ALTERATIONS IN THE DEVELOPMENT OF A M2 PHENOTYPE IN GLIAL CELLS:

ROTENONE *IN VITRO* MODEL OF PARKINSON'S DISEASE

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Abbreviations

ANOVA	Analysis of variance
Arg1	Arginase 1
BSA	Bovine serum albumin
CD206	Mannose receptor
Cox-2	Cyclooxygenase 2
FIZZ-1	Found in inflammatory zone 1
IL-1βRa	Interleukin 1 beta receptor antagonist
IL-4	Interleukin 4
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PBS	Phosphate buffer saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PI	Propidium ioide
qRT-PCR	Quantitative real time PCR
ROT	Rotenone
SEM	Standard error of the mean
TGF-β	Transforming growth factor beta
Ym-1	Chitinase 1

Abstract

Parkinson's disease is a chronic, progressive, disabling neurodegenerative disease of unknown cause. It turned out to be the second most common neurodegenerative disorder after Alzheimer Disease, and is characterized by motor and non-motor symptoms. As a multifactorial disease, it is characterized by dopaminergic degeneration, depositions of α-synuclein aggregates, and to run with an inflammatory process. The involvement of microglial cells on this chronic inflammation within the parenchyma is long accepted but its role is not fully understood. Both genetic and environmental factors have been postulated to play a role in the development of Parkinson's disease. Epidemiological studies show that the exposition to certain toxins, among them rotenone, is a risk factor to develop this neurodegenerative disease. Although the neurotoxic effect of rotenone has been widely studied in experimental approaches, the effect of rotenone on glial cells remains poorly characterized. The present work was aimed to study whether rotenone induced the expression of M2 markers in glial cells, and also whether rotenone interfered with the development of a M2 phenotype induced by Interleukin-4 in glial cells, using primary glial cultures. The results obtained reveal that rotenone did not induce the expression of M2 markers in glial cells *per se*. However, it dramatically inhibited the development of a M2 phenotype induced by Interleukin-4 in mixed glial cell cultures. On the contrary, rotenone did not modify Interleukin-4 – induced M2 phenotype in microglial cell cultures, suggesting the existence of alternative mechanisms to obtain energy of microglial cell cultures. However, phagocytic activity was impaired in microglial cell cultures. The results obtained suggest that rotenone impairs the immune response of glial cells and that glial cell dysfunction due to a direct action of certain toxins on glial cells may contribute to the development of neurodegeneration in PD.

1. Introduction

James Parkinson was the first to describe the disease that bears his name in "An Essay on the Shaking Palsy", published in 1817 (1). More than a century after the discovery of the disease, Parkinson's disease (PD) turned out to be the second most common neurodegenerative disorder after Alzheimer Disease (2) and its prevalence is estimated to be 329 per 100,000 people, with an annual incidence ranging from 16 to 19 per 100,000 (3). Moreover, it is expected to double by 2030 from the current estimates of 5 million patients worldwide (4). It is defined as a chronic, progressive, disabling neurodegenerative disease of unknown cause that presents motor impairments, autonomic dysfunction, and psychological and cognitive changes (5).

From the histopathological point of view, PD is characterized by the progressive degeneration of the dopaminergic cells of the substantia nigra (6) and the deposition of α-synuclein into Lewy bodies and Lewy neurites in many remaining neurons (4). Associated with the widespread neurodegeneration, the disease runs with an inflammatory process that evolves over many years, and it is almost exclusively dominated by the resident macrophages, the microglia (7). In the healthy brain, microglia is constantly monitoring their immediate environment palpating the surface of neurons and glia and it can be rapidly activated by various environmental changes (8), with a process called polarization (9). The cell turns into an activated state, changing its morphology, expressing receptors, and releasing products in all forms of central nervous system damage and diseases (7). This activation state of microglial cells is understood within a continuum of activation paradigms (10) that rapidly changes from one phenotype to another in a few hours. These phenotypes can be classified along a linear scale with two extremes, on which the M1 (or classical activation) state is associated with pro-inflammatory and pro-killing functions, and the M2 (or alternative activation) state is related with healing and scavenging processes (10).

A better understanding of PD was possible thank to the discovery of some toxins that were able to mimic the selective dopaminergic cell death of the disease. Overall, toxin-models have been the most extensively studied (11). A well-established model is for instance the use of the pesticide rotenone (5), that has been directly linked to idiopathic PC (12). This toxin is able to cross the blood brain barrier and inhibit the complex I of the electron transporter chain of the mitochondria, causing mitochondrial dysfunction (13). It also produces an oxidative and nitrative stress, inflammation, and a selective degeneration of nigrostriatal dopaminergic neurons (5).

Although the neurotoxic effect of rotenone has been widely studied, the effect of this toxin in glial cells remains poorly characterized. In the present project, we have studied the effect of rotenone on glial cells using *in vitro* approaches. We have studied how the microglial phenotype M2 develops and behaves in the presence of rotenone. In addition, we have focused on the CD200-CD200R1 system as a mechanism of inhibitory control of microglial activity.

2. Working hypothesis and objectives

Inflammation in PD is nowadays considered a characteristic of PD as much as the depletion of the dopaminergic cells or the α-synuclein aggregates. The involvement of microglia on this chronic inflammation within the parenchyma is long accepted. Unfortunately, its role is not fully understood. However, the CD200-CD200R1 ligand-receptor pair appears to be mandatory in the control of the microglial inflammatory response. In the central nervous system, the ligand is mainly expressed in neurons and astrocytes, while the receptor is expressed in microglial cells. The activation of the receptor has been found to down-regulate the inflammatory response of microglial cells (14).

Previous results of the research group show a decrease in the expression of the receptor CD200R1 in microglial cells in front of pro-inflammatory stimuli such as LPS or LPS+INF-γ (15, 16). They have also demonstrated that the exposure to neurotoxic agents can alter the expression of that receptor and its ligand in glial cell cultures, as well as the development of a M1 phenotype in microglial cells in response to a pro-inflammatory stimulus (17).

The aim of the present work was to study whether rotenone induces the expression of M2 markers in glial cells, and also whether rotenone interferes with the development of a M2 phenotype induced by Interleukin 4 (IL-4) (typical M2 stimulus) in glial cells, using *in vitro* approaches.

The specific objectives of this study were:

1. To assess the effect of rotenone on metabolic activity and cell viability in mixed glial and microglial cell cultures.

2. To determine morphological changes in astroglial and microglial cells in response to rotenone treatment.

3. To evaluate the effect of rotenone on the development of a M2 phenotype in glial cell cultures.

4. To determine the effect of rotenone on the CD200-CD200R1 system.

5. To analyse the effect of rotenone on the phagocytic activity of microglial cells.

3. Material and methods

Experiments were carried out in accordance with European Union directives (86/609/EU) and Spanish regulations (BOE 67/8509-12, 1988) on the use of laboratory animals, and were approved by the Ethics and Scientific Committees of Universitat de Barcelona and the Hospital Clínic Provincial de Barcelona.

Primary cell cultures

Primary mixed glial cell cultures were prepared from cerebral cortices of C57BL/6 mice (Charles River, Lyon, France) pups 1 to 3 days old as previously described by Enjarque-Ortiz et al. (18). After mechanical and chemical dissociation, cells were seeded in Dulbecco's modified Eagle medium-F-12 nutrient mixture supplemented with 10% heat-inactivated fetal bovine serum, 0.1% penicillin-streptomycin (Invitrogen), and 0.5 µg/mL amphotericin B (Fungizone®). Cells were suspended at a density of 350,000 cells/mL and cultured at 37°C in humidified 5% CO2/95% air. Medium was replaced every 7 days *in vitro* and the cultures were used at 21 days *in vitro* (see Supplementary Figure 1A).

Microglial cell cultures were prepared from mixed glial cell cultures by mild trypsinization according to the method of Saura et al. (19). With this mild trypsinization (0.06% Trypsin-EDTA solution, Invitrogen, Carlsbad, CA) of 25-35 minutes, we achieved the detachment of the upper layer formed by astrocytes, isolating the microglial cells that were firmly attached to the bottom of the well. This cells were predominantly microglia (>98%). Microglial cells were treated after 24 hours (Supp Fig 1B).

Treatments

In previous works of the group, different concentrations of the toxin rotenone were tested so as to avoid using concentrations that had a significant effect on cell viability. In the present work, cells were treated with 40 and 100 nM rotenone (Sigma-Aldrich), in absence or presence of recombinant mouse IL-4 (50 ng/mL; Creative BioMart®, Shirley, NY) during 24 hours.

