Final Project

Master in Quantitative biotechnology

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Co-cultured neurons and microglia treated with neuromelanin as a model to study the events leading to protein aggregation in Parkinson's disease

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Abbreviations

α-Syn	α-synuclein
ATRA	**All-trans retinoic acid**
DMEM	**Dulbecco's Modified Eagle Medium (DMEM)**
DMSO	**Dimethyl sulfoxide**
DPBS	**Dulbecco's phosphate buffered saline**
DTT	**Dithiothreitol**
EDTA	**Ethylenediaminetetraacetic acid**
ELISA	**Enzyme-linked immunosorbent assay**
EMEM	**Eagle's minimal essential medium**
EGFP	**Enhanced Green Fluorescent Protein**
FBS	**Fetal bovine serum**
LB	**Lewy bodies**
mRFP	**monomeric Red fluorescent protein**
NM	**Neuromelanin**
PD	**Parkinson's disease**
PVDF	**Polyvinylidene difluoride**
RIPA	**Radioimmunoprecipitation assay buffer**
SDS	**Sodium dodecyl sulfate**
SDS-PAGE	**Sodium dodecyl sulphate-polyacrilamide gel electrophoresis**
SN	**Substantia nigra**
TBST	**Tris Buffer Saline Tween**
WB	**Western blot**

Abstract

Parkinson's disease is a common neurodegenerative disease that affects more than 10 million people worldwide which causes motor abnormalities such as tremor, muscle stiffness, paucity of voluntary movements and postural instability. It is mainly characterized by neural degeneration in the substantia nigra of the mid brain. Misfolding and aggregation of alpha-synuclein (aSyn) is one of the major molecular hallmarks of the disease but it is not clear which are the initial molecular and cellular mechanisms leading to protein aggregation, although cellular stress caused by oxidative damage with the involvement of neuromelanin and microglia-mediated inflammatory responses appear to be involved. No experimental model has been able so far to accurately replicate the situation within the human brain. In this work, we have used co-cultures of in vitro-differentiated dopaminergic neurons (SH-SY5Y) together with an immortalized human microglia cell line (HMC3), treated with synthetic neuromelanin, to approach the initial processes leading to aSyn aggregation using 2D models.

1. Introduction

1.1. Parkinson's disease

1.1.1. Prevalence and background

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by disabling motor abnormalities such as tremor, muscle stiffness, paucity of voluntary movements and postural instability 1. PD is a slow and progressive parkinsonian syndrome that begins insidiously and usually affects one part of the body before spreading to another one2. Actually, according to some research, more than 10 million of people are suffering this disease worldwide, with thousands of newly diagnosed and undiagnosed cases every day 3. The determination of regional loss of neurons in post-mortem tissue has been quite difficult in past years due to an inaccuracy of immunological techniques4. Nevertheless, current advances in immuno-cytochemical techniques allow the study of the events related to Parkinson´s disease4.

1.1.2. Events related to Parkinson's disease

PD affects dopaminergic neurons located in the substantia nigra of the brain, promoting their death[5]. Misfolding and aggregation of alpha-synuclein (aSyn) is one of the major molecular hallmarks of the disease[6] but it is not clear which are the initial molecular and cellular mechanisms leading to protein aggregation, although cellular stress caused by oxidative damage with the involvement of neuromelanin and microglia-mediated inflammatory responses appear to be involved[7]. It is also known that there is a selective degeneration of brain cells containing high concentrations of neuromelanin[8]. We will explain in depth each event related to Parkinson's disease throughout the project remarking the key ideas and the possible evolution of further investigations.

1.2. Lewy bodies.

Lewy bodies (LBs) have been reported as the main pathological hallmark of Parkinson's disease[9]. The protein α-synuclein (α-Syn) is one of the major component of LBs and plays an important role in the formation of Lewy pathologies, including Alzheimer's disease (AD) and Parkinson's disease (PD)[10]. Apart from α-Syn, LBs are also composed in a lower proportion by neurofilaments, ubiquitin and p62, a protein linked to ubiquitin[11].

The process by which Lewy pathology arises and their roles in neurodegeneration is still unknown. One of the strongest hypothesis is that intraneuronal α-Syn forms as abnormal oligomers, probably induced by extracellular pathogenic aggregates that are taken up and transformed into β-sheet rich amyloid fibrils, which are the basis of the LBs[12].

1.2.1. α-synuclein

α-Syn is a small protein (14kDa) encoded by the SNCA gene which is abundantly expressed in presynaptic terminals of the central nervous system (CNS)[13]. This protein is characterized by an amphipathic lysine-rich amino terminus, which has a critical a role modulating the interaction between membranes, a non-amyloid component (NAC) central region and a disordered acidic carboxylic-terminal tail involved in regulating interactions with metals, small molecules and proteins.

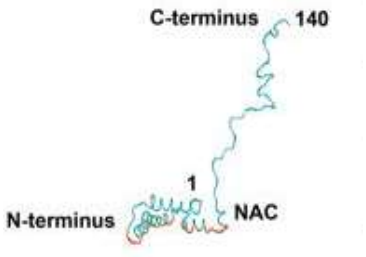

Figure 1. Biochemical structure of α-Synuclein[13].

Tipically, α-Syn exists in a dynamic equilibrium between unfolded monomers and α-helically-folded tetramers with a low propensity to aggregation[14]. The aggregation process of α-Syn involves a conformational change adopting a β-sheet-rich structure which facilitates its aggregation into oligomers, protofibrils and insoluble fibrils that end up accumulating in Lewy bodies[14]. It has been reported in recent studies that oligomers and protofibrils formed during the initial stages of the aggregation process are the potent neurotoxic species causing cell death in Parkinson's disease[15].

1.3. Neuromelanin

A progressive loss of neuromelanin (NM) in the substantia nigra (SN) of dopamine neurons has been associated with microgliosis and presence of extracellular NM[8]. Extracellular NM can activate microglia cells, promoting the production of superoxide, nitric oxide and pro-inflammatory factors such as IL-6 or TNF-α [8]. However, the main contribution of neuromelanin to PD pathogenesis remains unknown because, in contrast to humans, common laboratory animals lack this compound in a natural form and there are no accurate models to work with [7]. Neuromelanin can be isolated from human substantia nigra[16], with ethical/political concerns, or can be synthesized *in-vitro*[17] as we are going to prepare throughout this project.

Neuromelanin is a black pigment that is formed mainly by dopamine and L-cysteine co-polymerization which is accumulated in aging neuronal tissue [17].

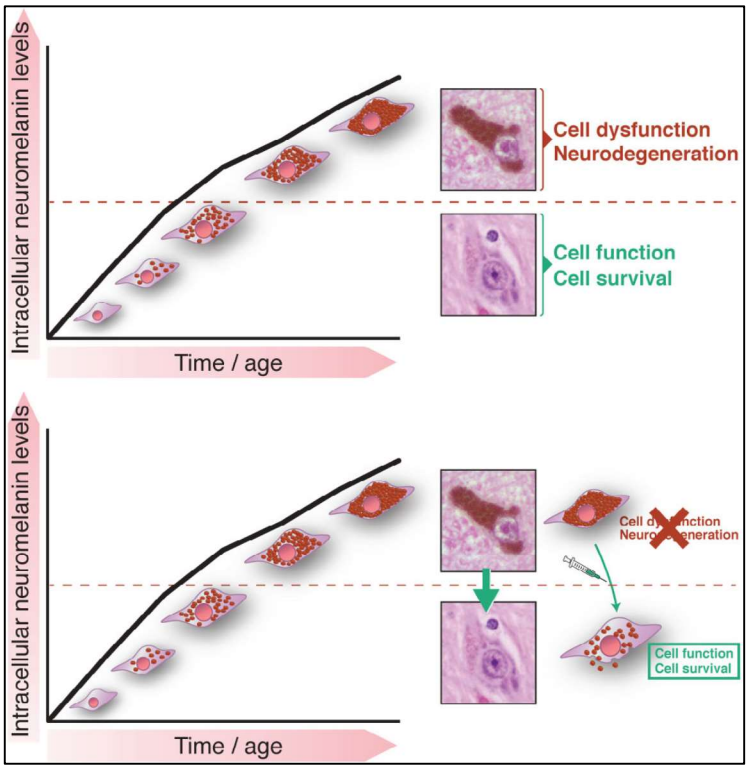

Figure 2. Hypothetical threshold of intracellular neuromelanin accumulation. Upper panel: cell dysfunction, neurodegeneration. Lower panel: rejuvenation strategies consisting on reducing neuromelanin levels. Taken from Vila (2019) [7].

Neuromelanin accumulation is represented in figure 2 in human tyrosinase (hTyr)-overexpressing rodents, remarking a red dotted line in which cell dysfunction and neurodegeneration starts [7]. In the lower panel, "rejuvenation strategies" are shown, which consist on the reduction of intracellular neuromelanin levels in order to prevent, delay cell dysfunction or neurodegeneration involved in Parkinson's disease and brain aging [7]. Moreover, neuromelanin is able to induce the activation of microglia cells causing them to produce an release superoxide, hydrogen peroxide, nitric oxide and pro-inflammatory factors [18].

1.4. Co-culture

Co-culture techniques consist on culturing two or more different types of cells together in the same culture device. This technique has been used over time for studying the interactions between cell populations, cell-cell interactions or for creating experimental models which can recreate natural systems as artificial tissues[19]. In addition, there are many biological systems that are developed with industrial and medical applications in mind involving co-culture methodologies such as tumor targeting, therapeutic delivery or bacterial cells engineered for killing pathogens[19].

Throughout the project, we will study the cell morphology, migration, intercellular contacts and α-synuclein aggregation in individual cell cultures o in co-cultures involving neurons (SH-SY5Y) and microglia (HMC3).

1.4.1. Microglia cells

Microglia are myeloid cells located in the brain parenchyma in charge of immunological response in the nervous sytem, regulation of synaptic architecture or neurogenesis that are related to several neurological disorders [18]. The use of a microglia cell line could accelerate many research programmes and reduce the large numbers of animals used for continuous cell preparations and animal experimentation[20]. It has been reported that microglia cells react to Lipopolysaccharide (LPS) stimulating immune responses by interacting with the membrane receptor CD14 to induce the generation of cytokines such as tumor necrosis factor (TNF)-α, interleukin 1 (IL-1), and IL-6[21]. Apart from LPS, the exposure to amyloid ß-peptides 25-35 triggered the secretion of IL-8 and TNF-α proteins[22], and the presence of certain cytokines as IL-1β and IFN-γ can promote the secretion of IL-6 into the medium[23].

1.4.2. SH-SY5Y cell line

Typically, SH-SY5Y cells have been employed to study the molecular and cellular mechanisms underlying the effects of some of the Parkinson's disease (PD)-related toxins, to perform functional studies on familial PD genes, and to test neuroprotective compounds for PD treatment[24]. Nevertheless, this cell line is obtained as a neuroblastoma derivative and is not purely dopaminergic so physiological characteristics can differ from those of dopaminergic neurons[24]. SH-SY5Y cells can be differentiated into dopaminergic neurons stressing cells using all-trans retinoic acid (ATRA) in low serum media for 7-10 days[25].

1.4.3. 2D and 3D models

Currently, an improvement of the models to study neurodegeneration issues is necessary. Traditionally, mixed glial cell cultures in 2 dimensions (Petri dish) have been used in order to investigate this type of events [26]. Although flat, 2 dimensional (2D) cell cultures have predominated so far and a lot of valuable information has been obtained from them, recent research has been changing toward the use of three-dimensional (3D) cell cultures due to a more realistic biochemical and biomechanical microenvironment[27], because cells on a rigid plastic (2D) are in an abnormal environment that can affect their properties such as morphology, function and reactivity [26].

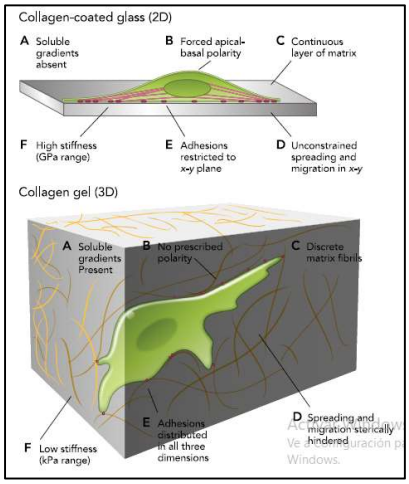

Figure 3. Microenvironment differences culturing cells in 2D and 3D. Mechanical and chemical environment culturing in 2D (upper panel) and culturing in 3D (lower panel). Taken from Duval et al (2017) [27]

Figure 3 shows that cells in 2D and 3D interact differently with their environment due to differences in the mechanical or chemical cues that they experience.

Some differences in cell shape, proliferation, motility, or activation markers for microglia cells when the type of culture is modified have been reported, as it is observed in figure 4 [26].

Phenotype	Microglia (in vivo)	Microglia (2D)	Microglia (3D)
Cell shape	Small cell bodies with complex, fine ramifications	Amoeboid	Increased ramification
Proliferation	Mostly only under pathological conditions and tissue trauma	Cells proliferate in 2D culture	Proliferation generally reduced compared to 2D
Motility	Cells migrate mostly only under pathological conditions and tissue trauma; cell ramifications are motile	Cells show some basal motility in 2D culture	Comprehensive data not yet available
Activation markers	Proinflammatory genes increased under pathological conditions and tissue trauma	Increased basal expression in some immune and motility genes	Comprehensive data not yet available

Figure 4. Summary of the major phenotypic differences between microglia cells when are *in vivo* and cultured in 2D and 3D[26].

