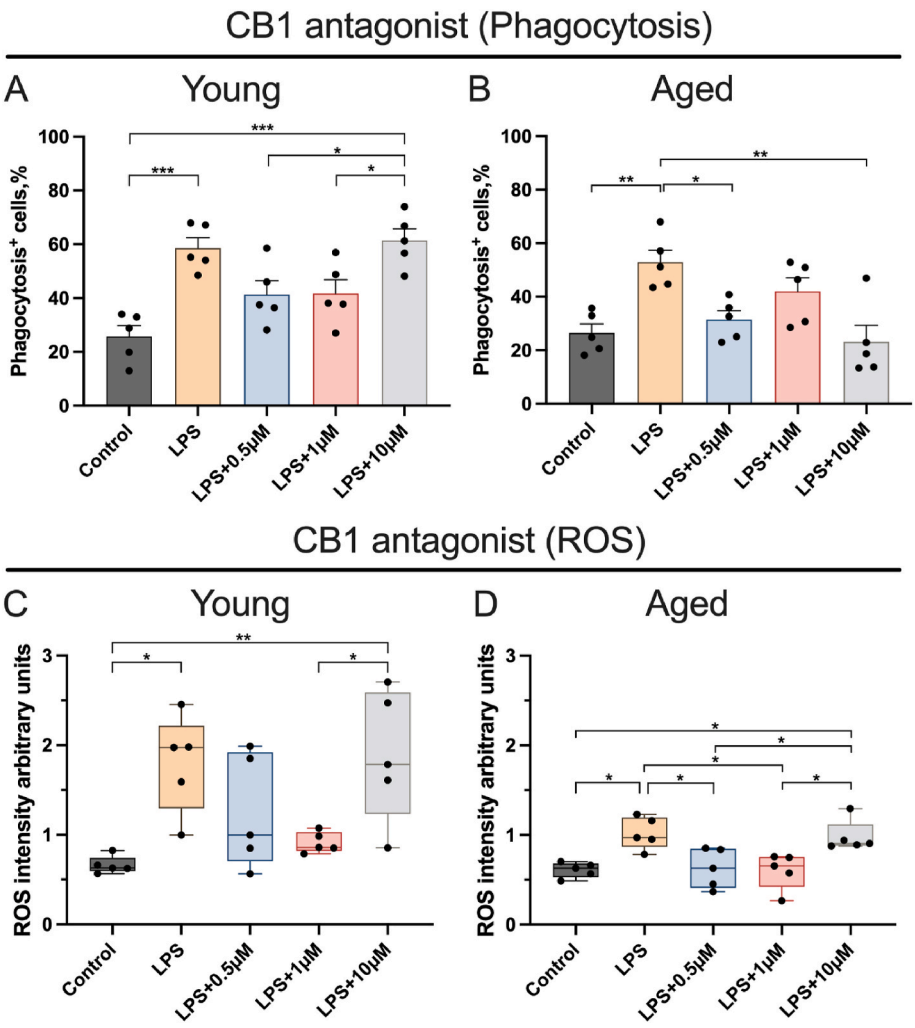

Fig. 4. Evaluation of the functionality of microglia. (A). The average percentage of young mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells ***p < 0.001; *p < 0.05. (B). The average percentage of aged mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells **p < 0.01; *p < 0.05. (C). Average Log10 transformed ROS fluorescence intensity in young mice microglia. Data are presented as boxplots **p < 0.01; *p < 0.05. (D). Average Log10 transformed ROS fluorescence intensity in aged mice microglia. Data are presented as boxplots *p < 0.05.

One-way ANOVA was used [$F_{(4,15)} = 5.279$; $p = 0.0074$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control. However, when CB_2 agonist was added, the cells showed a significant increase in phagocytic activity only in 0.5 μM ($p = 0.0061$) and 1 μM ($p = 0.0196$) compared to the control. Among the LPS groups, no such significant increase in phagocytosis was observed compared to LPS (Fig. 5A and Fig. S6A).

- Microglial oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4,20)} = 27.69$; $p < 0.001$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS only in the LPS-treated microglia ($p = 0.001$) and 10 μM ($p = 0.018$) concentration compared to control. Interestingly, among the LPS groups, 0.5 μM ($p = 0.001$) and 1 μM ($p = 0.001$) and 10 μM ($p = 0.001$) showed a significant reduction in ROS production compared to LPS-treated microglia. In addition, the cells showed a significant difference between 0.5 μM and 10 μM ($p = 0.0344$) LPS groups (Fig. 5C and Fig. S6B).