Metabolic activity and cell viability assays

To determine metabolic activity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used. 24 h after treatment, MTT (5 mg/mL; Sigma-Aldrich) was added during the last 30 or 90 minutes (mixed glial and microglial cells respectively) of incubation. Then, the medium was removed and dimethil sulfoxide was added to dissolve the dark blue crystals formed. The reduction of MTT was estimated by optical density at 570 and 650 nm using a microplate reader (Multiskan Spectrum, Thermo Electron Corporation, Waltham, CA).

Cells were also labelled with propidium ioide (PI; 7.5 µg/mL; Molecular Probes) and Hoechst 33342 (3 µg/mL; Molecular Probes) for 10 minutes to label nuclei of death and live cells respectivelly. Images were obtained under a fluorescence microscope using a 4X objective (microglial cultures) or a 10X objective (mixed glial cultures) using an Olympus IX70 microscope (Olympus, Okoya, Japan) and a digital camera (CC-12, Olympus Soft

Imaging Solutions GmbH, Hamburg, Germany). Results were expressed as percentage of control.

Immunofluorescence

In order to observe morphological changes, we performed single and double immunofluorescence. After 24 hours of the treatment, cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffer (PBS) 0.1 M for 15 minutes at room temperature. Cells were rinsed with PBS with 1% bovine serum albumin (BSA), 0.3% Triton X-100, and 10% normal goat serum for 20 minutes and then incubated over-night at 4°C with primary antibodies (see Supplementary Table 1). After being rinsed with PBS, cells were incubated for 1 hour at room temperature with secondary antibodies (Supp Table 1). All antibodies were diluted in PBS 0.1 M, 1% BSA, 0.3% Triton X-100, and 10% NGS. Images of the fluorescence labelling were obtained using the same Olympus microscope employed for the IP/Hoechst method.

Qualitative real time PCR (qRT-PCR)

qRT-PCR was used to quantify mRNA expression in cell cultures. Total RNA was isolated using High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) for mixed glial cultures and PureLink RNA micro Kit (Invitrogen) for microglial cell cultures and stored at -80°C. RNA was then reverse transcribed with random primers using Transcriptor Reverse Transcriptase (Roche Diagnostics) and stored at -20°C. Three nanograms of cDNA were used to perform qRT-PCR. The primers used (Integrated DNA Technology, IDT, Skokie, IL) are shown in the supplementary data (Supp Table 2). IQ SYBR Green SuperMix (Bio-Rad Laboratories, Hercules, CA) was used in 15 µL of final volume, using an iCyclerMyIQ apparatus (Bio-Rad Laboratories). Samples were run for 40 cycles (95°C for 15 seconds, 60°C for 30 seconds, 72°C for 15 seconds. Relative gene expression values were calculated using the 2-ΔΔCt method with the CFX software (Bio-Rad Laboratories). Ct values were corrected according to the reference genes actin and S18.

Western blot

For protein isolation, every well was washed with cold PBS. Cells were scraped and recovered in 100 µL of RIPA buffer (1% Igepal CA-630, 5 mg/mL sodium deoxycholate, 1 mg/mL sodium dodecyl phosphate and protease inhibitor cocktail Complete®, Roche Diagnostics, in PBS) for mixed glial cells. Samples were sonicated, centrifuged for 10 minutes at 10,400g at 4°C and the supernatant was collected and stored at -20°C. Protein concentration was determined by the Bradford assay.

Western blot analyses of total protein extracts were performed with 30 µg of protein subjected to SDS-PAGE on a 12% polyacrylamide minigel, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were incubated with primary (4°C, overnight) and horseradish peroxidase-labelled secondary (1 hour at room temperature) antibodies (Supp Table 3). The signal was developed with the western blotting detection kit WesternBrith™ Sirius HRP substrate (Advansta, CA) and images were obtained using a VersaDoc System (Bio-Rad Laboratories). Data are expressed as the ratio between the band intensity of the protein of interest and the loading control protein (actin).

Phagocytic activity

To assess the phagocytic activity of microglial cells, red fluorescent latex beads (FluoSpheres® Carboxylate-Modified Microspheres, Termofisher Scientific) of 0.2 µm were added to the medium 23 hours after treatment during 60 minutes. After being rinsed with PBS, microglial cell cultures were fixed with 4% PFA for 15 minutes and stained with immunofluorescence against IBA-1 (Supp Table 1). Images were taken with the with the Olympus microscope. The results are expressed as the percentage of phagocytic and non-phagocytic cells, the average number of beads per cell and the percentage of cells with 1 to 4 beads (cells with low phagocytic activity) versus the cells with more than 4 beads (cells with high phagocytic activity).

Statistical analysis

Results are presented as the mean + standard error of the mean (SEM) values. Statistical analyses were performed with the software GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Student t-test was used to compare two groups and one-way or two-way analysis of variance (ANOVA) followed by the Newman-Keuls Multiple Comparison Test or the Bonferroni post-test were used to compare three or more groups. Values of $P < 0.05$ were considered statistically significant.

4. Results

Rotenone treatment induced alterations in metabolic activity but not in cell viability in mixed glial and microglial cell cultures

We evaluated the metabolic activity of the cell cultures with the MTT assay and the cell death using the IP/Hoechst labelling. Hoechst labels all cells with blue fluorescence and IP only enters in the death cells, with red fluorescence.

The analysis of the MTT reduction in mixed glial cultures, showed significant alterations at the two concentrations of rotenone used, in the presence of IL-4, compared with the control at 24 hours (Fig. 1A). Microglial cell cultures showed a significant increase in metabolic activity after any of the treatments, compared with the control at 24 hours (Fig. 1B).

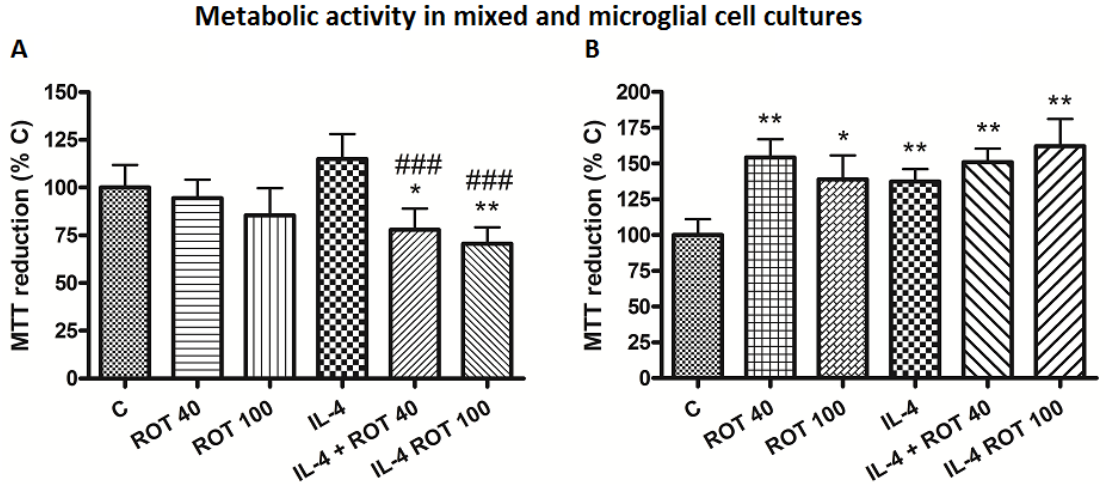


Figure 1. Metabolic activity in glial cell cultures. MTT reduction in mixed glial cell cultures **(A)** and microglial cell cultures **(B)** exposed to rotenone (ROT), in the absence and the presence of IL-4. Bars are means ± SEM of n=4-5 cell cultures. One-way ANOVA (repeated measures) and Newman-Keuls post-test (*p<0.05 and **p<0.01, compared with the control group; ###p<0.001 compared with the IL-4 group).

Regarding PI/Hoechst labelling, no cell death was observed caused by the treatments either in the mixed or in the microglial cell cultures (Supp Fig. 2A-D). Further analyses of the proliferation in microglial cell cultures were done. The results showed no significant proliferation in those cell cultures (Supp Fig. 2E).