Even though 3D cell cultures are a more realistic approximation to *in vivo* conditions, too many challenges remain such as including the tissue-tissue surface, the mechanical microenvironment and the spatiotemporal distribution of oxygen, nutrients, and metabolic waste [27].

1.5. Caco-2 cell line

Although our major objectives in this work were focussed on the establishment of HMC3 and SH-SY5Y co-cultures as a novel model, since the HMC3 cells took much longer that expected to arrive to the lab, we started working in another project related to the transmission of misfolded proteins from the gut to the nervous system. For this project, we used the Caco-2 cell line.

The human intestinal Caco-2 cell line has been used over the last twenty years as a model for studying the intestinal barrier[28]. It is a human colon adenocarcinoma cell line that differentiates to form a confluent epithelial cell monolayer consisting of columnar and polarized cells, resembling the small intestine inner cell layer[28].

One idea was to study the transmission of α-synuclein from gut to the brain using transwell cultures, also known as inserts. Accumulation and aggregation of α-synuclein could be spread from the gastrointestinal tract to the brain via the vagus nerve as it has been reported[29]. Besides it, colonic injection of preformed fibrils (PFFs) can induce PD pathologies, motor dysfunctions and cognitive impairment, being more neurotoxic, compact, and aggregating more quickly along the vagus nerve than the individual fragments' fibrils[29].

Transwell cultures can be designed in two different ways, culturing cells in contact and without contact, depending on the information seeked, as it is shown in figure 5[30]:

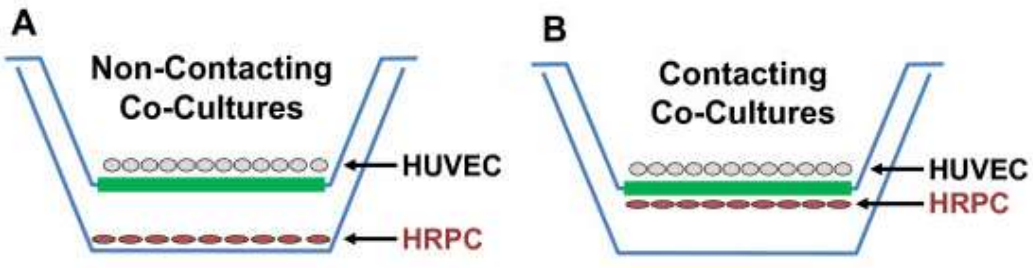

Figure 5. Scheme of two different modes of transwell co-culture system. A) Non-contact: Human retinal progenitor cells (HRPC) and human umbilical vein endothelial cells (HUVEC) cells co-cultured in two different compartments (insert membrane and well); cells may communicate through the pore of the membrane. B) Contact: both cells may be cultured on the insert membrane thus allowing extensive and direct cell-cell interactions. Taken from Kumar et Al (2012) [31]

2. Hypothesis and specific aims

Since no experimental model has been able so far to accurately replicate the situation within the human brain, in an attempt to get closer to that objective we have considered the following aims:

- Use of co-cultures of dopaminergic neurons (SH-S5Y5) together with an immortalized human microglia cell line (HMC3) to approach the initial processes leading to aSyn aggregation.

- Determine cell morphology, migration, intercellular contacts and possible α-Syn aggregation in the co-colture by fluorescence microscopy using fluorescently labeled α-Syn to get more knowledge on the usefulness of SH-S5Y5 and HMC3 cells interaction as a model for Parkinson's disease.

- Synthesize *in-vitro* neuromelanin and analyze its potential capacity to activate microglia cells and its neural toxicity in our model.

- Induce inflammation in HMC3 cells using a combination of cytokines such as IL-1β and IFN-γ, comparing the results with the treatment using synthesized neuromelanin.

- Perform a preliminary study of Caco-2 cells viability for further experiments involving transwell cultures.

3. Materials and methods

3.1. Cell culture

3.1.1.Cell lines

All experiments related to cell culture were carried out in a laminar flow cabinet in sterile conditions. Different adherent cell lines were used throughout this project:

- **SH-SY5Y**: it is a cell line which is often used as an in vitro model of neuronal function and differentiation. It is a sub-clone of the original cell line SK-N-SH, which was isolated from a four-year-old female with neuroblastoma.
- **SH-SY5Y-SNCA**: a SH-SY5Y cell line harboring SNCA cDNA cassette that allows for induction of controlled α-synuclein expression.
- **Caco-2**: a human colon adenocarcinoma cell line that differentiates to form a confluent epithelial cell monolayer consisting of columnar and polarized cells, resembling the small intestine inner cell layer.
- **HMC3**: a human microglia cell line which was established through SV-40 immortalization of human microglial cells in 1995.

3.1.2. Maintenance of cultured cells

Cells were grown in T25 and T75 flasks inside an incubator at 37˚C, in a 5% CO2 atmosphere and were passaged before confluence was reached. While SH-SY5Y and Caco-2 cell lines were grown in DMEM medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM), HMC3 cells were grown in EMEM medium, supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL) and L-glutamine (2 mM).

3.1.3. Passaging cells

Cell passaging was carried out washing the cells with DPBS 1X after removing the medium, adding 3 or 5 mL of trypsin depending on the T flask size for 5 minutes in order to detach the cells. Then, supplemented medium was added to inactivate trypsin and cells were centrifuged for 5 minutes at 500*g*, before resuspension in fresh media and plating.

3.1.4. Cryopreservation of cell lines

Different cell lines were treated with dimethyl sulfoxide (DMSO) as a cryoprotectant. Cell lines were stored containing supplemented medium + 10% DMSO in Mr. Frosty freezing container at -80˚C during 24h. Then, cells were kept at -196˚C in liquid nitrogen until needed. Mr. Frosty freezing container is a system able to cool the cells with a rate of -1˚C/minute, the optimal rate for cell preservation. When needed, cells were thawed by rapid incubation in a 37˚C water bath.

3.2. Synthesis of neuromelanin

Neuromelanin was synthesized from dopamine and L-cysteine in a stoichiometric relation 6:1, respectively, incubated for 72h at 37ºC and pH 7.4 in a phosphate buffer. After mixing 1.5 g of dopamine with 232 mg of L-cysteine and incubation with agitation, a black solid product was obtained.

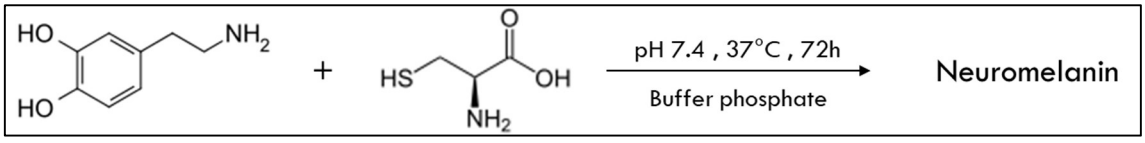

Figure 6. Synthesis of *in-vitro* neuromelanin. Dopamine and L-cysteine in a stoichiometric relation 6:1 are transformed into neuromelanin after incubating 3 days, at 37ºC, pH 7.4 in a phosphate buffer. [32]

The black solid product was centrifuged at 16.*000 x g i*n a JA 10 rotor at 4˚C for 60 minutes, the pellet was washed with 1% acetic acid once and then with distilled water

twice and stored at 4°C. The black solid product was weighed in an analytical balance after drying at room temperature for 96 hours in order to quantify the neuromelanin. Finally, 10mL of neuromelanin solved in water at a concentration of 17 mg/mL was obtained.

3.3. Staining cells

Different stains were used to visualize cell organelles using a fluorescence microscope. Cells were incubated at 37°C and 5% CO_2 for 30 min in Hank´s balanced salt solution (HBSS) using different cell-dyes at specific concentration. Then, medium was washed several times using phosphate buffer, DPBS 1X, to avoid background signals when taking photos with a Leica DMI6000B fluorescence microscope.

3.4. SH-SY5Y differentiation to neurons

Differentiation of SH-SY5Y cell line to neurons was carried out seeding cells at a density of $1.25 \cdot 10^4$ cells/cm^2 in multiwell plates for 24 hours until cells were attached to the surface. The amount of FBS in the medium was decreased from 10% to 5% and all-trans retinoic acid (ATRA) at 30 µM was added when cells were attached in order to induce differentiation. Then, cells were incubated at 37°C in a 5% CO_2 atmosphere for 7-10 days.

3.5. Cell transfection

Gene Juice (Novagen) and Lipofectamine 3000 (Invitrogen) were used as transfection reagents for introducing exogenous DNA into different cells. Transfections were carried out in multiwell plates, seeding cells 24 hours before being transfected.

- Gene Juice is a non-lipid based chemical transfection reagent optimized for maximum transfection efficiency, ease-of-use, and minimal cytotoxicity on a wide variety of mammalian cells. It was used at a plasmid DNA / Gene Juice ratio of 1:3 (0,5µg of DNA).

- Lipofectamine 3000 uses most advanced lipid nanoparticle technology to provide superior transfection performance with improved application outcomes and reproducible results. In our case, it was used at a plasmid DNA / Lipofectamine 3000 reagent ratio of 1.5:1 and 3:1 (0,25µg or 0,50µg of DNA).

The suitability of each transfection reagent for each cell line was analyzed.

3.6. Western Blot

3.6.1. Protein extraction

In order to analyze protein expression, cells were detached from the surface using trypsin for 5 minutes. Trypsin was inactivated using complete medium and the cell suspension was centrifuged for 5 minute at 500*g*. The pellet was washed with DPBS 1X twice and resuspended in 50 µL of lysis buffer RIPA (150 mM NaCl, 50 mM Tris-HCl pH 8, 1% Tritón X-100, 0,5% sodium deoxycholate, 0,1% SDS) and protease inhibitors. Samples were incubated 30 minutes in an ice bath and centrifuged again for 30 minutes, 18.000g and 4°C. The supernatant was collected (50 µL) and 50 µL of SDS-PAGE loading buffer 2X (0,125 M Tris pH 6.8, 4% SDS, 20% glycerol, 0,04 mg/mL bromophenol blue, 75 mg/mL DTT) was added. Then, samples were boiled at 100 ºC for 5 minutes and loaded in an electrophoresis gel or stored at -80 ºC until used.

3.6.2. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE)

The running and the stacking phases of the gel were prepared at concentrations of polyacrylamide of 10% and 4%, respectively. Electrophoresis was carried out in electrophoresis buffer (0,38 M Glycine, 25 mM Tris-Base, 1 g/L SDS, pH 8,3) at 100V for the first 30 minutes and then the voltage was increased at 120V until the smaller proteins have migrated to the bottom of the gel. PageRuler™ Prestained Protein Ladder 10-180 kDa (Thermo Scientific) was used as a molecular mass marker.

3.6.3 Blotting

When electrophoresis had finished, transfer of proteins from the gel to a membrane was carried out. PVDF membranes (Inmobilon-P, Merck) activated with methanol were used for transferring proteins using a transblot, at 4°C with a constant amperage of 200 mA for 90 minutes in blotting buffer (0,38M glycine, 25mM Tris-Base, 20% methanol).

3.6.4. Incubation with antibodies and visualization.

Before incubating the membrane with antibodies, unspecific sites were blocked with a 1% skimmed milk solution in TBST 1X (20 mM Tris-Base, 150 mM NaCl, 0,1% Tween-20, pH 7,6. Then, membranes were incubated with the primary antibody at the appropriate dilution (table 1) at 4°C overnight. Later, they were washed for 5 minutes with TBST 1X three times. Afterwards, unspecific sites were blocked again using 1% casein solution and membrane was incubated with secondary antibodies for at least 1h. The membrane was washed with TBST 1X three times before developing using enhanced chemiluminescence (ECL) by addition of luminol and H_2O_2 (Quimioluminiscence SuperSignal™ West Pico Plus kit (Thermo Scientific) to the membrane at a ratio 1:1 and incubating for 5 minutes. A ChemiDoc apparatus (Bio-Rad) was used to visualize the results.

Table 1.Primary and secondary antibodies used to detect proteins by western blot

Primary antibody	Reference	Host	Dilution
α-synuclein	ab138501	Rabbit	1:10.000
Anti-GAPDH	60004-1-Ig	Mouse	1:50.000

Secondary antibody	Reference	Host	Dilution
Goat Anti-Rabbit IgG	AP307P	Goat	1:10.000
Rabbit Anti-mouse IgG	AP160-KC	Rabbit	1:10.000

3.7. Enzyme-Linked ImmunoSorbent Assay (ELISA)

Human IL-6 Uncoated ELISA from Invitrogen was used for quantitative detection of Human IL-6. First of all, the required amount of wells in a Corning™ Costar™ 9018 ELISA plate were coated with capture antibody in PBS 1X and incubated overnight at 4°C. After that, wells were washed 3 times with PBS allowing time for soaking (1 minute) during each wash step. Then, wells were blocked with ELISA/ELISPOT Diluent (1X) and incubated at room temperature for 1 hour. After washing once with PBS, a standard curve for a total of 8 points was prepared performing 2-fold serial dilutions of the top standards. At the same time, samples were added to their corresponding wells (including control and blank wells). The plate was incubated at room temperature for 2 hours or overnight at 4°C for maximum sensitivity. Afterwards, wells were washed 3 times and the plate was incubated for 1 hour with detection antibody. Again, wells were washed 3 times and Streptavidin-HRP solution was added and incubated at room temperature for 30 minutes. After washing 5 times and soaking for 1 to 2 minutes, the wells were incubated for 15 minutes with TMB (3,3', 5,5;-tetramethylbenzidine) solution. H_2SO_4 2N was used to stop the reaction. Absorbance was read at 450nm and 570nm in a microplate reader (Fludia T70). The values from 570nm were substracted from those of 450nm.