3.1.6. CB_2 receptor agonist JWH-133: aged mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,15)} = 24.0$; $p = 0.001$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to the control ($p = 0.001$). Interestingly, with the addition of CB_2 receptor agonist, cells showed a significant increase in phagocytic activity in all concentrations 0.5 μM ($p = 0.001$), 1 μM ($p = 0.001$), and 10 μM ($p = 0.001$) compared to control. Among the LPS-treated groups no such significant increase in phagocytosis was observed compared to LPS (Fig. 5B and Fig. S7A).

- Microglial oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4, 20)} = 4.107$; $p < 0.0137$], and the subsequent Tukey's post-hoc test showed a steady increase in the production of ROS in LPS-treated microglia ($p = 0.388$), 1 μM ($p = 0.0151$) and 10 μM ($p = 0.0345$) concentrations compared to control (Fig. 5D and Fig. S7B).

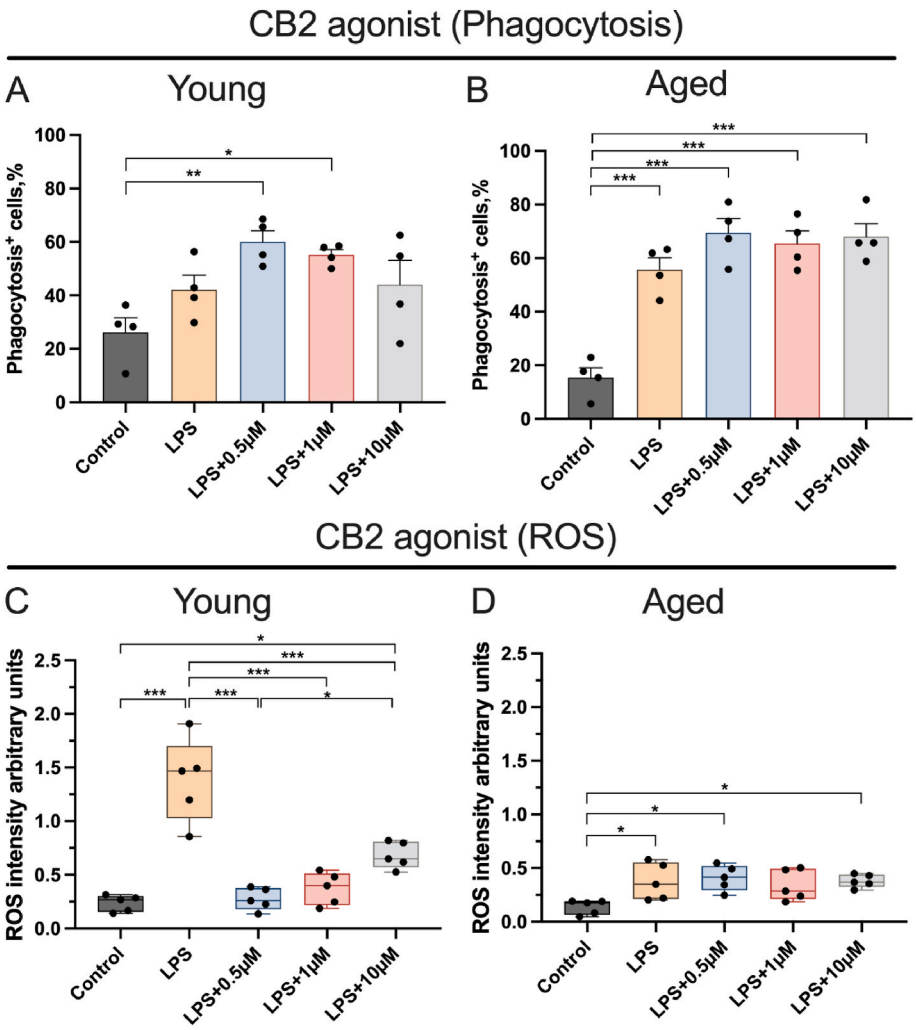

Fig. 5. Evaluation of the functionality of microglia. (A). The average percentage of young mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells **p < 0.01; *p < 0.05. (B). The average percentage of aged mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells ***p < 0.001. (C). Average Log10 transformed ROS fluorescence intensity in young mice microglia. Data are presented as boxplots ***p < 0.001; *p < 0.05. (D). Average Log10 transformed ROS fluorescence intensity in aged mice microglia. Data are presented as boxplots *p < 0.05.