Microglial cells change their morphology to a more amoeboid form in front of rotenone

To determine morphological changes of the cells in response to the different treatments, in the microglial cell cultures we have used an immunofluorescence staining against IBA-1 and in the mixed glial cultures a double staining against GFAP and CD-68+IBA-1 for astrocytes and microglia respectively (Supp Table 1).

Whereas neither astrocytes nor microglial cells did present evident morphological changes in the mixed glial cell cultures, in response to rotenone, in absence and presence of

IL-4 (Supp Fig. 3A and 3B), the microglial cell cultures did. The microglial cells changed its morphology to a more rounded or "ameboid" form with retraction and shortening of their projections. Compared with the control group, IL-4 did not produce apparent modifications, only a trend in the elongation of the projections. Moreover, when microglial cells were exposed to IL-4 and Rotenone, they preserved their projections, compared to the Rotenone group (Fig. 2).

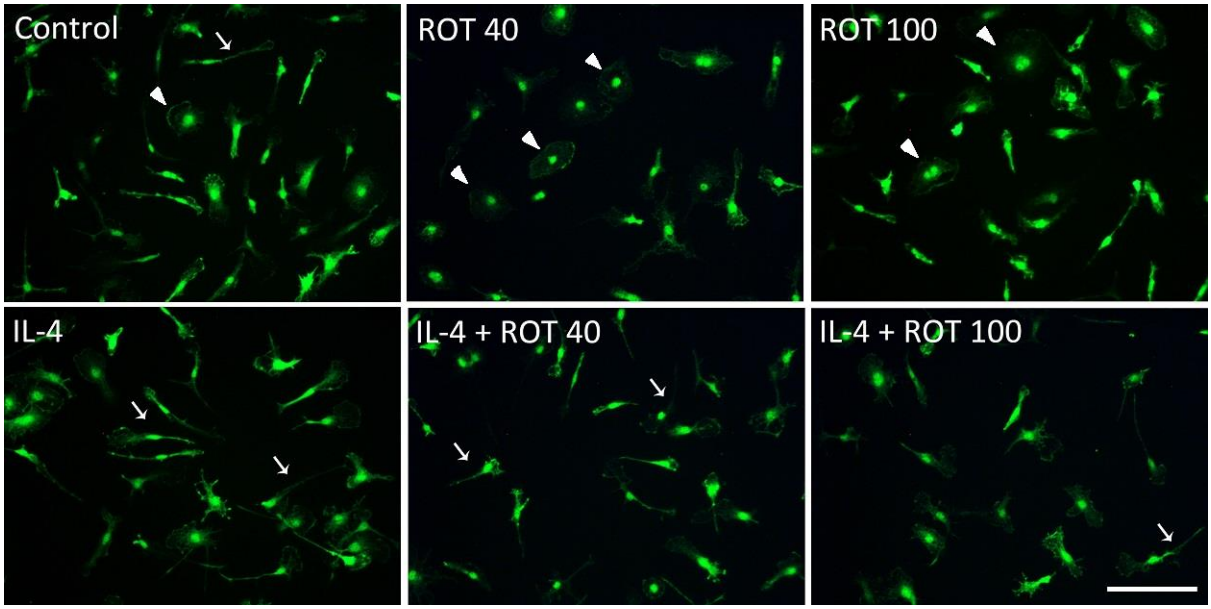


Figure 2. IBA-1 immunolabelling in microglial cell cultures. Microglial cell culture treated with rotenone (ROT), in the absence and the presence of IL-4. Arrowheads show the ameboid cells; and arrows show the elongated projections. Scale bar: 100 µm.

The mRNA expression of M2 phenotype markers induced by IL-4 is altered by rotenone in mixed glial cell cultures but not in microglial cell cultures

In order to assess the alternative M2 phenotype, we have studied the expression of several markers such as arginase 1 (Arg1), transforming growth factor β (TGF-β), mannose receptor (CD206), found in inflammatory zone-1 (FIZZ-1), Ym-1 (Chitinase), IL-10, IL-1β receptor antagonist (IL-1Ra), and cyclooxygenase-2 (Cox-2).

In mixed glial cultures, rotenone treatment altered IL-10 and Cox-2 mRNA expression, compared with the control group. IL-10 mRNA expression showed a decrease in response to rotenone (Fig. 3F, *p<0.05 for ROT 100 and **p<0.01 for ROT 40). In contrast, Cox-2 mRNA expression increased (Fig. 3H, ***p<0.001 for ROT 100). Regarding the treatment with IL-4, it showed a significant increase in the mRNA expression of the M2 markers: Arg1 (Fig. 3A, ***p<0.001), TFG-β (Fig. 3B, *p<0.05), CD206 (Fig. 3C, *p<0.05), FIZZ-1 (Fig. 3D, ***p<0.001), and IL-1βRa (Fig. 3G, **p<0.01) compared with the control group. This increase was inhibited when we combined IL-4 with rotenone, compared with the IL-4 group, for Arg1 (###p<0.001), TGF-β (##p<0.01), FIZZ-1 (###p<0.001), CD206 (#p<0.05 for ROT 100), IL-10 (#p<0.05), and IL-1βRa (##p<0.01). The expression levels of Ym-1 did not change significantly in response to treatments, although a trend to increase was observed after IL-4 treatment (Fig. 3E).