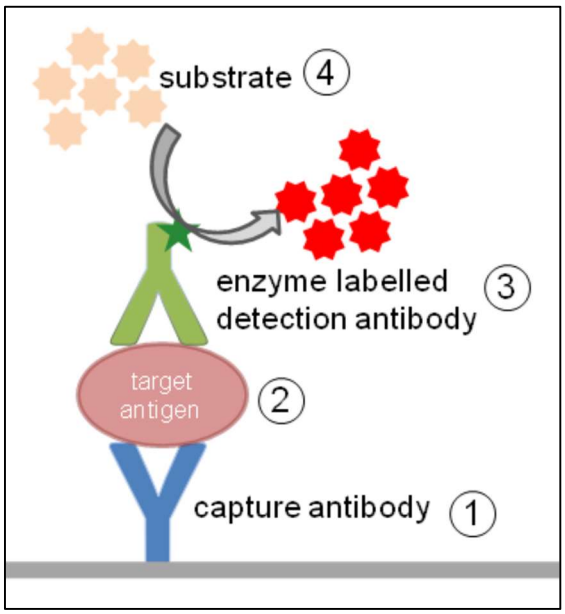

Figure 7. Representative scheme of Enzyme-linked immunosorbent assay (ELISA). Taken from Claire Horlock, Imperial College London, UK.

3.8. Co-culturing HMC3 and SH-SY5Y cells

Method 1: HMC3 and SH-SY5Y cells were seeded at 1,25x10^4 cells/cm^2 separately in 12- or 24-well plates in DMEM medium. After 24 hours, cells were incubated at 37˚C and 5% CO_2 during 30 min in Hank´s balanced salt solution (HBSS) using suitable cell-dyes at specific concentrations (Mitotracker-CMXROS or Hoechst in the case of HMC3 cells and Calcein AM for SH-SY5Y cells). Then, HMC3 and SH-SY5Y cells media were washed for 30 minutes twice using a phosphate buffer, DPBS 1X. After staining, HMC3 wells were trypsinized to detach them from the surface and centrifuged for 5 minutes at 500 x *g*. Prior to co-culture, HMC3 cells were washed once with DPBS 1X to eliminate completely possible traces of trypsin and resuspended in 1mL of DMEM. Finally, SH-SY5Y cells medium was removed and HMC3 cells were plated on top of SH-SY5Y ones.

Mehtod 2: HMC3 and SH-SY5Y-SNCA cells were initially seeded and stained as in Method 1. Afterwards, SH-SY5Y-SNCA cells were transfected with plasmid pJAC332 (EGFP,α-Syn) using Lipofectamine 3000 at a plasmid DNA / Lipofectamine ratio of 1.5:1. After 24 hours, SH-SY5Y-SNCA cells were trypsinized and centrifuged for 5 minutes at 500 x *g*. The co-culture was then established as in Method 1.

3.9. Microscopy

Visualization of cells, transfections and other treatments were carried out using fluorescence microscope Leica DMI600B. For acquiring pictures, bright field and fluorescence filters (shown in table 2) were used. In experiments *in vivo* (Time lapse microscopy) pictures were taken every 15 minutes for 24 hours using bright field wavelength at different Z planes (±9 µm respect to center).

Table 2. Filters in fluorescence microscope.

FILTER	EXCITATION	DICROICO	EMISSION
A4	BP 360/40	400	BP 470/40
GFP	BP 470/40	500	BP 525/50
TX2	BP 560/40	595	BP 645/75
Y5	BP 620/60	660	BP 700/75

4. Results

4.1. Staining Caco-2 cells

Before starting the major experiments, Caco-2 cells were used to learn basic methodologies in cell culture laboratories and to optimize the conditions for future experiments.

Information about Caco-2 cells staining will be really useful in further experiments involving the co-culture of HMC3 and SH-SY5Y cell lines because they will be stained individually previous to co-culturing. Since the morphologies of HMC3 and SH-SY5Y cell lines are quite similar, fibroblasts, they should be stained individually before plating them together in order to distinguish them.

Caco-2 cells were seeded at a density of $4x10^4$ cells per well in 24 well plates and they were stained with different cell-dyes at the following concentrations:

Table 3. Cell-dye concentrations for staining Caco-2 cells

Cell dye	Stock concentration	Working concentration
Calceina AM	1mM	5µM
Cell mask	5mg/mL	5µg/mL
ER tracker	10µM	0,5µM
Mitotracker-RED	1mM	250nM
Mitotracker-GREEN	100µM	100nM
Mitrotracker-CMXROS	1mM	250nM
DAPI	2mg/mL	10µg/mL

After incubating for 30 minutes in Hank's balanced salt solution (HBSS), pictures were taken using the fluorescence microscope, obtaining the results shown in figure 8.

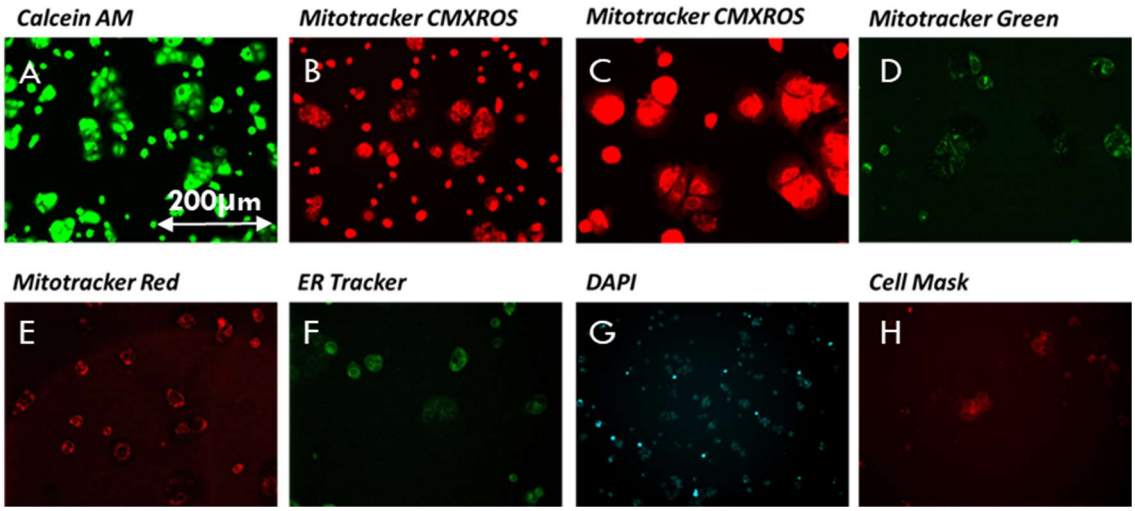

Figure 8. Staining Caco-2 cells using different cell-dyes. A) Stained using Calcein AM. B) and C) stained with Mitotracker-CMXROS. D) stained using Mitotracker Green. E) Stained with Mitotracker-Red. F) Stained using ER Tracker. G) Stained with DAPI. H) Stained using Cell Mask.

Looking at the pictures, it is possible to say that Calcein AM and Mitotracker-CMXROS could be a good option to stain Caco-2 cells but they should be used at lower concentrations or adjusting gain/exposure in the microscope because the signal is too saturated. Calcein AM is a cell-permeant dye that can be used to determine cell viability in most eukaryotic cells. In live cells the nonfluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. MitoTracker CMXROS is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent on membrane potential. The results for Mitotracker-RED and Mitotracker-Green show that they could be a good option to appreciate dotted mitochondria in cells but there is too much background in the images so they were discarded. Same happens with Cell Mask, ER tracker and DAPI, results are not good enough to follow staining experiments at those concentrations/conditions.

4.2. Viability of Caco-2 cells

Viability of Caco-2 cells stained with different selected cell dyes was studied in order to obtain information about the best cell-dye in terms of mortality and efficiency. Apart from studying the viability of Caco-2 cells stained with Calcein AM and Mitrotracker-CMXROS, Hoechst 33342 was also used, a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. Cells were seeded at a concentration of $1x10^5$ cells/mL in 6 well plates and were detached from the surface using trypsin to count the number of cells using a Neubauer chamber. Results are shown in figure 9.

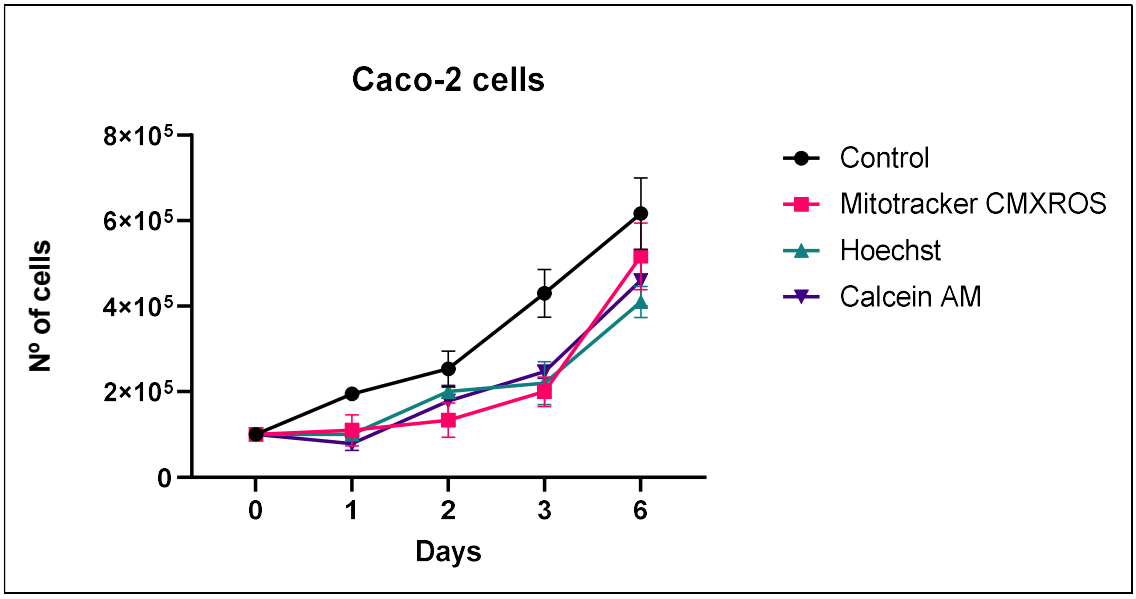

Figure 9. Proliferation of Caco-2 cells stained with different cell-dyes (error bars represent SD, n=3).

As a result, it is observed that Caco-2 cells stained with Mitrotracker-CMXROS, Hoechst 33342 and Calcein AM continue growing over time in a similar way. To confirm this, an ANOVA test was performed, a statistical analysis which measures if there is a significant difference between treatments. In this case, no significant differences were obtained among different cell-dyes. Nevertheless, there are significant differences

among the growth rate of stained cells and the control, stained cells proliferation is lower than cells without treatment. In this experiment, the concentration of Mitotracker-CMXROS and Calcein AM used for the viability study was reduced 10 and 5 times, respectively, compared to previous staining. Apart from these dyes, Hoechst 33342 was also used to specifically stain the nuclei of living or fixed cells and tissues.

Table 4. Cell-dye concentrations for staining Caco-2 cells.

Cell dye	Stock concentration	Working concentration
Calcein AM	1mM	1µM
Mitrotracker-CMXROS	1mM	25nM
Hoescht 33342	10 mg/mL	250 ng/mL

Pictures of cells were taken every 24 hours in order to evaluate for how long the staining remains. Results are shown in figure 10.