3.1.7. CB_2 receptor antagonist SR144528: young mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,15)} = 1.342$; $p = 0.3001$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control. However, with the addition of CB_2 receptor antagonist, cells showed no significant increase in phagocytic activity in all LPS groups compared to control (Fig. 6A and Fig. S8A).

- Microglial oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4, 20)} = 19.74$; $p < 0.001$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS in LPS-treated microglia ($p = 0.0001$) and 1000 nM ($p = 0.0446$) compared to control. Among the LPS groups, all concentrations 50 nM ($p = 0.001$), 100 nM ($p = 0.001$), and 1000 nM ($p = 0.0014$) showed a significant reduction in ROS production compared to LPS-treated microglia (Fig. 6C and Fig. S8B).

3.1.8. CB_2 receptor antagonist SR144528: aged mice

- The phagocytic properties of microglia

One-way ANOVA was used [$F_{(4,15)} = 27.45$; $p = 0.001$] and the subsequent Tukey's post-hoc test showed that phagocytic activity was increased in the presence of LPS compared to control ($p = 0.001$). However, with the addition of CB_2 receptor antagonist, cells showed a significant increase in phagocytic activity in all concentrations 50 nM ($p = 0.001$), 100 nM ($p = 0.001$), and 1000 nM ($p = 0.001$) LPS groups compared to control (Fig. 6B and Fig. S9A).

- Microglial oxidative stress

One-way ANOVA was used for statistical analysis [$F_{(4,20)} = 5.667$; $p < 0.0032$], and the subsequent Tukey's post-hoc test showed a steep increase in the production of ROS in LPS-treated microglia ($p = 0.0042$) and 50 nM ($p = 0.0045$) compared to control. Among the LPS groups, no significance was observed in ROS production compared to LPS-treated microglia (Fig. 6D and Fig. S9B).