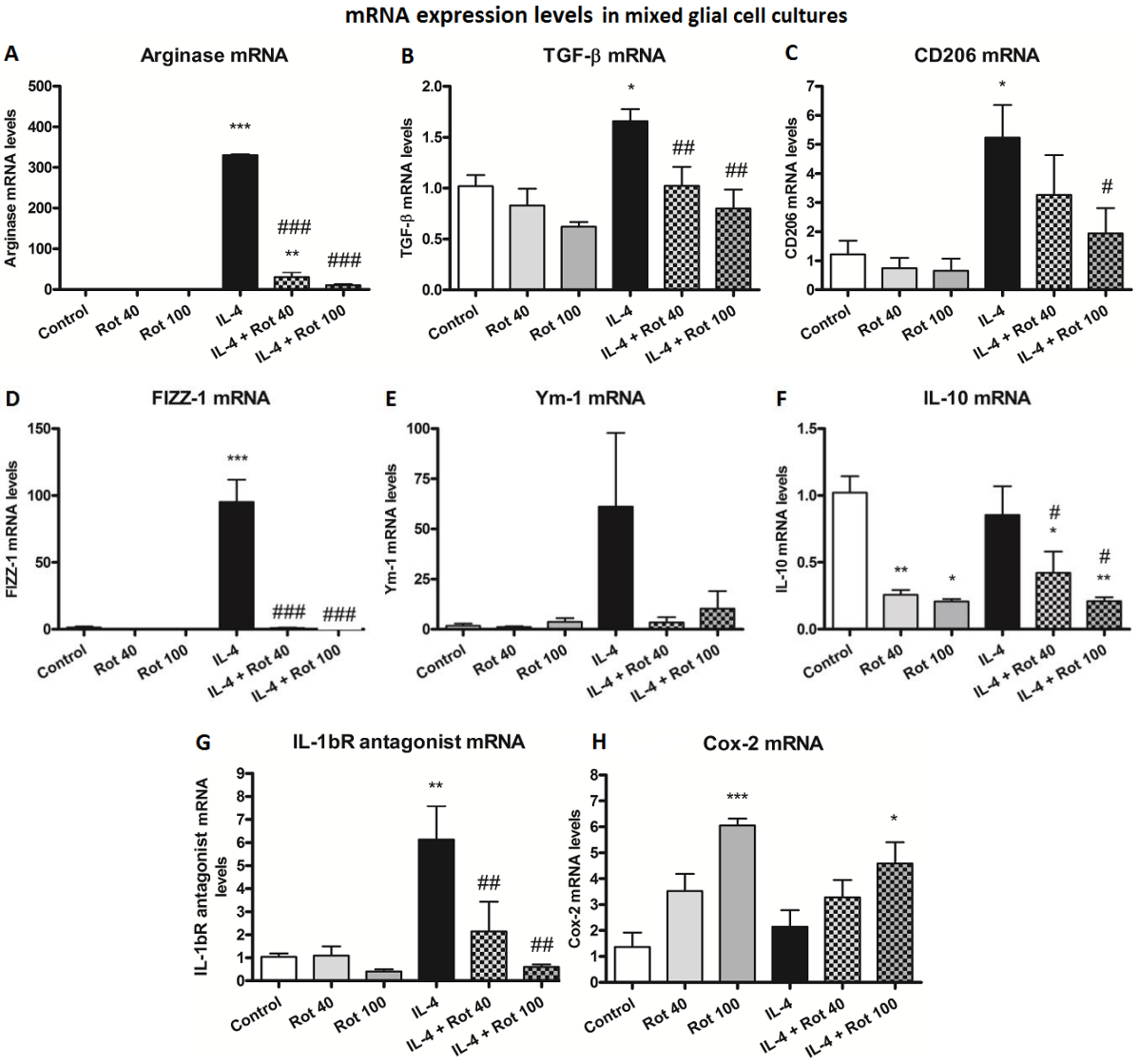


Figure 3. mRNA expression levels of M2 phenotype markers in mixed glial cell cultures exposed to rotenone (ROT) in the absence and the presence of IL-4. mRNA of arginase 1 **(A)**, TGF-β **(B)**, CD206 **(C)**, FIZZ-1 **(D)**, Ym-1 **(E)**, IL-10 **(F)**, IL-1βRa **(G)**, and Cox-2 **(H)**. Bars are means ± SEM of n=4 cell cultures. One-way ANOVA and Newman-Keuls post-test (*p<0.05, **p<0.01 and ***p<0.001 compared with the control group; #p<0.05, ##p<0.01 and ###p<0.001 compared with the IL-4 group). Internal normalization controls: actin and S18.

On the other hand, microglial cell cultures treated with rotenone did not suffer any alteration on the mRNA expression of M2 markers, in comparison with the control group. IL-4 treatment induced the mRNA expression of two M2 markers: Arg1 (*p<0.05) and CD206 (***p<0.001) compared to the control group (Fig. 4A and 4B respectively). These increases were not modified by rotenone. The mRNA expression of the other markers can be found in Supp Fig. 4.

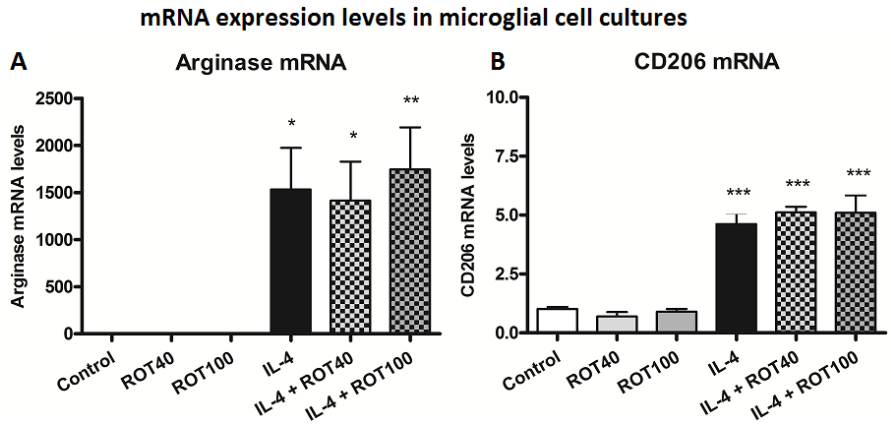


Figure 4. mRNA expression levels of M2 phenotype markers in microglial cultures exposed to rotenone (ROT) in the absence and the presence of IL-4. mRNA expression of arginase 1 **(A)** and CD206 **(B)**. Bars are means ± SEM of n=4 cell cultures. One-way ANOVA and Newman-Keuls post-test (*p<0.05, **p<0.01 and ***p<0.001 compared with the control group). Internal normalization controls: actin and S18.

The protein expression of M2 phenotype markers induced by IL-4 is also altered by rotenone in mixed glial cell cultures

In parallel to the determination of mRNA expression of M2 markers, western blot assays were carried out in mixed glial cell cultures to study the protein expression of Arg1 (Fig. 5A) and CD206 (Fig. 5B). In response to rotenone, the protein expression of Arg1 or CD206 was not altered compared with the control group. IL-4 treatment induced a clear increase in the expression of Arg1 and CD206 in comparison to control group (***p<0.001). However, rotenone inhibited the expression of Arg1 and CD206 induced by IL-4 (###p<0.001). Representative western blot images are presented in Fig. 5C.

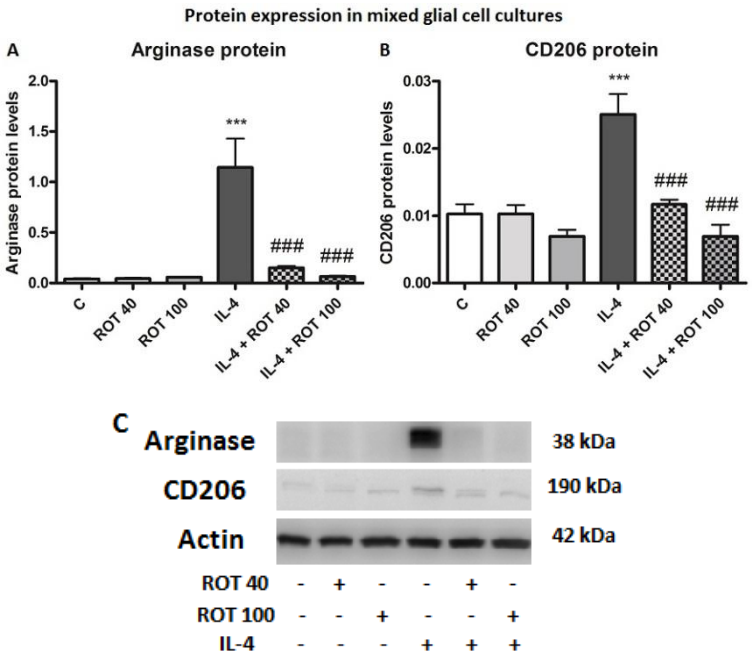


Figure 5. Protein expression in mixed glial cell cultures exposed to rotenone (ROT) in the absence and presence of IL-4. Protein expression of arginase 1 **(A)** and CD206 **(B)** and representative western blot images of both markers **(C)**. Bars are means ± SEM of n=4 cell cultures. One-way ANOVA and Newman-Keuls post-test (***p<0.001 compared with the control group; ###p<0.001 compared with the IL-4 group). Internal normalization controls: actin.

Rotenone alters the expression of the CD200-CD200R1 system in mixed glial cultures but not in microglial cultures

Another M2 phenotype marker is the known as CD200-CD200R1 system. An alteration in the mRNA expression of this system in mixed glial cell cultures was observed 24 hours after the treatment with two different concentrations of rotenone, in absence and presence of IL-4. Both mRNA forms of the CD200 ligand (CD200full and CD200tr mRNA) presented a decrease compared with the control group (Fig. 6A and 6B). The decrease of gene expression of CD200full and CD200tr was observed with both concentrations of the toxin (*p<0.05 and ***p<0.001 respectively). A decrease for both ligands was also observed in response to IL-4 (*p<0.05). The treatment of rotenone in presence of IL-4 followed the same tendency but only with statistical significance in the case of the CD200tr mRNA (*p<0.01), compared with the control group.

In contrast, rotenone treatment did not alter the expression of the receptor, CD200R1, in any concentration, compared with the control group. Only IL-4 treatment increased its expression (*p<0.05), but the exposure of IL-4 together with rotenone showed a trend to diminish it (Fig.6C).

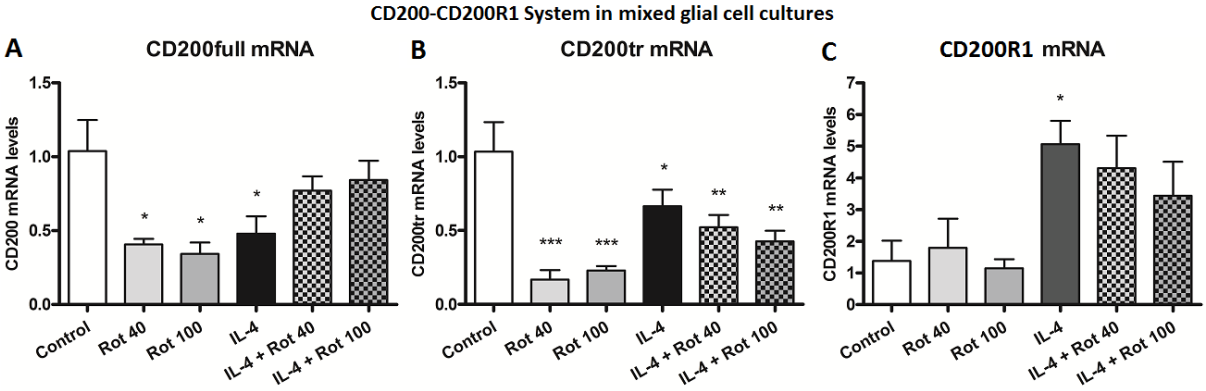


Figure 6. mRNA expression of the CD200-CD200R1 system in mixed glial cell cultures in response rotenone (ROT) in the absence and the presence of IL-4. Representation of the mRNA expression levels of the CD200full **(A)**, CD200tr **(B)**, and CD200R1 **(C)**. Bars are means ± SEM of n=4 cell cultures. One-way ANOVA and Newman-Keuls post-test (*p<0.05, **p<0.01 and ***p<0.001 compared with the control group). Internal normalization controls: actin and S18.