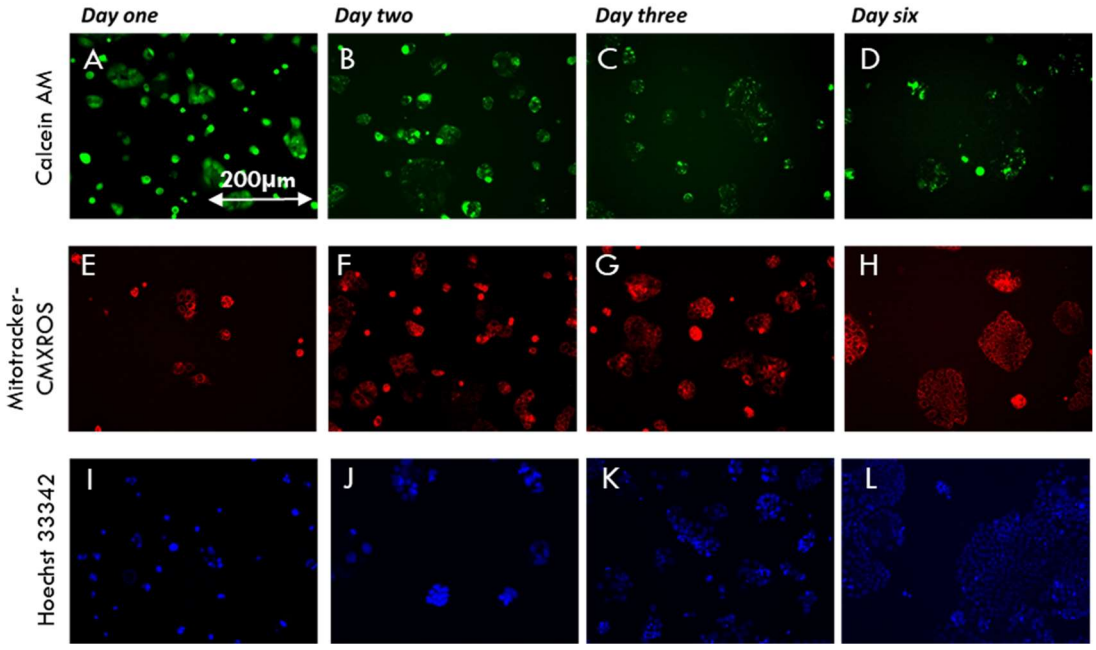

Figure 10. Evolution of Caco-2 cells staining over the time. Pictures A-D correspond to cells stained using Calcein AM after 24h, 48h, 72h and 144h respectively. E-H correspond to cells stained with Mitotracker-CMXROS after 24h, 48h, 72h and 144h respectively. I-L correspond to cell stained using Hoechst 3342 after 24h, 48h, 72h and 144h respectively.

The results indicate that Calcein AM, Mitotracker CMXROS and Hoechst are good options to stain Caco-2 cells due to low mortality and the staining preservation over time. After some days, cells should be washed with DPBS 1X to reduce background because dead cells could release traces of the cell-dye to the medium.

4.3. Viability of HMC3 cells in different media

Before co-culturing HMC3 and SH-SY5Y cell lines, it is necessary to determine if both cell lines can grow adequately in the same medium. SH-SY5Y cells are recommended to be grown in DMEM (Dulbecco's Modified Eagle's Medium) whereas EMEM (Eagle's minimal essential medium) is the best option for HMC3 cells. For that reason, a proliferation study comparing these two different media was carried out. Cells were seeded at a concentration of $5x10^4$ cells/mL in 6 well plates and were detached from the surface using trypsin. Cells were counted at different times to determine their proliferation, obtaining the results illustrated in figure 11.

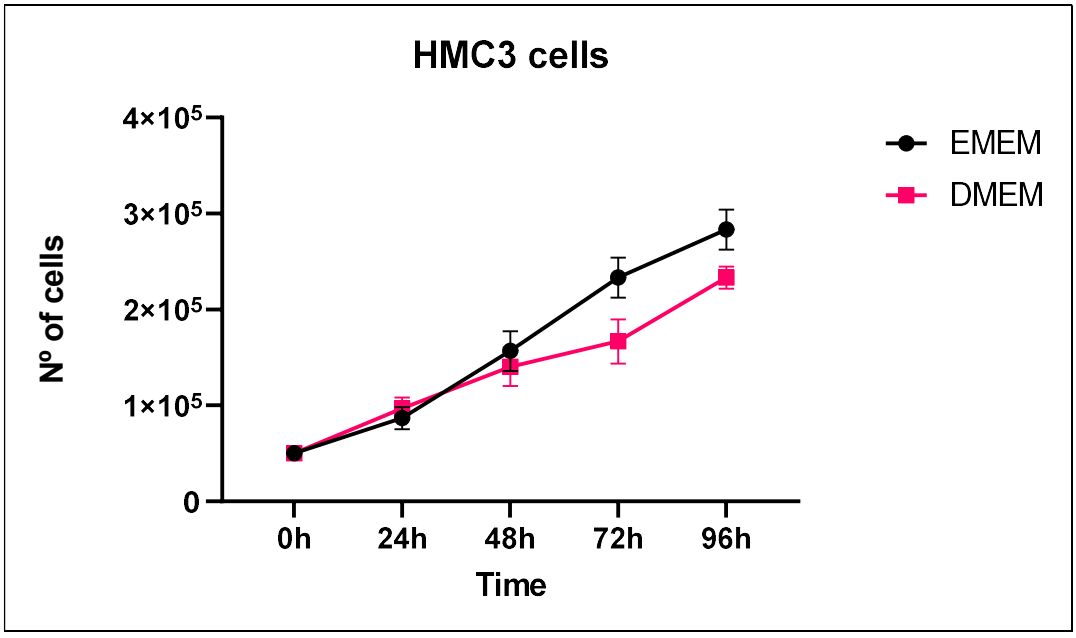

Figure 11. Proliferation of HMC3 cells grown in different media (EMEM and DMEM).

At first sight, it seems that HMC3 cells grow a little better in EMEM than in DMEM. Nevertheless, it should be analysed if differences are significant or not from a statistical point of view. For that purpose, an independent *t test* was carried out, a statistical procedure that compares the averages/means of two independent or unrelated groups to determine if there is a significant difference between them. As a result, it was obtained that there was no significant difference in using EMEM or DMEM media for growing HMC3 cells.

4.4. Viability and morphology of HMC3 cells with different stains

Since the morphologies of HMC3 and undifferentiated SH-SY5Y cell lines are quite similar, both have a fibroblast morphology, distinction in the co-culture could be a bit complicated. For that reason, it is necessary to stain different cell lines individually with a suitable cell dye previous to co-culturing.

Taking into account previous data of Caco-2 cells staining, Calcein AM, Mitrotracker-CMXROS and Hoechst cell dyes were used to obtain information about viability and morphology of HMC3 cells.

Table 5. Cell-dye concentrations for staining HMC3 cells

Cell dye	Stock concentration	Working concentration
Calceina AM	1mM	1µM
Mitrotracker-CMXROS	1mM	25nM
Hoescht	10 mg/mL	250 ng/mL

Cells were seeded at a concentration of $5x10^4$ cells/mL in 6 well plates, detached and counted at different times. Results are shown in figure 12.

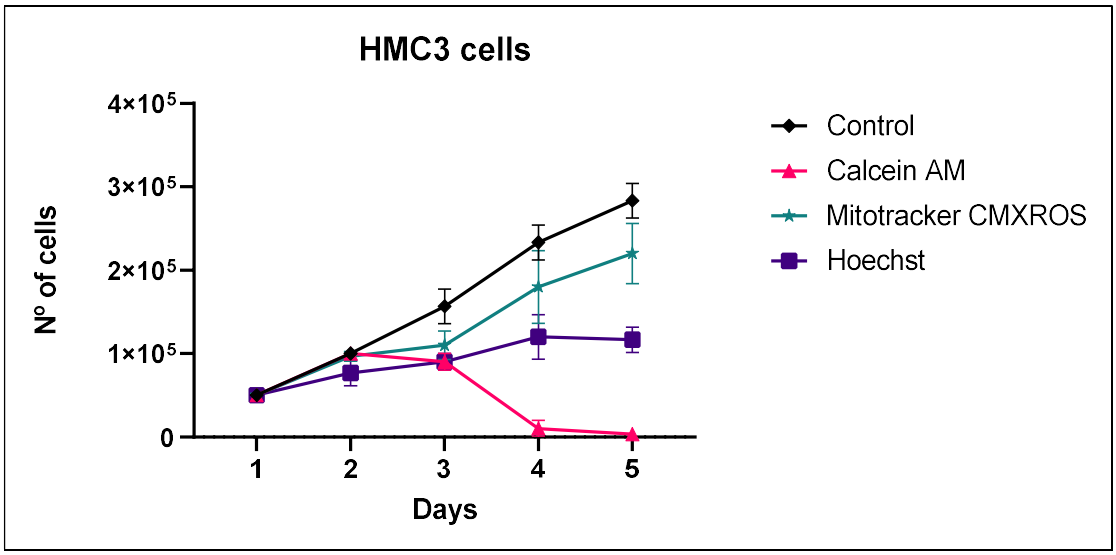

Figure 12. Proliferation of HMC3 cells stained with different dyes.

The results indicate that Mitotracker-CMXROS seems to be the best cell-dye in terms of cell viability whereas Calcein AM it is not a good option because it causes cell death after 2-3 days. Apart from viability results, fluorescence microscopy pictures were taken at different times to verify that cells are still stained after some days. It is possible to observe that cell staining remains over time in the case of Mitotracker-CMXROS and Hoechst and how cells stained with Calcein AM dye after few days. Results are shown in figure 13:

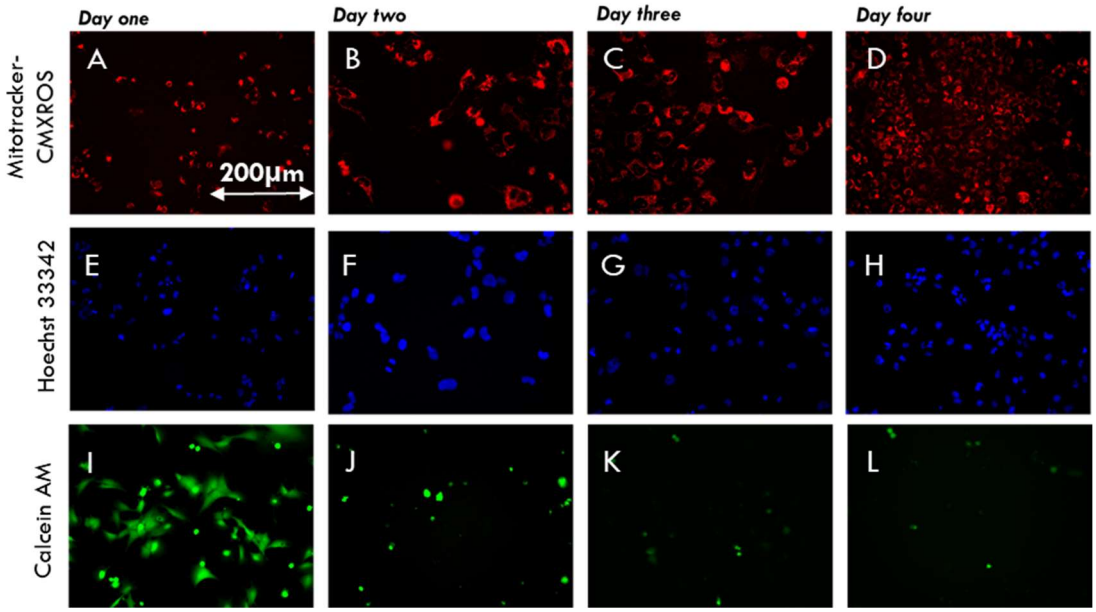

Figure 13. Evolution of HMC3 cell staining over the time. Pictures A-D correspond to cells stained using Mitotracker-CMXROS after 24h, 48h, 72h and 96h respectively. E-H correspond to cells stained with Hoechst 3342 after 24h, 48h, 72h and 96h respectively. I-L correspond to cell stained using Calcein AM after 24h, 48h, 72h and 144h respectively.

4.5. Transfection of HMC3 cells

HMC3 cells were transfected to overexpress α-synuclein using Lipofectamine 3000, which is supposed to deliver exceptional transfection efficiency into the widest range of difficult-to-transfect and common cell types with improved cell viability.

The possible formation of protein aggregates was studied, one of the main molecular events in Parkinson´s disease, using plasmids pJAC321 (pCMV-Myc-mRFP-aSyn) and pJAC332 (EGFP-αSyn). Plasmids structure is shown in figure 14:

Cells were seeded at the following different densities in 24 well plates to optimize transfection conditions:

- $5x10^4$ cells/well
- $8x10^4$ cells/well
- $1x10^5$ cells/well

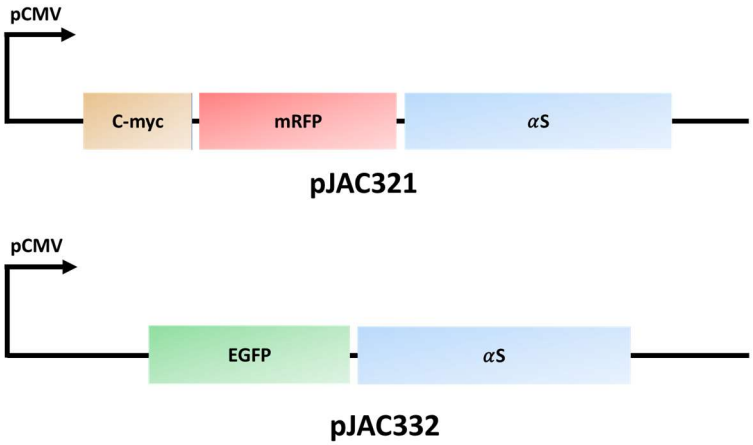

Figure 14. Structure of plasmids pJAC321 and pJAC332

Lipofectamine 3000 reagent was used at a ratio 1,5:1 (reagent/DNA). Results are shown in figure 15.