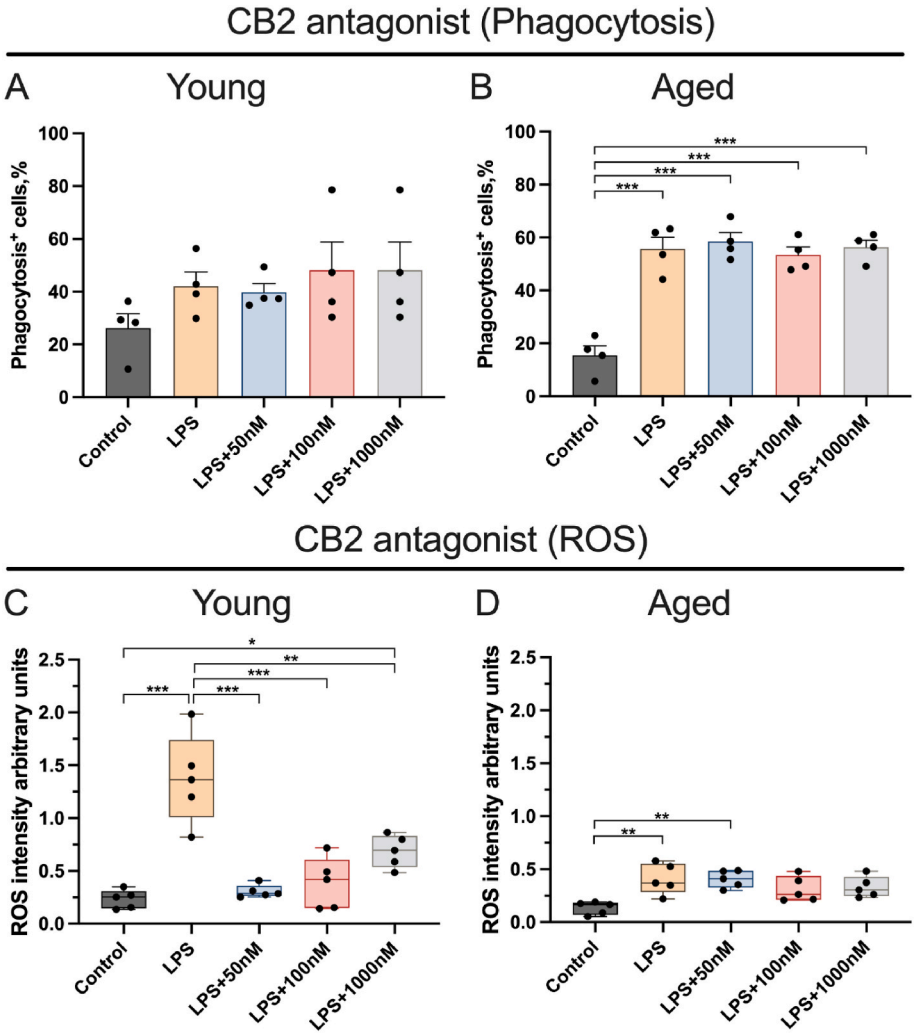

Fig. 6. Evaluation of the functionality of microglia. (A). The average percentage of young mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells. (B). The average percentage of aged mice microglia involved in phagocytosis by ingesting at least 10 beads. Data are presented as mean ± SEM of the percentage of CD11b + latex beads + cells ***p < 0.001. (C). Average Log10 transformed ROS fluorescence intensity in young mice microglia. Data are presented as boxplots ***p < 0.001; **p < 0.01; *p < 0.05. (D). Average Log10 transformed ROS fluorescence intensity in aged mice microglia. Data are presented as boxplots **p < 0.05.

3.2. Evaluation of gene expression

- CB_1 receptor (Cnr1)

Regarding the expression of *Cnr1*, we found no significant difference in the interaction between treatment and age, as revealed by two-way ANOVA. However, independently age [$F_{(1,16)} = 49.15$; $p = 0.001$], and treatment age [$F_{(1,16)} = 7.664$; $p = 0.0138$], showed a significant difference between LPS-treated microglia and non-LPS- treated microglia (Fig. 7A). Between age groups, we only see a significant increase in *Cnr1* gene expression in non-LPS-treated microglia ($p = 0.0348$). In young animals, a significant upregulation of the *Cnr1* gene expression ($p = 0.001$) was observed in LPS-treated microglia compared to non-LPS-treated microglia. Similar results were observed in aged animals where *Cnr1* expression was upregulated in +LPS-treated microglia ($p = 0.0068$) compared to non-LPS treated microglia (Fig. 7A).

- CB_2 receptor (*Cnr2*)

Concerning the expression of the *Cnr2*, a significant difference in age was revealed by two-way ANOVA [$F_{(1,16)} = 44.46$; $p = 0.0001$]. Between age, any significant differences were not observed (Fig. 7B). However, between young mice significant upregulation of the *Cnr2* gene expression ($p = 0.001$) was observed in LPS-treated microglia compared to non-LPS-treated microglia. Significant difference was also observed among aged mice where *Cnr2* expression in LPS-treated microglia ($p = 0.044$) was upregulated compared to non-LPS-treated microglia (Fig. 7B).

3.3. Evaluation of protein expression

- CB_1 receptor

Regarding the expression of the CB_1 receptor protein, we found no significant difference in the interaction between treatment and age, as revealed by two-way ANOVA. A significant difference with age was revealed by two-way ANOVA [$F_{(1,20)} = 8.238$; $p = 0.01$]. With age, we only observed a significant increase CB_1 receptor level only in LPS treated aged microglia cells compared to young microglia cells ($p = 0.05$). Although LPS stimulated cells showed an increase in CB_1 receptor expression, no significance was observed in young and aged microglia cells (Fig. 8A).

- CB_2 receptor

Regarding the expression of the CB_2 receptor protein, we found no significant difference in the interaction between treatment and age, as revealed by two-way ANOVA. However, independently they had a significant effect with age [$F_{(1,20)} = 19.43$; $p = 0.001$] and treatment [$F_{(1,20)} = 13.89$; $p = 0.01$]. In young microglia cells, significant increase in CB_2 receptor levels were determined in LPS treated cells ($p = 0.05$). Although increase levels in LPS treated aged microglia, no significance was obtained. However, with age we observed significant increased levels were observed in non-LPS aged microglia compared to non-LPS young microglia ($p = 0.05$) (Fig. 8B).

4. Discussion

The activation and functionality of microglia in the context of inflammation has been studied and documented over the years, but little is known about their modulation (Young and Denovan-Wright, 2022a). Neuroinflammation mediated by microglia is sometimes considered a double-edged sword. Therefore, there is a need to modulate the cells to reduce/elicit an immune response (Santiago et al., 2017). The ECS can influence the activation and regulation of microglia cells through the modulation of CB_1 and CB_2 receptors (Beins et al., 2021; Ruiz de Martín Esteban et al., 2022). In our research, we seek to explore the effects of synthetic cannabinoid compounds on microglia functionality during neuroinflammation in ageing mice.