Regarding the microglial cells, since those cells do not express the ligand, we only analyzed the expression of CD200R1 mRNA. The expression of CD200R1 mRNA was not altered in response to rotenone treatment, in the absence and presence of IL-4 (Fig 7).

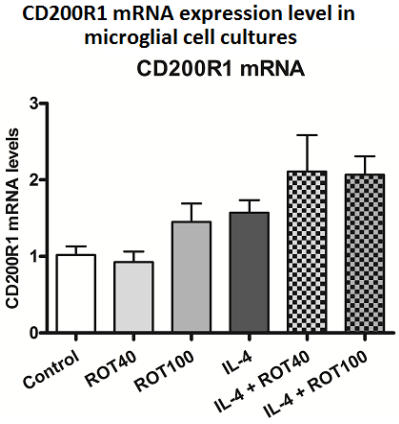


Figure 7. CD200R1 mRNA expression levels in microglial cell cultures in response to rotenone (ROT) in the presence and absence of IL-4. Bars are means ± SEM of n=4 cultures. Internal normalization controls: actin and S18.

The phagocytic activity of microglial cells is diminished in response to rotenone

The phagocytic activity of microglial cells was tested using red fluorescence latex beads (Fig. 9A). The percentages of non-phagocytic (Fig. 9B) and average of beads per cell in phagocytic cells (Fig. 9C) were not altered in response to rotenone, in the absence and the presence of IL-4. However, when we classified the cells into two groups according to their phagocytic capacity (cells with 1 to 4 beads: low phagocytic capacity; cells with more than 4 beads: high phagocytic capacity), the treatment with rotenone showed an increase in the percentage of cells with low phagocytic capacity and a decrease in the percentage cells with high phagocytic capacity, compared with the control group (Fig.9D, **p<0.01).

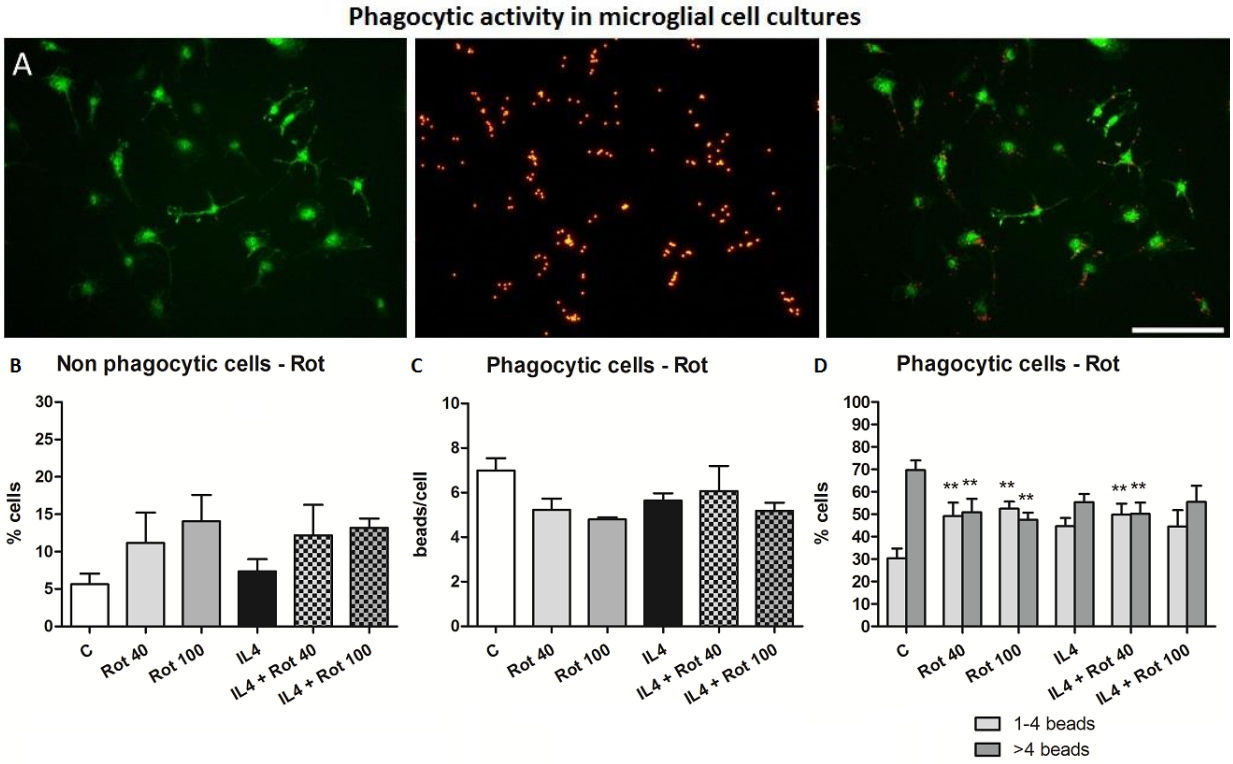


Figure 3. Phagocytic activity of microglial cells in response to rotenone (ROT), in absence and presence of IL-4. Images of control cells immunolabelled against IBA-1 (green) and the latex beads (red) **(A)**. Scale bar: 100 µm. Percentage of non-phagocytic **(B)**, average of beads per cell in phagocytic cells **(C)**, and percentage of cells classified by its phagocytic capacity, from 1 to 4 beads and more than 4 beads per cell **(D)**. Bars are means ± SEM of n=4 cell cultures. Two-way ANOVA and Bonferroni post-test (*p<0.05 and **p<0.01 compared with the control group).

5. Discussion

In the present work, we studied the effect of rotenone on glial cells, focusing on the expression of markers of the microglial M2 phenotype and the CD200-CD200R1 system. To this end, we treated the cells with 40 and 100 nM rotenone and we determined morphological changes by immunocytochemistry, and functional changes by mRNA and protein expression, and phagocytosis. We also evaluated whether rotenone could interfere with the development of a M2 phenotype by IL-4 (typical M2 stimulus) treatment.

As it has been explained, the two concentrations of rotenone used were chosen so as not to induce significant cell death in the glial cell cultures, at least after 24 h. This was evaluated by PI and Hoechst labelling, to detect the nuclei of death cells and live cells respectively. The percentage of PI labelled nuclei observed at 24 hours of the treatments did not significantly differ from the control situation, in both mixed and microglial cell cultures.

Rotenone is an inhibitor of the electron transporter chain of the mitochondria (13), and consequently it interferes with the ATP production and the metabolic activity of the cells. The electron transporter chain is mandatory to perform the oxidative phosphorylation as well as driving the pyruvate into the Krebs cycle (20). A mitochondrial dysfunction means difficulties to obtain ATP, at least with the Krebs cycle. We performed the MTT assay in order to check whether the concentrations of rotenone used in the present study were able to induce alterations in the metabolic activity of glial cells. The results obtained revealed alterations in the metabolic activity of mixed glial cultures and microglial cultures 24 h after rotenone treatment. Thus, we observed a significant decrease in metabolic activity in mixed glial cultures treated with IL-4 plus rotenone. This decrease in metabolic activity was not due to cell death. Surprisingly, microglial cell cultures showed an increase in metabolic activity when compared with the control group, and this increase was not due to proliferation of microglial cells.

Microglial cells use alternative mechanisms to obtain energy, apart from the Krebs cycle. It is long accepted that macrophages obtain ATP from various sources with clear differences between M1 and M2 phenotypes. In M1 macrophages, a shift to aerobic glycolysis is induced upon activation, which involves an increase in glucose uptake as well as the conversion of pyruvate to lactate. This is associated to an attenuation of the respiratory chain activity (20). On the contrary, M2 macrophages obtain much of their energy from fatty oxidation and oxidative metabolism, and have low glycolysis rates (20, 21). They can induce the expression of constituents of the electron transport chain that will perform oxidative phosphorylation as well as driving the pyruvate into the Krebs cycle. The results obtained suggest that obtaining energy is compromised in mixed glial cell cultures but not in microglial cell cultures.