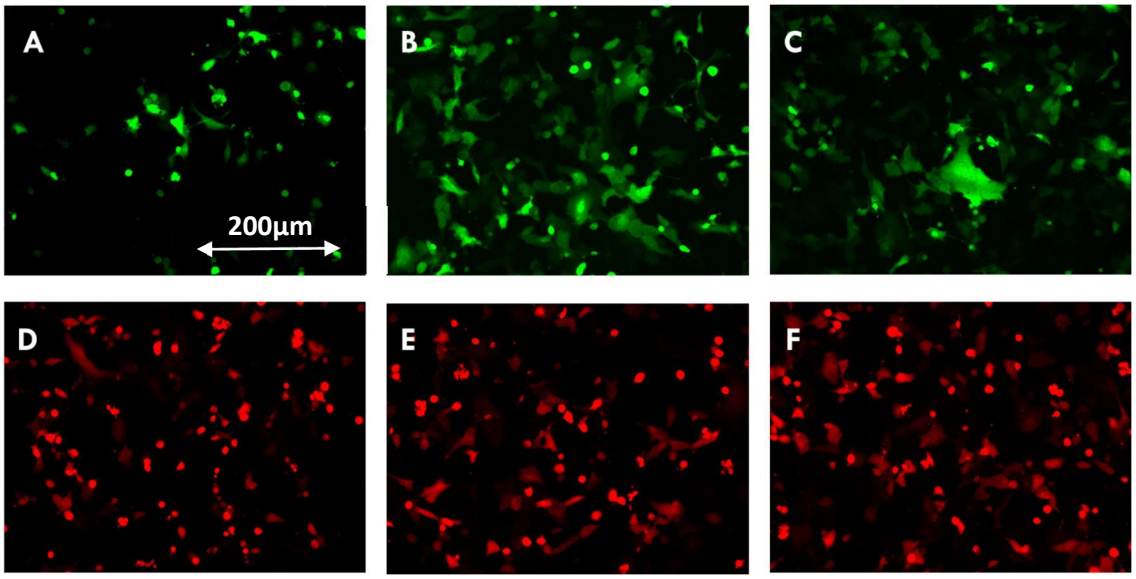

Figure 15. Transfection of HMC3 cells using pJAC332 (A-C) and pJAC321 (D-F). Cell density for transfection was $5x10^4$ (A,D), $8x10^4$ (B, E) and $1x10^5$ (C, F). Transfected cells show green fluorescence in (A-C) and red fluorescence in (D-F).

Calculation of transfection efficiency was carried out by image segmentation (selecting the objects of interest over the background), taking into account the ratio between transfected and all cells. While Hoechst was used to stain all cells in blue (transfected and non-transfected), transfected cells had their own fluorescence. In the case of

plasmid pJAC332, transfected cells were green due to green-fluorescent protein (GFP) whereas the protein encoded in plasmid pJAC321 is red due to mRFP. After segmentation three different pictures of the highest cell density ($1x10^5$), a transfection efficiency of 58,2 ± 4,3% was obtained using plasmid pJAC332.

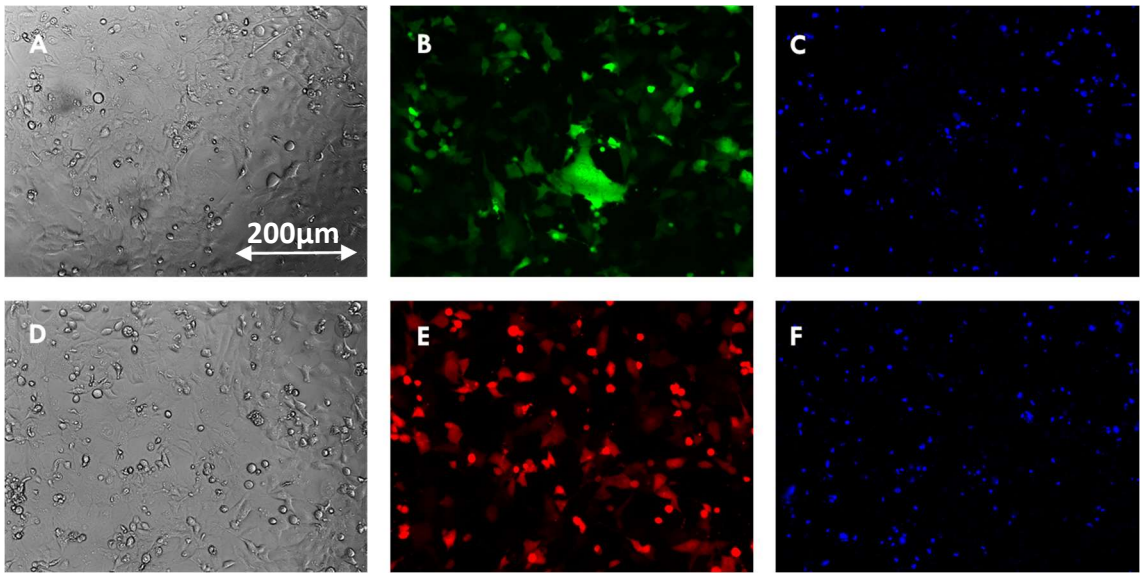

Figure 16. Transfection of HMC3 cells using pJAC332 and pJAC321 plasmids. Picture A correspond to transfected cells with pJAC332 in bright field, B) transfected cells with pJAC332 showing green fluorescence and C) transfected cells with pJAC332 stained with Hoechst. Picture D correspond to transfected cells with pJAC321 in bright field, E) transfected cells with pJAC321 showing red fluorescence and F) transfected cells with pJAC321 stained with Hoechst.

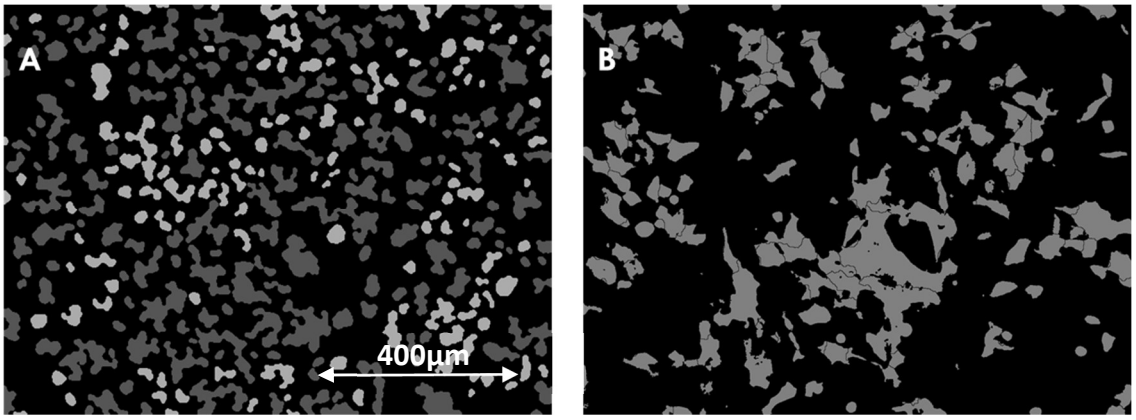

Figure 17. Segmentation of HMC3 cells depending on transfection. While A) corresponds to staining of all cells, B) represents only transfected cells.

4.6. Quantifying transfection by western blot

Proteins were extracted from transfection samples using lysis buffer RIPA containing a cocktail of protease inhibitors. 15 µL of each sample were analyzed by SDS-PAGE and 5 µL of PageRuler™ Prestained Protein Ladder 10-180 kDa was used as a molecular weight marker. Two gels were used to normalize signals, the first one was used to quantify α-synuclein and the second one, to quantify a housekeeping protein, GAPDH in this case.

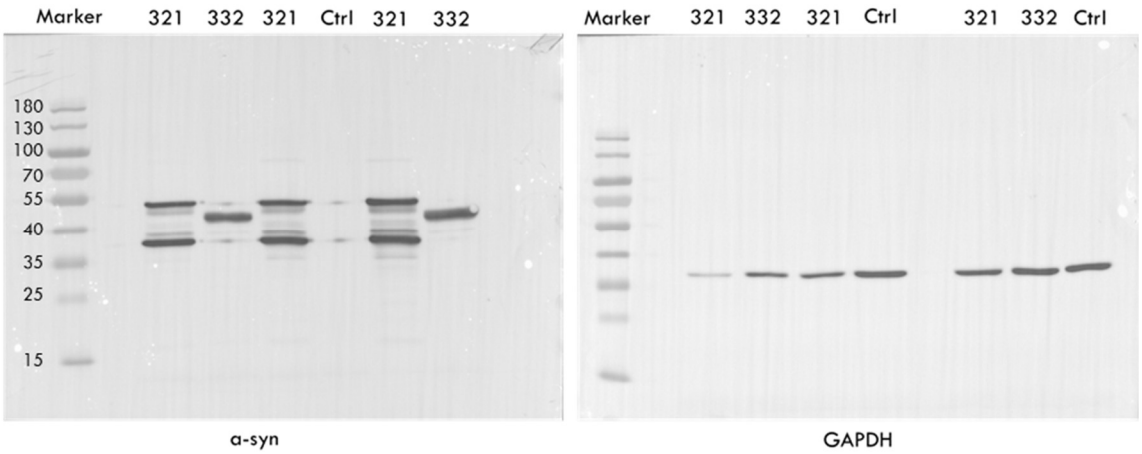

Figure 18. SDS-PAGE of HMC3 cells transfected with α-synuclein. Bands corresponding to α-synuclein (left panel) and bands corresponding to GAPDH (right panel). The sizes (in kDa) of molecular mass markers run alongside are indicated on the left.

ImageJ (Fiji) software was used to quantify protein bands in different gels. For this purpose, the first step is inverting image colors. Then, rectangular areas covering whole protein bands of interest are selected and their intensity is analysed (a base line correction of each peak is needed to calculate accurately area/intensity). In the case of α-synuclein gel, specifically plasmid pJAC321, there are two major bands corresponding α-synuclein. The reason for that remains unknown and is under investigation in the lab since the plasmid sequence is correct. Therefore, for quantifying protein, the two bands were selected. The first band corresponding to GAPDH was discarded due to an error loading the sample into the gel. After quantifying bands, we obtained the results shown in figure 19.

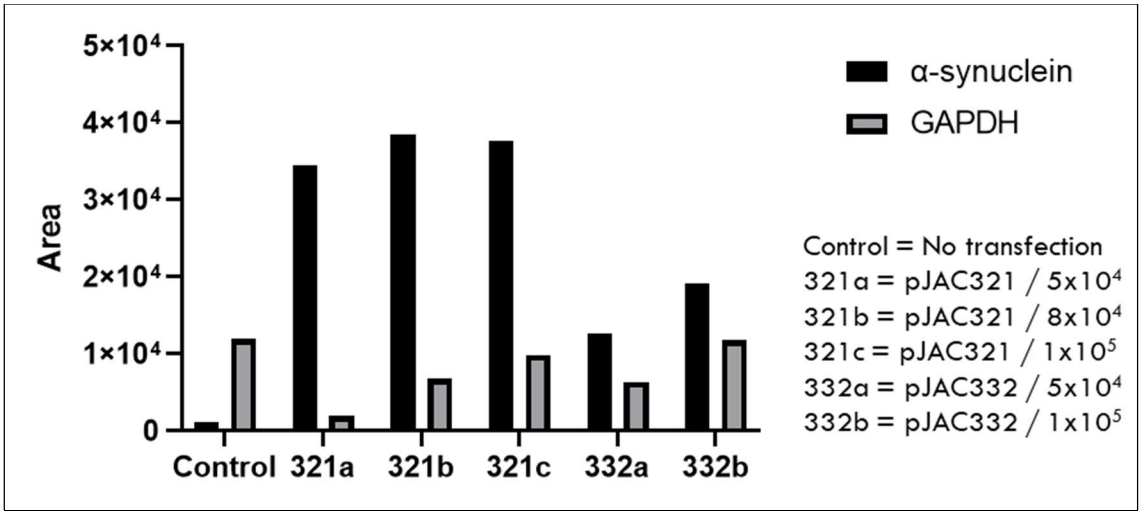

Figure 19. Quantification of HMC3 cells transfection with different plasmids at different cell densities.

Once the signal is normalized, it is possible to conclude that transfection efficiency is similar using plasmid pJAC332 at cell densities between $5x10^4$ and $1x10^5$. Transfection efficiency of plasmid pJAC321 seems to be a bit better compared to plasmid pJAC332. Nevertheless, no statistical conclusions can be obtained because samples were measured only once. For further experiments, as they work properly, pJAC332 and pJAC321 plasmids will be used equally.

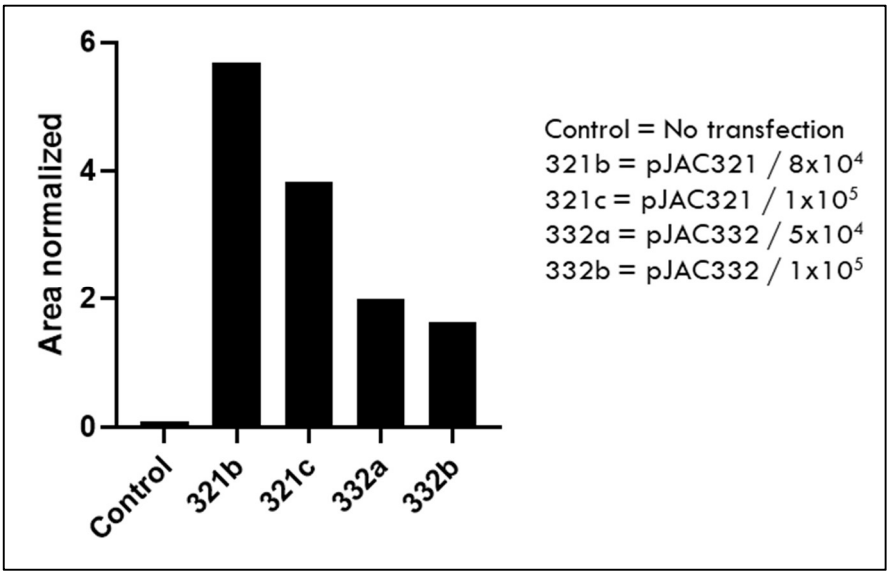

Figure 20. Quantification of protein expression in transfected HMC3 cells after signal normalization with respect to a housekeeping gene (GAPDH).