Ageing is characterized by systemic inflammation and is driven in the brain (Zhu et al., 2023) particularly by microglia-associated neuroinflammation. Therefore, the ECS is activated as a negative feedback mechanism to modulate microglia-mediated inflammation. CB_1 and CB_2 receptors are important endocannabinoid receptors in microglia, that play a crucial role in the modulating inflammation (Kilaru and Chapma, 2020; Skaper and Di Marzo, 2012). Their activation promotes the inhibition of inflammation along with wound healing and tissue repair (Yuan et al., 2023). However, in young animals, these receptors are only expressed at low levels, which is due to a more homeostatic brain environment, as young animals are usually not exposed to prolonged stress, trauma, or any type of pathology that leads to inflammation (Haspula and Clark, 2020). On the other hand, ageing mice demonstrated a significant upregulation of CB_1 and CB_2 receptors with/without stimulation. This is due to the fact that the ageing brain usually tends to have a disrupted system, as ageing is associated with various

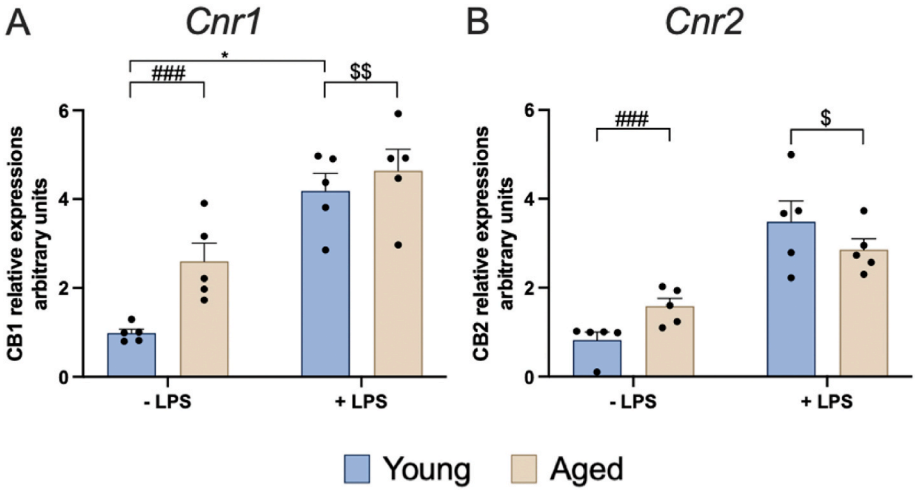

Fig. 7. Evaluation of microglia cannabinoid receptor gene expression. (A). Graphical representation of *Cnr1* expression showing a significant fold increase in expression in LPS-treated microglia compared to non-LPS-treated microglia. Data are presented as mean ± SEM of relative expression. $^{###}p < 0.001$; $^{\$\$}p < 0.01$; $^{*}p < 0.05$. (B). Graphical representation of *Cnr2* expression showing a significant fold increase in expression in LPS-treated microglia compared to non-LPS-treated microglia. Data are presented as mean ± SEM of relative expression. $^{###}p < 0.001$; $^{\$}p < 0.05$.