Glial cells have the ability to change its morphology in response to pathologic conditions. In the control brain, microglia show a ramified morphology. In contrast, under pathological conditions, microglial cells are activated, and change to a more rounded form termed “ameboid” (14). This process has been suggested to help microglia to invade lesions (8). With immunofluorescence techniques, the morphology of the cell can be drawn to observe any change in response to stimuli. Several morphologies were observed in control

microglial cultures, but most of cells showed elongated forms with few projections. In response to rotenone treatment, we observed an ameboid transformation, with retraction and shortening of the projections. The morphology of IL-4-treated cells did not differ from that of control cells. Interestingly, cells treated with rotenone and IL-4, turn its shape into a similar morphology to the cells of the control group, compared with the cells treated only with rotenone. Regarding the mixed glial cell cultures, neither astrocytes nor microglia presented evident morphological changes when treated with rotenone, in absence and presence of IL-4.

We next studied whether rotenone could induce the expression of M2 phenotype markers in glial cells, as well as whether rotenone could interfere with the development of the M2 phenotype induced by the typical M2 stimulus IL-4. We looked at the expression of several M2 markers: Arg1, TGF-β, CD206, FIZZ-1, Ym-1, Il-10, IL-1bRa and Cox-2. These markers have been shown to be over-expressed in response to IL-4, as an anti-inflammatory response of microglial cells (7, 8, 10, 22). We have obtained different results in mixed glial cultures and microglial cultures. In mixed glial cultures, rotenone treatment resulted in a decrease in IL-10 mRNA expression and an increase in Cox-2 mRNA expression. When we treated the cells with IL-4, the expression of most of the typical M2 markers drastically increased compared with the control group. However, rotenone treatment significantly inhibited the mRNA expression of M2 markers induced by IL-4. These effects were corroborated at the level of protein expression in the case of Arg-1 and CD206. In microglial cultures, rotenone treatment did not induce significant changes in the expression of the M2 markers tested. IL-4-treated microglial cell cultures showed a significant increase in the Arg-1 and CD206 mRNA expression, effect that was abrogated in the presence of rotenone. These results show that rotenone treatment results in an impairment of the M2 response in glial cells, and suggest that rotenone exposition affects the immune response of glial cells, interfering with the development of the appropriate response after an M2 stimulus. An impaired M2 response could result in a dysfunctional regulation of the inflammatory response, as well as improper wound healing and scavenging processes.

It is known that macrophages need most of its energy to sustain phagocytic and secretory activity, with a direct involvement in the transcriptional regulation of the immune response (20). The M2 macrophage secretory program is energetically demanding, both in terms of intensity and duration, and oxidative metabolism is best suited to meet the bioenergetics demands for long-term macrophage activation (21). Even though rotenone impairs oxidative metabolism, the expression of M2 markers induced by IL-4 is not affected by rotenone in microglial cell cultures. These results suggest that microglial cells are able to handle the effect of rotenone on metabolic supply through alternative mechanisms. Indeed, MTT results showed an increase in metabolic activity in rotenone-treated microglial cell cultures. On the contrary, a different scenario was observed in mixed glial cultures. In these cultures, the increase in the expression of M2 markers induced by IL-4 was dramatically inhibited by rotenone. Furthermore, MTT results showed a decrease in metabolic activity in mixed glial cultures exposed to rotenone, which could explain the lack of energy to perform and maintain this M2 secretory program.

We additionally studied the effect of rotenone on the CD200-CD200R1 system. CD200R1 is a microglial inhibitory receptor involved in the control of the inflammatory response and its expression has been associated to alterative macrophage activation (23).

Astrocyte CD200 and neuronal CD200 interact with the receptor to maintain microglial cells in a quiescent/surveillant state. In the mixed glial cell cultures, we observed alterations on this system when we treated cells with rotenone, in absence and presence of IL-4. Both forms of the ligand (full and truncated) showed a decrease on their mRNA expression when cells were exposed to rotenone, while the expression of CD200R1 mRNA was not modified. These results suggest that rotenone-treated astrocytes would facilitate the development of a pro-inflammatory phenotype in microglial cells. CD200 has a pivotal role on inflammation. It has been observed that its over-expression establishes an anti-inflammatory milieu in a mouse model of Alzheimer 's disease (24). In contrast, the lack of the ligand CD200 exhibited, in CD200-deficient mice model, an increase on microglial response (25). IL-4-treatment inhibits CD200full and CD200tr mRNA expression and increases CD200R1 mRNA in mixed glial cell cultures. IL-4, as prototypical M2 phenotype stimulus (7, 8, 10, 22), appears to enhance the amount of receptors to recognise those reduced ligands, promoting the development of a M2 phenotype in mixed glial cultures. Rotenone-treatment did not significantly modify the pattern of expression of CD200 and CD200R1 induced by IL-4. Since the microglial cells only express the receptor, we only analyzed CD200R1 expression in microglial cell cultures. Although no significant alterations in CD200R1 mRNA expression were detected in microglial cell cultures after the treatments, a trend to increase the expression was observed after 100 nM rotenone or IL-4 treatment, which became more evident after IL-4 plus rotenone treatment.

Microglial cells, as macrophages, have the ability to phagocyte. We have studied how the phagocytic activity of these cells may be altered in the presence of rotenone. We observed that while rotenone did not induce significant alterations in the percentage of phagocytic cells (although a trend to decrease was observed), the phagocytic capacity of the cells was modified. Thus, rotenone treatment significantly increased the number of cells with low phagocytic capacity (<4 beads/cell) and decreased the number of cells with high phagocytic capacity (>4 beads/cell). This effect was observed both in the presence and in the absence of IL-4. Phagocytosis is a very energetically demanding process (21), and despite the increase in metabolic activity observed in microglial cell cultures, alterations in the mechanisms to obtain energy may be responsible for the impaired phagocytic activity observed.

In summary, the results obtained show that rotenone did not induce the expression of M2 markers in glial cells *per se*. However, rotenone dramatically inhibited the development of a M2 phenotype induced by IL-4 in mixed glial cell cultures. This effect may be due to the observed decrease in metabolic activity of mixed glial cell cultures treated with rotenone. On the contrary, rotenone did not modify IL-4 - induced M2 phenotype in microglial cell cultures, where rotenone did not decrease metabolic activity either. Indeed, the increase in metabolic activity observed in rotenone-treated microglial cells cultures suggests the existence of alternative mechanisms to obtain energy. Nevertheless, phagocytic activity was impaired by rotenone in microglial cells. Altogether, these results show that rotenone impairs the immune response of glial cells, suggesting that glial cell dysfunction could be involved in the development of neurodegeneration in PD.

6. Conclusions

The conclusions obtained from the present work are the following:

1. The **metabolic supply** is impaired in mixed glial cell cultures but not in microglial cell cultures in response to rotenone.

2. Microglial cells change their **morphology** to a more ameboid form in response to rotenone.

3. The **M2 phenotype** induced by IL-4 is drastically inhibited by rotenone in mixed glial cell cultures, and maintained in microglial cell cultures.

4. The **CD200-CD200R1 system** is altered in response to rotenone in mixed glial cell cultures.

5. **Phagocytic activity** is impaired in microglial cells in response to rotenone.