4.7. Induction of inflammation

4.7.1. Activation of microglia cells using IL-1β and IFN-γ

Cytokines IL-1ß and IFN-γ were used to induce inflammation of HMC3 cells as a positive control[33]. For measuring inflammation, IL-6 secreted in the medium was detected by Enzyme-Linked ImmunoSorbent Assay (ELISA) using Human IL-6 Uncoated ELISA (Invitrogen). The activation of microglia cells using only IFN-γ or the combination of IFN-γ and IL-1ß were compared.

HMC3 cells were seeded at $1x10^5$ cells/mL in 12 well plates. After 24 hours of treatment with cytokines at a concentration of 10 ng/mL each, the cell supernatant was collected and stored at -20°C until analysed.

Since the standard curve covers a range of concentrations between 1,5 – 200 pg/mL, previous sample dilutions should be made to avoid saturating the absorbance signal. Each sample was measured three times and analysed without dilution, at 1:10 dilution and at 1:50 dilution. After removing the value corresponding to 200 pg/mL (it is an outlier), we obtained the calibration curve shown in figure 21.

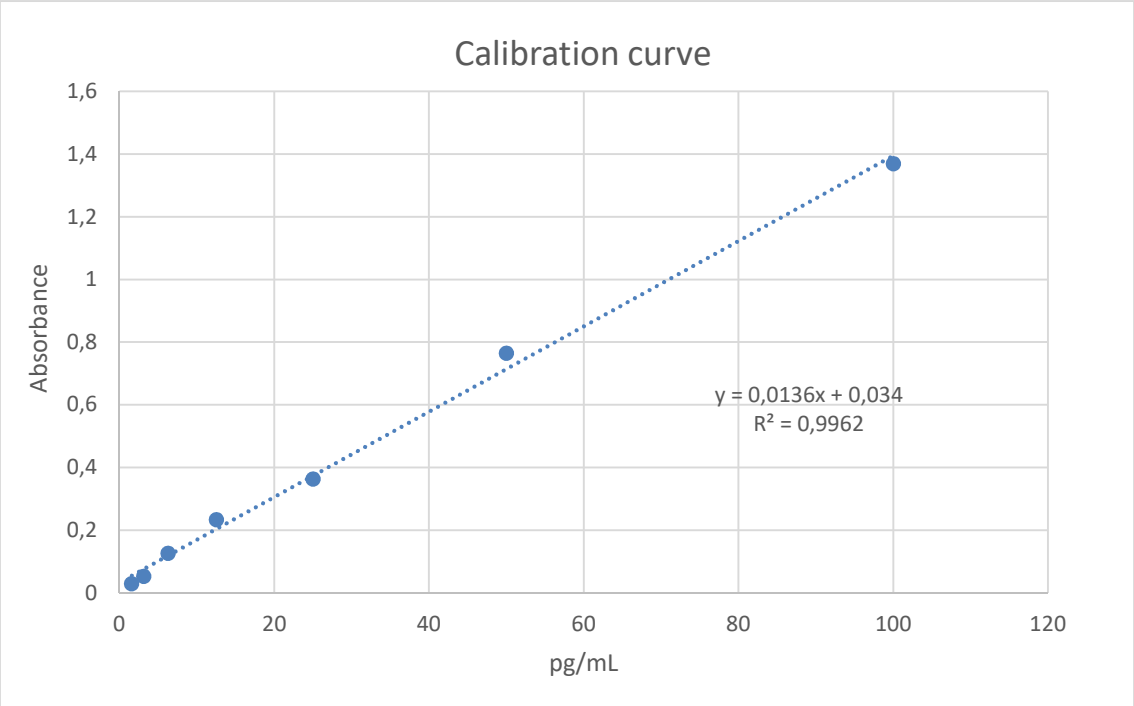

Figure 21. Calibration curve of IL-6 standard

In this case, samples without dilution and 1:10 dilution were discarded due to a saturation in the signal and the concentration of IL-6 was obtained by interpolating absorbance values from the 1:50 dilution (*Table 6*).

Table 6. IL-6 released results for IFN-γ and IL-1β treatments measured by ELISA.

Sample	IL-6 concentration (pg/mL)	SD
Control	139.3	9,76
IFN-γ	861.65	187,18
IFN-γ + IL-1β	3317.8	382,67

Looking at table 5, it can be observed that treated microglia cells are activated 6 times with respect to untreated ones (control) using IFN-γ whereas they are activated 24 times using the combination of IFN-γ and IL-1β with respect to the control.

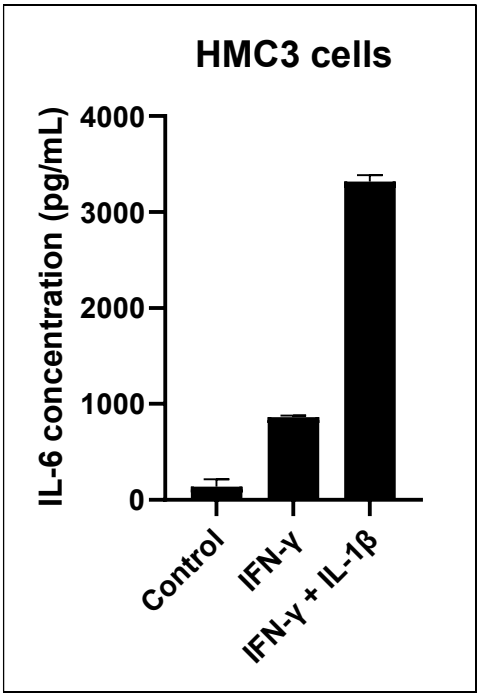

Figure 22. IL-6 concentration using only IFN-γ and a combination of IFN-γ + IL-1β.

Once it was demonstrated that a combination of IFN-γ and IL-1β at a concentration of 10 ng/mL each can activate microglia cells, we studied if cytokines IFN-γ and IL-1ß have negative effects on HMC3 and SH-SY5Y cell viability over time (because these cytokines will be present in the medium while co-culturing). Cell morphology after treatment appears to be normal and cytokines did not promote cell death in the case of SH-SY5Y cells (at least in the first 72 hours) as it is shown in figure 23.

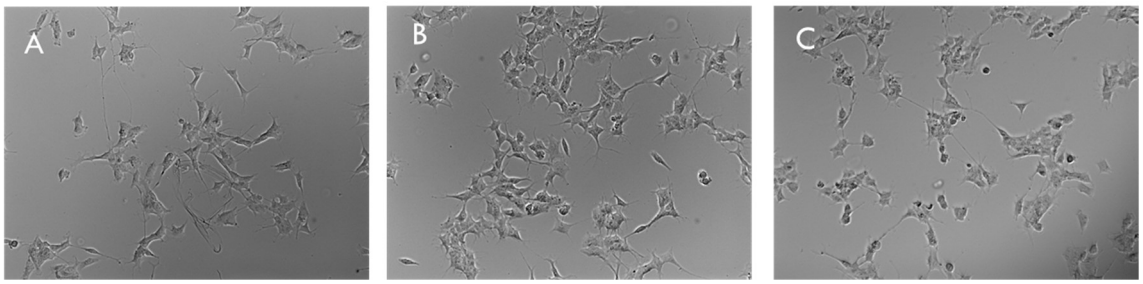

Figure 23. SH-SY5Y-SNCA cells without treatment (A). SH-SY5Y-SNCA cells treated with IFN-γ and IL-1β 10 ng/mL (B,C). Pictures taken in bright field.

Since just looking at cell pictures is not enough to be sure that cytokines do not promote cell death, a cell viability assay using resazurin (7-Hydroxy3H-henoxazin-3-one 10-oxide) was performed. Resazurin cell viability assay is a sensitive fluorescent assay that detects cellular metabolic activity. Resazurin is a blue non-fluorescent dye until it is irreversibly reduced to the pink colored and highly red fluorescent resorufin by dehydrogenase enzymes in metabolically active cells. Fluorescent signal was measured using 530-560 nm excitation wavelength and 590 nm emission wavelength.

The results, performing an ANOVA test, indicate that there is no statistically significant difference in cell viability when a combination of IFN-γ and IL-1ß at a concentration of 10 ng/mL each is added to SH-SY5Y and HMC3 cells, as it is shown in figure 24.

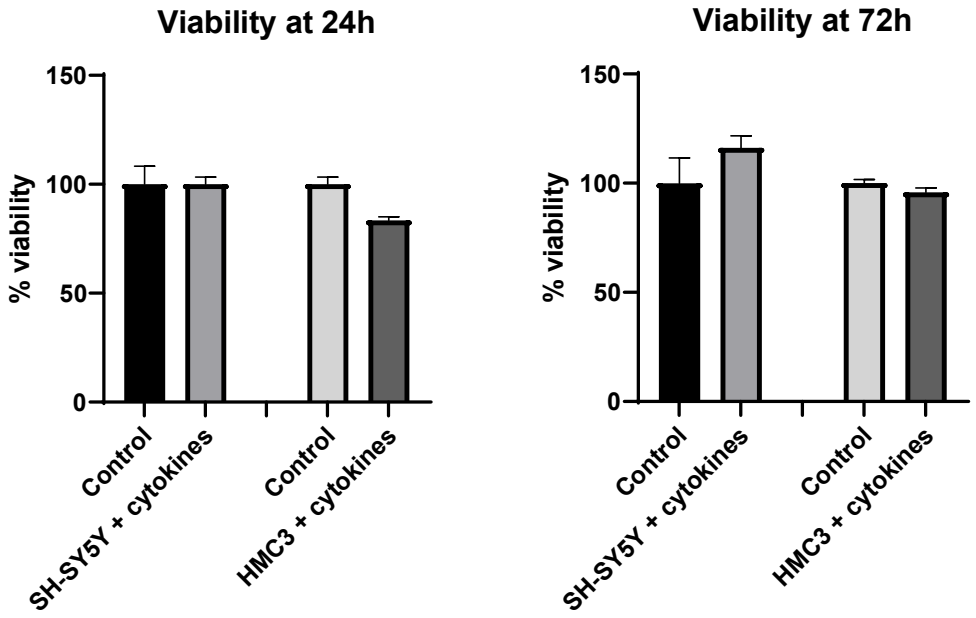

Figure 24. Viability of SH-SY5Y and HMC3 cells treated with a combination of IFN-γ and IL-1β 10ng/mL each one after 24 and 72 hours.

Taking into account previous results, for further experiments involving the activation of microglia cells a combination of IFN-γ and IL-1β at a concentration of 10 ng/mL each could be used.

4.7.2. Activation of microglia cells using neuromelanin

Neuromelanin was studied as an inductor of inflammation in microglia cells. Synthesized neuromelanin (NM), which was prepared at a concentration of 17 mg/mL, was used to attempt to induce inflammation in microglia cells in a wide concentration range. Inflammation was carried out diluting several times stock solution between 1:100 - 1:2.000 and measuring IL-6 release from cells treated with 85 µg/mL (NM1), 17 µg/mL (NM2) and 1,7 µg/mL (NM3).

HMC3 cells were seeded at $1x10^5$ cells/mL in 12-well plates. After 24 hours of treatment, the supernatant was collected and stored at -20°C until analyzed.

Following the same process as done with the ELISA positive control (HMC3 cells treated with cytokines), each sample was measured three times and analysed without dilution,

1:10 dilution and 1:50 dilution. After removing the value corresponding to 200 pg/mL (an outlier), the calibration curve is shown in figure 25.

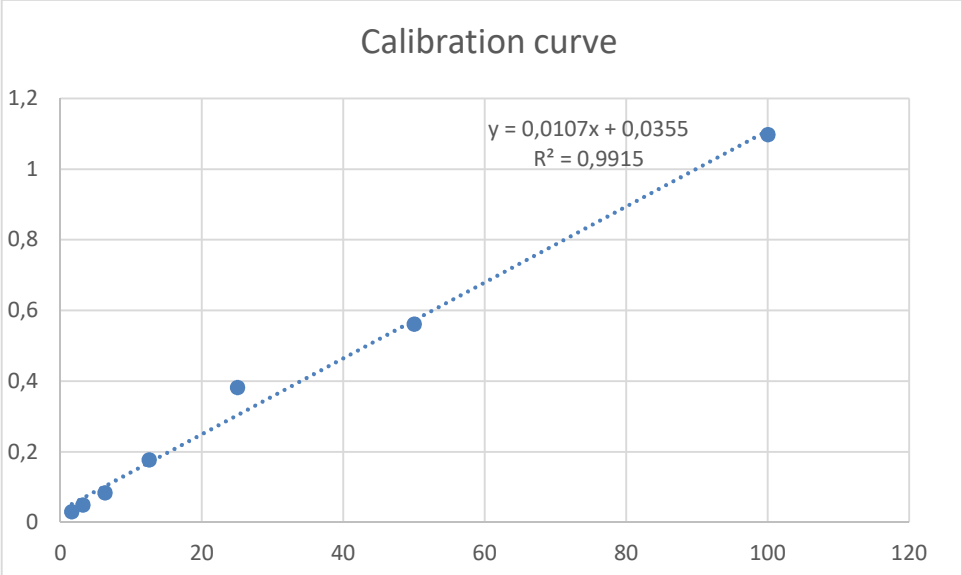

Figure 25. Calibration curve of IL-6 standard.