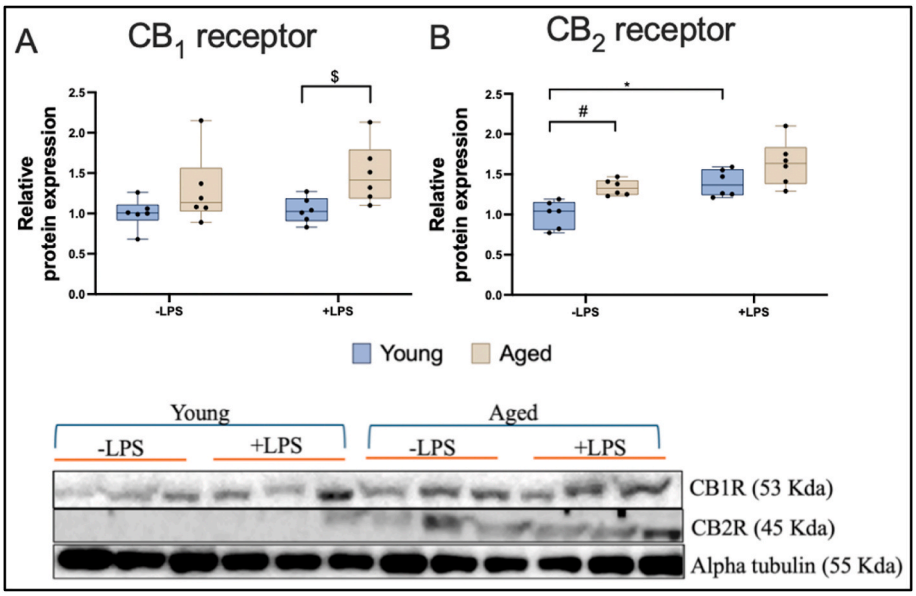

Fig. 8. Evaluation of microglia cannabinoid receptor protein expression. (A). Graphical representation of CB_1 receptor protein showing a significant fold increase in expression in LPS-treated compared to non-LPS-treated in young and aged microglia cells. Data are presented as box plot of relative expression. $^{*}p < 0.05$. (B). Graphical representation of CB_2 receptor protein showing a significant fold increase in expression in LPS-treated compared to non-LPS-treated in young and aged microglia cells. Data are presented as mean ± SEM of relative expression. $^{\#}p < 0.05$; $^{*}p < 0.05$.

pathological problems, especially microglia-mediated proinflammation (Sanguino-Gómez et al., 2022; Simpson and Oliver, 2020). Therefore, upregulation of the ECS has been shown to influence the anti-inflammatory state of microglia (Barrie and Manolios, 2017).

In young animals, the expression of the ECS components is inactive (Ginsburg and Hensler, 2022), as evidenced by low expression of the CB_1 receptor. Thus, the immune response is downregulated with respect to phagocytosis. However, the opposite effect is observed in old animals. Interestingly, although CB_1 is known an anti-inflammatory receptor, it showed quite the opposite effect on oxidative stress in young and aged animals. In the presence of the antagonist, the expression of CB_1 receptor is similar in all cases due to the regular immune response. Furthermore, the data on the immune response in young animals using the antagonist were inconclusive. In aged animals, though the cells were treated with the antagonist, there was an increase in the immune response along with the increased oxidative stress, indicating a possible reduction in anti-inflammatory or tissue repair of the microglia (Fig. 9). These unusual microglia response is characterized by the presence of excessive oxidative stress, as previously it was pointed out (von Bernhardi et al., 2015).

CB_2 receptor in the presence of the agonist in young animals, decreased its phagocytic activity and subsequently reduced oxidative stress. The presence of extracellular LPS triggered activation of microglia in young animals, upregulating their inflammation and thus reducing their phagocytosis. In contrast, the opposite effect was observed in aged animals as the ECS is activated, and the modulation of oxidative stress during age-related long-term inflammation was also demonstrated (Simpson and Oliver, 2020). In the presence of the antagonist, there is an upregulation of both phagocytosis and ROS in young animals, which occurs in young animals as a result of excessive production of ROS, an important marker for a long-term pro-inflammatory immune response (Fig. 9) (Mittal et al., 2014). On the other hand, ECS expression is upregulated in aged animals due to a dysfunctional cellular state caused by long-term neuroinflammation, as prolonged inflammation leads to a failure of cellular functionality (Bilkei-Gorzo, 2012).