Supplementary Data

Supplementary Table 1: Antibodies used in Immunofluorescence

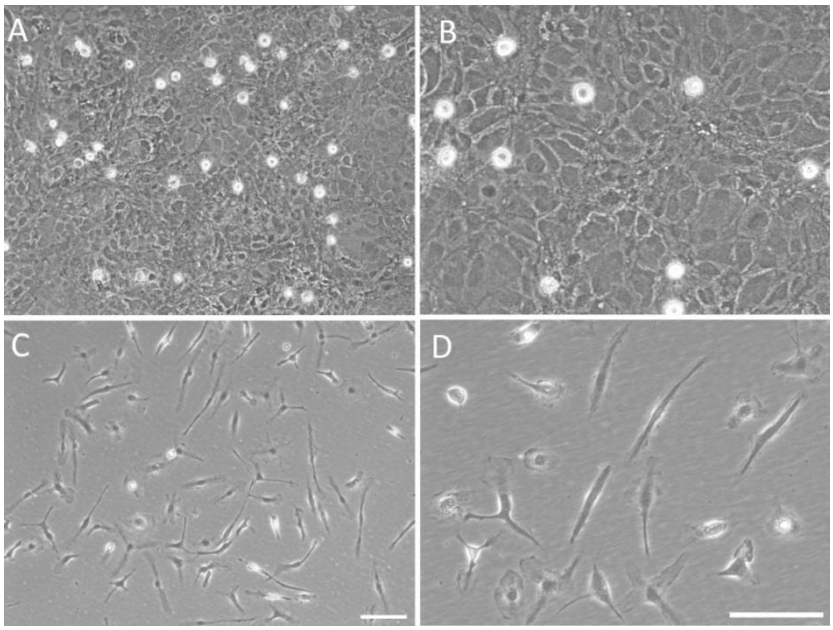
Immunofluorescence						
Primary antibody	**Species**	**Company**	**Dilution**	**Secondary antibody**	**Company**	**Dilution**
GFAP	Mouse	DAKO	1/3000	Alexa 488 donkey anti-mouse	Invitrogen™	1/1000
IBA-1	Rabbit	WAKO	1/500	Alexa 594 donkey anti-rabbit	Invitrogen™	1/1000
				Alexa 488 donkey anti-rabbit	Invitrogen™	1/1000
CD-68	Rat	AbDSerotec	1/1000	Alexa 594 donkey anti-rat	Invitrogen™	1/1000

Supplementary Table 2: Primers of the genes analysed by qRT-PCR

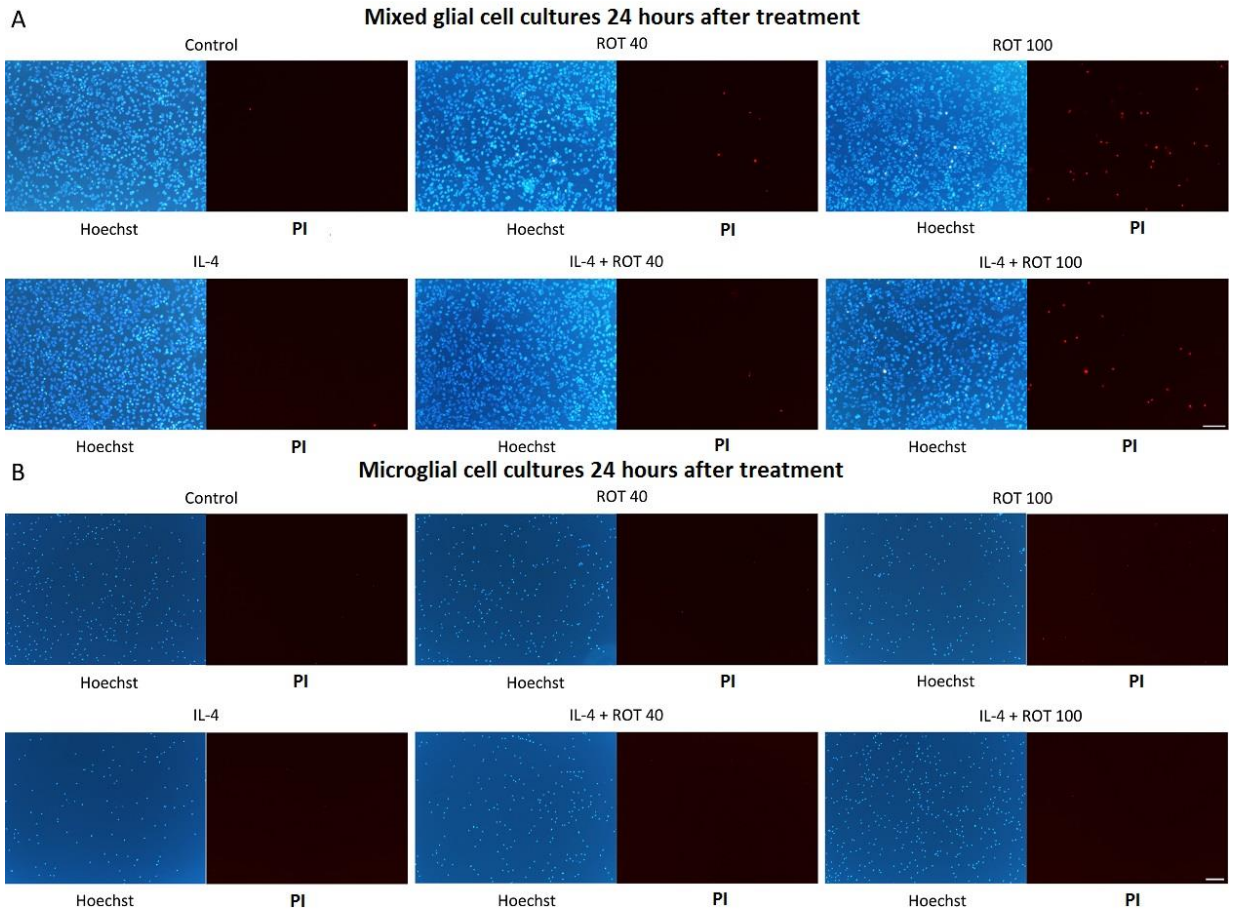
Murine			
Target mRNA	**Accession number**	**Forward primer (5'→3')**	**Reverse primer (5'→3')**
CD200Full	NM_010818.3	GGGCATGGCAGCAGTAGCG	TGTGCAGCGCCTTTCTTTC
CD200tr	#	GATGGGCAGTCTGTGGAAGTG	GAGAACATCGTAAGGATGCAGTTG
CD200R1	NM_021325.3	AGGAGGATGAAATGCAGCCTTA	TGCCTCCACCTTAGTCACAGTATC
Arginase	NM_007482.3	TTGCGAGACGTAGACCCTGG	CAAAGCTCAGGTGAATCGGC
TGF-β	NM_011577.1	TGCGCTTGCAGAGATTAAAA	AGCCCTGTATTCCGTCTCCT
CD206	NM_008625	TCTTTTACGACAAGTTGGGGTCAG	ATCATTCCGTTCACCAGAGGG
FIZZ-1	NM_020509.3	TCCCAGTGAATACTGATGAGA	CCACTCTGGATCTCCCAAGA
Ym-1	NM_009892.3	GGGCATACCTTTATCCTGAG	CCACTGAAGTCATCCATGTC
IL-10	NM_010548.2	TGAATTCCCTGGGTGAGAAG	ACACCTTGGTCTTGGAGCTT
IL-1βRa	NM_001159562.1	AGGCCCCACCACCAGCTTTGAGTC	TCACCCAGATGGCAGAGGCAACAA
Cox-2	NM_011198.3	TGCAGAATTGAAAGCCCTCT	CCCCAAAGATAGCATCTGGA
Actin	NM_007393.5	CAACGAGCGGTTCCGATG	GCCACAGGATTCTATACCCA
S18	NM_003286.2	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

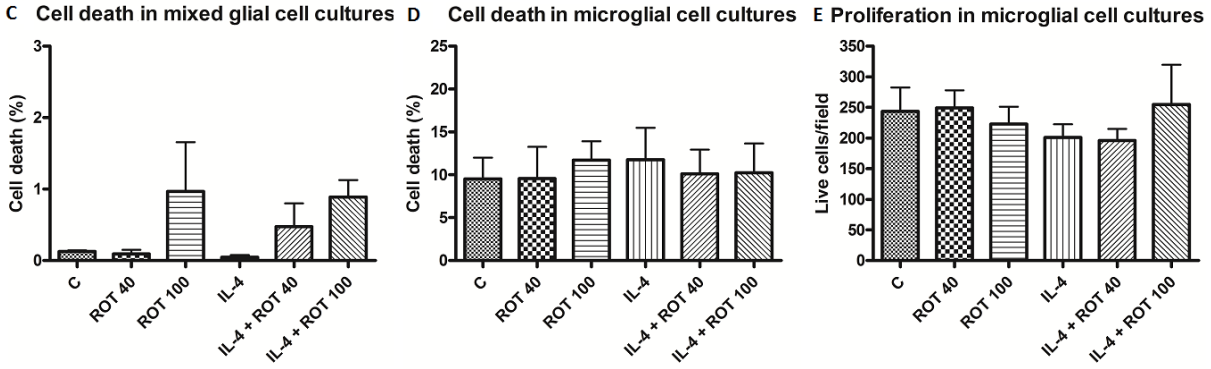
Supplementary Table 3: Antibodies used in Western blot assay