After interpolating absorbance values into the calibration curve, concentration values of IL-6 in samples can be obtained and are summarized in table 7 (values for sample without dilution and 1:50 dilution were discarded since they did not adjust into the calibration curve).

Table 7. Released IL-6 from HMC3 cells treated with different amounts of neuromelanin measured by ELISA.

Sample	IL-6 concentration (pg/mL)	SD
Control	638,94	130,36
NM 1 (85 µg/mL)	876,64	10,10
NM 2 (17 µg/mL)	773,83	112,52
NM 3 (1,7 µg/mL)	712,15	85,61

At first sight, it appears there is a little difference between control and NM 1 samples. Nevertheless, standard deviation of control is really high (around 20%) so it was statistically analysed. An ANOVA test was conducted to evaluate if differences were significative.

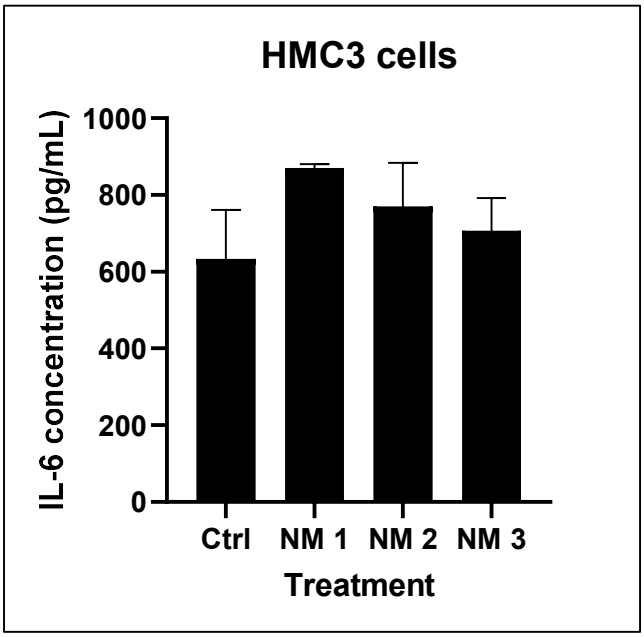

Figure 26. IL-6 concentration using different amounts of neuromelanin (NM).

Taking into consideration a confidence interval (CI) of 95%, the results indicate that differences between treatments are not significant and that synthesized neuromelanin do not activate microglia cells under de tested conditions.

Apart from analysing the secretion of IL-6 after adding the inductor of inflammation, we also studied if there were changes in cell morphology and how cells react after several days of treatment. Some pictures were taken after 7 days of neuromelanin treatment and it could be said that HMC3 cells seem to continue growing without problems until confluence as can be seen in figure 27.

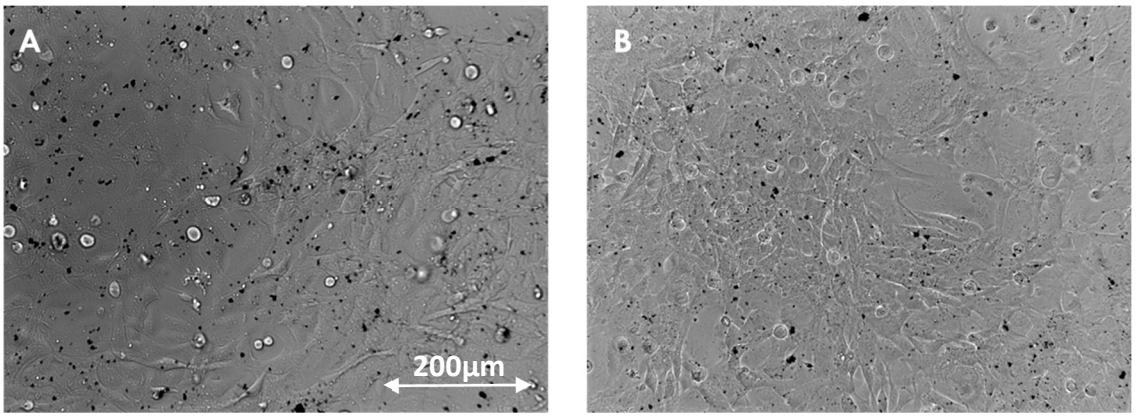

Figure 27. HMC3 cells treated with neuromelanin after 7 days. Pictures taken in bright field.

To check that neuromelanin did not promote cell death, a viability assay was performed in HMC3 cells using resazurin. Surprisingly, results indicate that metabolic activity of HMC3 cells decrease around 80% and 90% when they are treated with neuromelanin after 24 and 72 hours respectively as it can be seen in figure 28.

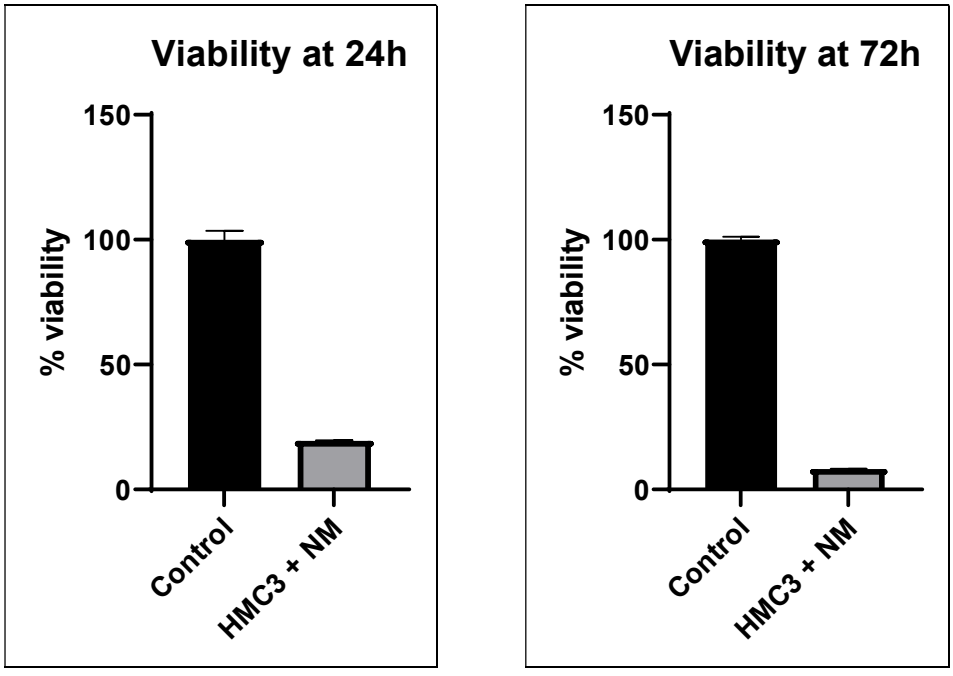

Figure 28. Viability of HMC3 cells treated with NM 85 µg/mL after 24 and 72 hours.

An *in vivo* experiment was carried out to clarify what was happening in HMC3 cells treated with neuromelanin because they appear to continue growing but the metabolic activity decreases considerably. Cells pictures were taken every 15 minutes for 24 hours using in bright field at different Z planes (±9 µm respect to center). Results are simplified in figure 29.

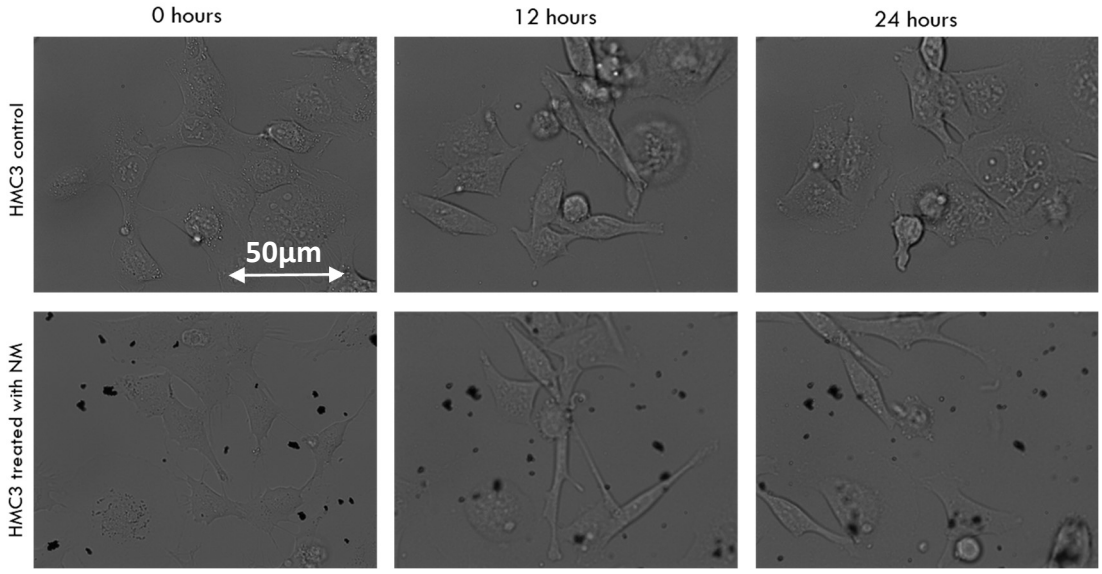

Figure 29. Time lapse microscopy of HMC3 cells treated with NM. Cell pictures of HMC3 cells without treatment (top) after 0,12 and 24 hours and HMC3 cells treated with neuromelanin (bottom) after 0,12 and 24 hours.

All in all, the presence of neuromelanin seems to delay HMC3 cells proliferation but it does not promote cell death at least during the first 24 hours.

4.7.3. Effect of neuromelanin on SH-SY5Y-SNCA cells

The effect of synthesized neuromelanin on SH-SY5Y5-SNCA cells was also studied in order to gain information about the effects on viability and metabolic activity of this compound for future experiments co-culturing HMC3 and SH-SY5Y-SNCA cells. As a result, it was observed that after using different concentrations of neuromelanin, (85

µg/mL, 17 µg/mL and 1,7 µg/mL), it promotes cell death and most cells are dead after 48 hours as it is shown in figure 30.

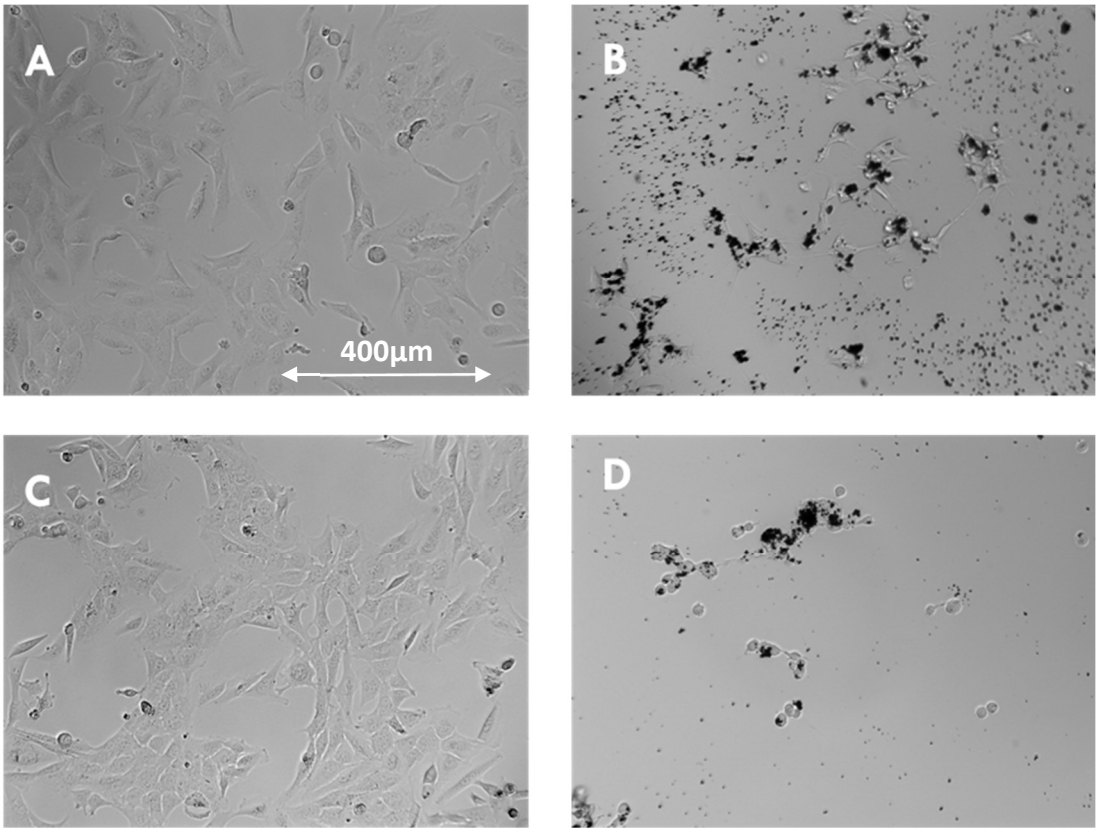

Figure 30. Effect of NM on SH-SY5Y-SNCA cells. Cells after 24 hours without treatment (A) and treated with neuromelanin (B). Cells after 48 hours without treatment (C) and treated with neuromelanin (D). Cells were washed three times with DPBS 1X before taking photos at 48 hours. Pictures are taken in bright field.