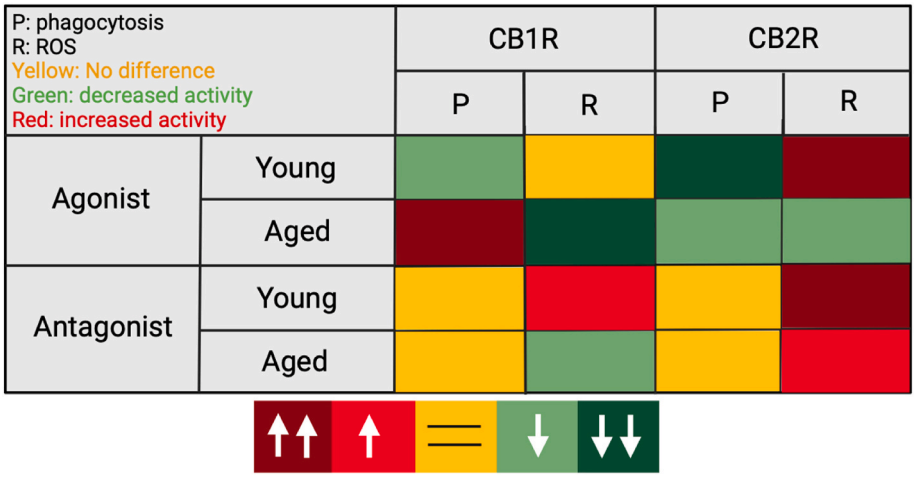

Fig. 9. Summary of the results. Darker the colour indicates a stronger effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In support of these findings, preliminary analysis of ERK/pERK signalling also indicated age-dependent modulation following cannabinoid treatment (data not shown), further underscoring the involvement of downstream pathways in ECS-mediated microglial responses. The ERK signalling pathway is implicated during the activation of ECS and CB1 and CB2 mediated modulation during ageing (Ribeiro et al., 2013). CB1 activation enhances ERK-dependent neuroprotective and antioxidative responses in the hippocampus (Young and Denovan-Wright, 2022b), while CB2 regulates ERK1/2 to mitigate neuroinflammation (Merighi et al., 2012). The age-related expression of the receptor function disrupts ERK dynamics, impairing memory and promoting neurodegeneration (Paloczi et al., 2018; Tadijan et al., 2022).

Overall, we have shown that young mice in the presence of the agonist for both CB_1 and CB_2 receptors demonstrated a decrease in inflammation and oxidative stress. As animals aged, various systemic pathologies associated with ageing have been shown to significantly contribute to increased inflammation and decreased oxidative stress in both CB_1 and CB_2 receptor expression when treated with the agonist. The presence of extracellular LPS triggered activation of microglia in young animals and led to an upregulation of inflammation and thus a reduction in phagocytosis. In the presence of antagonists for both CB_1 and CB_2 receptors in young animals, the data showed an increase in immune response and oxidative stress in the presence of LPS. Interestingly, treatment with an antagonist in aged mice showed an increase in inflammation and oxidative stress via the CB_1 receptor and a decrease in inflammation and oxidative stress in the case of the CB_2 receptor (Fig. 9).

5. Conclusions

The study showed that agonists and antagonists of the CB_1 and CB_2 receptors significantly influence the functionality of the microglia. In young animals, CB_1 receptor activation reduced phagocytic activity, whereas it was increased in aged animals after LPS pretreatment. CB_2 receptor activation was more effective in regulating microglial phagocytosis in both young and aged mice. Treatment with the antagonist in young and aged mice inactivated the CB_1 receptor and prevented phagocytosis in the presence of LPS, whereas microglial functionality was not modulated by the CB_2 receptor antagonist in young and aged mice, indicating the importance of CB_2 receptor activation in the microglia-mediated immune response. Both CB_1 and CB_2 receptor modulators affected oxidative stress during inflammation. CB_1 receptor agonists were more effective in reducing oxidative stress in young animals, while CB_2 receptor antagonists were more beneficial in aged animals, indicating age-specific responses. The expression of CB_1 and CB_2 receptors increased during inflammation in both young and aged animals. However, the upregulation was more significant in aged animals, suggesting ongoing inflammation processes even before LPS administration. Ultimately, the results suggest that synthetic cannabinoids could be used in the future to treat age-related pathologies such as neurodegenerative diseases.

CRediT authorship contribution statement

Akshay Kumar Vijaya: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Greta Krisikaitytė:** Investigation, Data curation. **Simonas Kuras:** Investigation, Formal analysis, Data curation. **Daiva Baltriukienė:** Writing – review & editing, Project administration, Conceptualization. **Aurelijus Burokas:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data availablity statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Lithuanian State Food and Veterinary Service (No. G2-104).

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abbreviations

ACEA	arachidonyl-2′-chloroacetamide
CB_1	cannabinoid type 1
CB_2	cannabinoid type 2
DAPI	4′,6-diamidino-2-phenylindole
ECS	the endocannabinoid system
JWH-133	dimethylbutyl-deoxy-Delta-8-tetrahydrocannabinol
LPS	lipopolysaccharides
ROS	reactive oxygen species
SR144528	5-(4-Chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo [2.2.1] heptan-2-yl]-1H-pyrazole-3-carboxamide

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2025.177705.

Data availability

Data will be made available on request.

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