Western blot						
Primary antibody	**Species**	**Company**	**Dilution**	**Secondary antibody**	**Company**	**Dilution**
Arginase	Goat	Santa Cruz 18354	1/250	IgG-HRP mouse anti-goat/sheep	SIGMA-ALDRICH®	1/2000
CD206	Rabbit	Abcam	1/1000	IgG-HRP donkey anti-rabbit	GE	1/5000
Actin	Mouse	Sigma-Aldrich	1/40000	IgG-HRP goat anti-mouse	BIO-RAD	1/5000

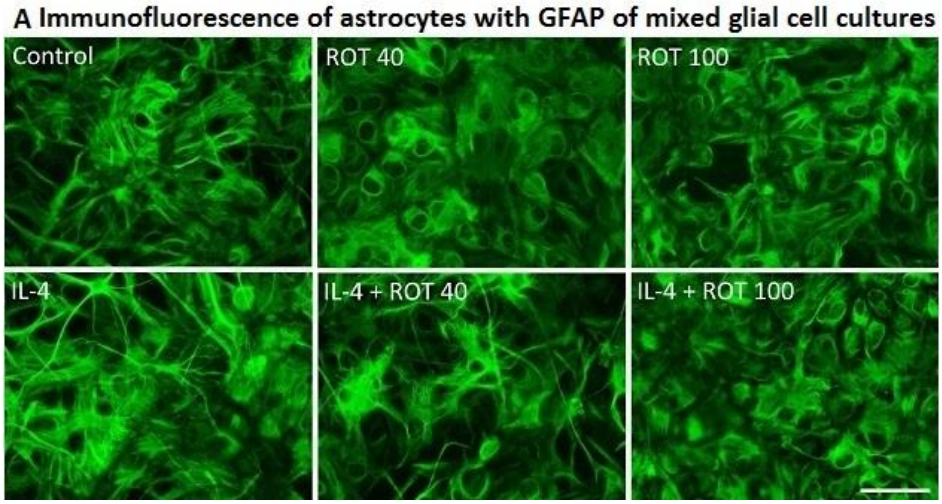


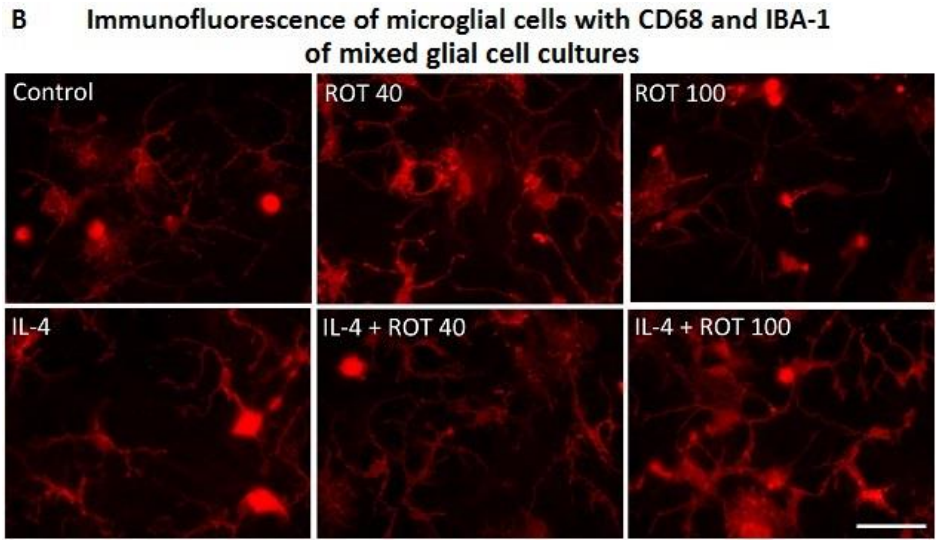
Supplementary Figure 1. Phase-contrast images of control glial cultures. (A) and **(B)** show mixed glial cell cultures. **(C)** and **(D)** show microglial cell cultures. Scale bars: 100 µm.



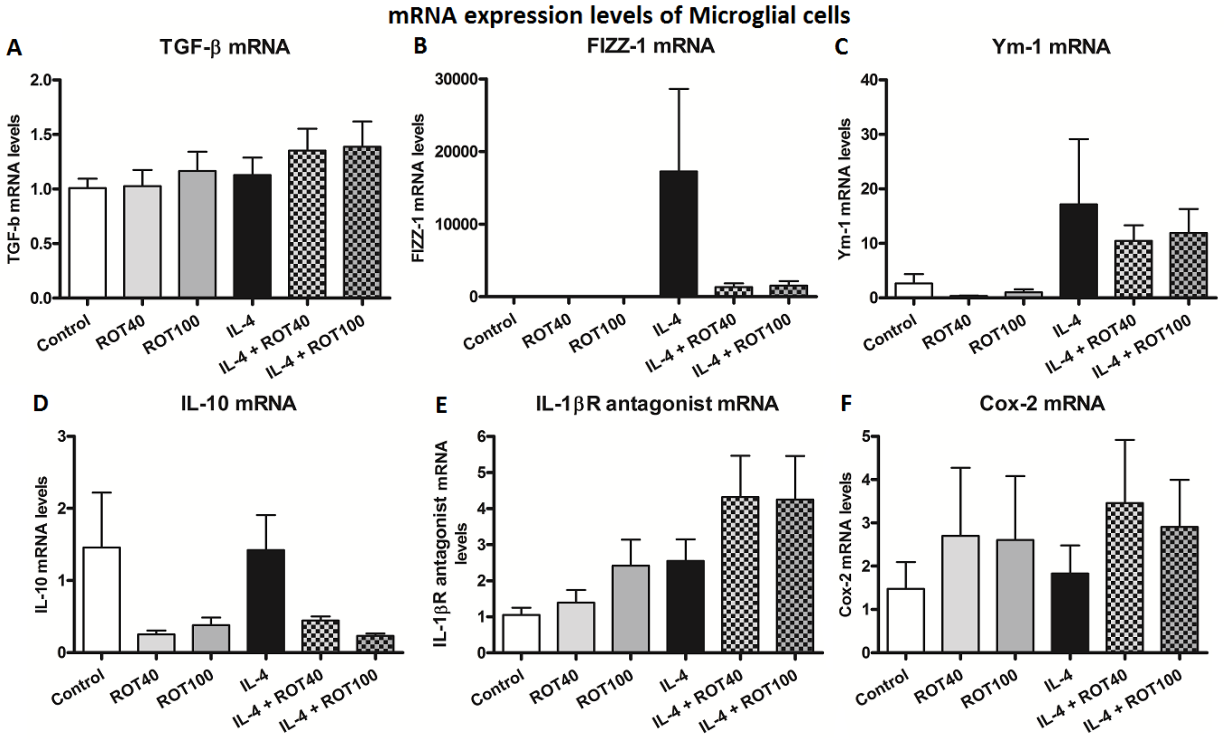


Supplementary Figure 2. Hoechst and PI staining in glial cell cultures in response to treatments. Images and percentage of cells of mixeld glial **(A, C)** and microglial cell cultures **(B, D)** showed no significant cell death due to rotenone, in absence and presence of IL-4. Scale bar: 100 µm and 200 µm respectively. Microglial cell cultures revealed no proliferation due to treatments **(E)**, represented as number of cells per field. Bars are means ± SEM of n=4 cell cultures.





Supplementary Figure 3. Images of the double immunostaining of mixed glial cell cultures treated with rotenone (ROT), in the absence and the presence of IL-4. Images of the astrocytes with immunofluorescence against GFAP (green) **(A)** and microglial cells with immunofluorescence against CD-68 and IBA-1 (red) **(B)** in mixed glial cell cultures. Scale bars: 100 µm.



Supplementary Figure 4. mRNA expression levels of M2 phenotype markers in microglial cell cultures exposed to rotenone (ROT) in the absence and the presence of IL-4.mRNA expression of TGF-β **(A)**, FIZZ-1 **(B)**, Ym-1 **(C)**, IL-10 **(D)**, IL-βRa **(E)**, and Cox-2 **(F)**. Bars are means ± SEM of n=4 cell cultures. Internal normalization controls: actin and S18.

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