SH-SY5Y-SNCA cells seem to capture neuromelanin from the medium, forming big aggregates of this compound inside the cell. An *in vivo* experiment was carried out to analyse what was happening in cells treated with neuromelanin in the first 24 hours. Cell pictures were taken every 15 minutes for 24 hours using the microscope in bright field at different Z planes (±9 µm respect to center). Results are shown in a simplified way in figure 31.

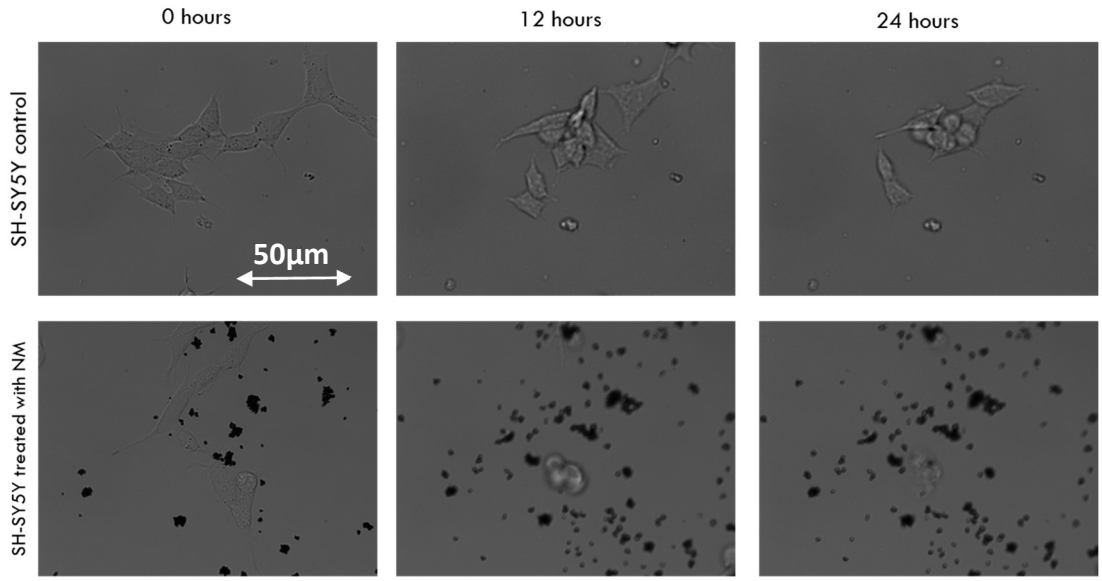

Figure 31. Time lapse of SH-SY5Y-SNCA cells treated with NM. Cell pictures of SH-SY5Y-SNCA cells without treatment (top) after 0,12 and 24 hours and SH-SY5Y-SNCA cells treated with neuromelanin (bottom) after 0,12 and 24 hours.

Neuromelanin promotes cell death very quickly and SH-SY5Y cells do not capture neuromelanin at least in the first 24 hours of the experiment.

SH-SY5Y cell death was verified performing a viability assay similar to previous cases, using resazurin. Results after 24 hours of treatment are shown in figure 32.

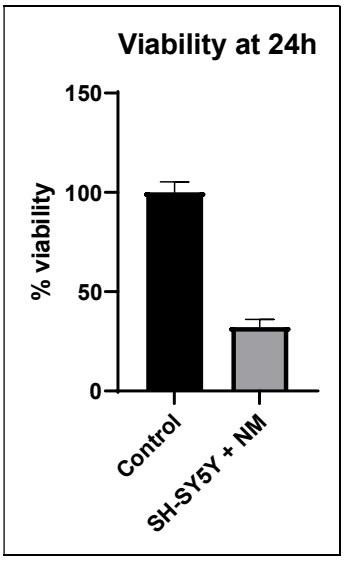

Figure 32. Viability of SH-SY5Y cells treated with NM 85 µg/mL.

Due to the inefficacy to activate HMC3 cells as well as the killing effect on SH-SY5Y5 cells, it is not a good idea to promote inflammation using synthesized neuromelanin in the co-culture so other inductors of inflammation were used as it is going to be shown in the following section.

4.7.4. α-Synuclein as an inductor of inflammation

Similar to cytokines and neuromelanin experiments, the effect of α-Synuclein to induce inflammation in microglia cells was studied. It is believed that monomers, fibrils or α-Syn aggregates can activate microglia cells in a different way[34,35]. The activation of monomeric α-Syn at two different concentrations was measured by ELISA as done before, quantifying secreted IL-6. Results are shown in figure 33.

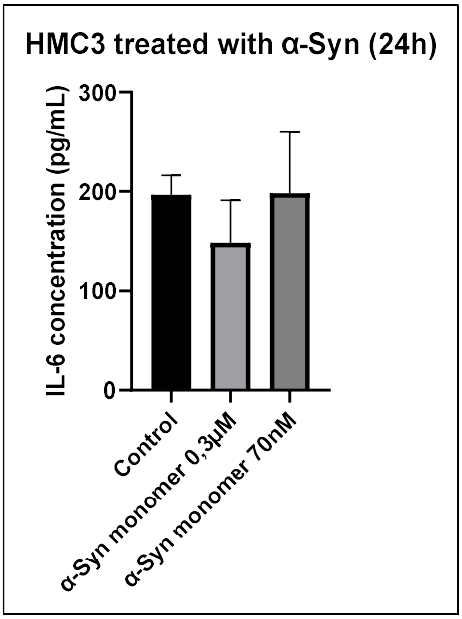

Figure 33. IL-6 concentration using different concentrations of monomeric α-Syn.

In our conditions, monomeric α-Syn did not activate microglia cells at least during the first 24 hours and cell viability, measured using a resazurin assay, did not change significatively compared to control as it can be seen in figure 34.

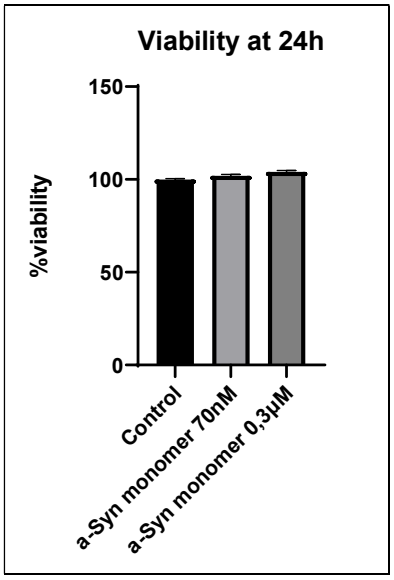

Figure 34. Viability of HMC3 cells using different amounts of α-Syn after 24h

4.8. Inducing inflammation in transfected HMC3 cells

Once it was demonstrated that IFN-γ and IL-1β cytokines have the ability to activate HMC3 cells in our conditions, it is possible to study its immunological response in certain situations. The first one is to analyse if there is some reaction in HMC3 cells when α-synuclein is overexpressed and cells are treated with IFN-γ and IL-1β. The inflammation of microglia cells induced by these cytokines could be involved in α-synuclein aggregation inside the cell or it could affect the elimination of this protein.

Cells were seeded at a density of $5x10^4$ cells/well in 24 well plates using EMEM as culture medium. They were transfected with plasmid pJAC332 (EGFP-αSyn) using Lipofectamine 3000 at a ratio 1,5:1 (reagent/DNA). After 24 hours of transfection, HMC3 cells were activated using IFN-γ and IL-1β at a concentration of 10 ng/mL each. Pictures were taken after 48 hours of activation and some are shown in figure 35.

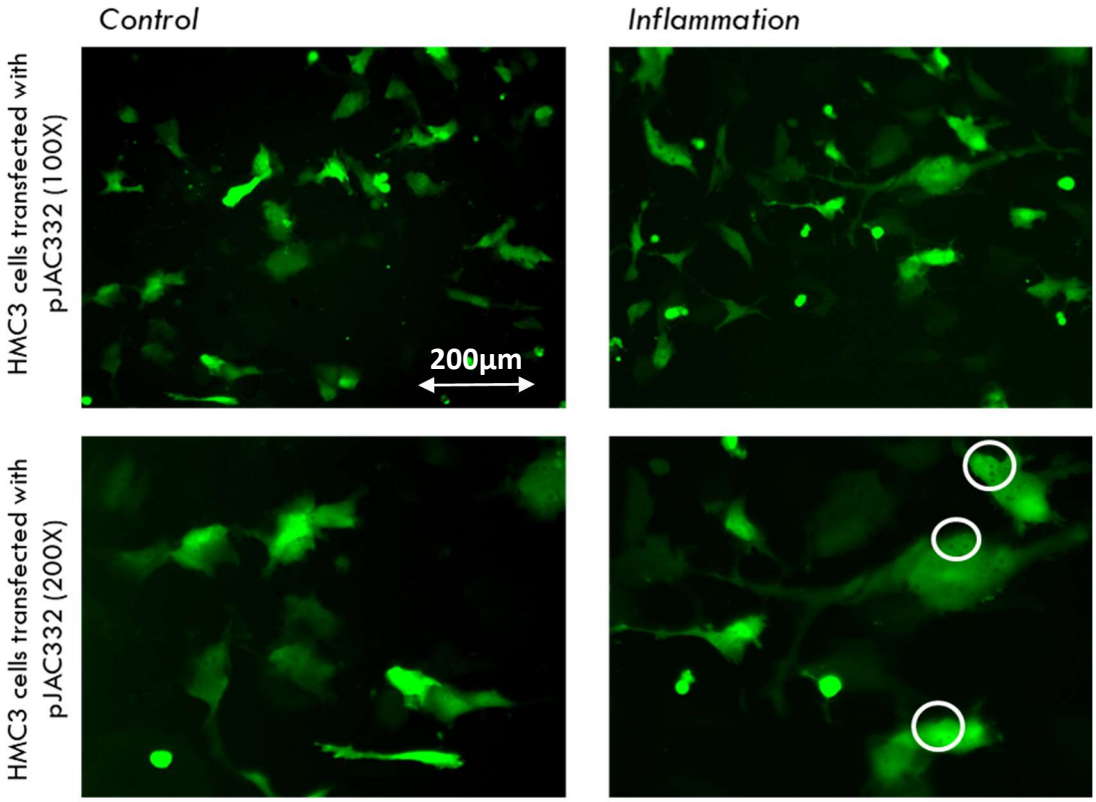

Figure 35. Transfection of HMC3 cells with plasmid pJAC332 after 48 hours. HMC3 cells without inflammation (left) and inducing inflammation (right). Pictures at the bottom were made using 200X objective. White circles point out black dots inside the cell.

There was no observable difference in the behaviour of transfected cells with or without inflammation. Viability of control and treated HMC3 cells seems to be quite similar after 48 hours as well as their morphology and α-synuclein aggregates inside the cytoplasm are not appreciated. Nevertheless, an increase in the formation of "black dots" (circled in white in figure 35) inside the cells was observed after treating transfected microglia cells with IFN-γ and IL-1ß cytokines.

4.9. Co-culture of HMC3 and SH-SY5Y-SNCA cell line

4.9.1. Analysis of dye exchange among cells

Due to morphology similarities between HMC3 and SH-SY5Y-SNCA cell lines, it is really important to be able to distinguish both type of cells when co-culturing. As it has been said before, one idea could be to stain cells with different dyes that remain over time. The possibility that cells release the dye to the medium naturally or when they die, which could be taken up by other cells, should be contemplated because it could generate problems at the time of distinguishing both initially labelled and non-labelled cells. For that purpose, the possible exchange of dye between both groups of cells should be analysed.

HMC3 and SH-SY5Y-SNCA cells were seeded at a density of $5x10^4$ cells/mL in 12-well plates and stained with Hoechst and Mitotracker-CMXROS (HMC3 cells) or Calcein AM (SH-SY5Y-SNCA cells) at the concentrations shown in table 8. Cells were incubated with dyes for 30 minutes at 37°C in an atmosphere of 5% CO_2. Then, they were washed twice, incubated for 30 minutes with DPBS 1X and incubated with soft agitation for 5 minutes before co-culture. HMC3 cells were detached from the surface using tripsyn, centrifuged for 5 minutes, and added on top of SH-SY5Y-SNA cells. Results are shown in figure 36.

Table 8. Cell- dye concentrations for staining HMC3 cells (Hoechst and Mitotracker-CMXROS) and SH-SY5Y-SNCA cells (Calcein AM).

Cell dye	Stock concentration	Working concentration
Calceine AM	1mM	0,5µM
Mitrotracker-CMXROS	1mM	25nM
Hoechst	10 mg/mL	250 ng/mL

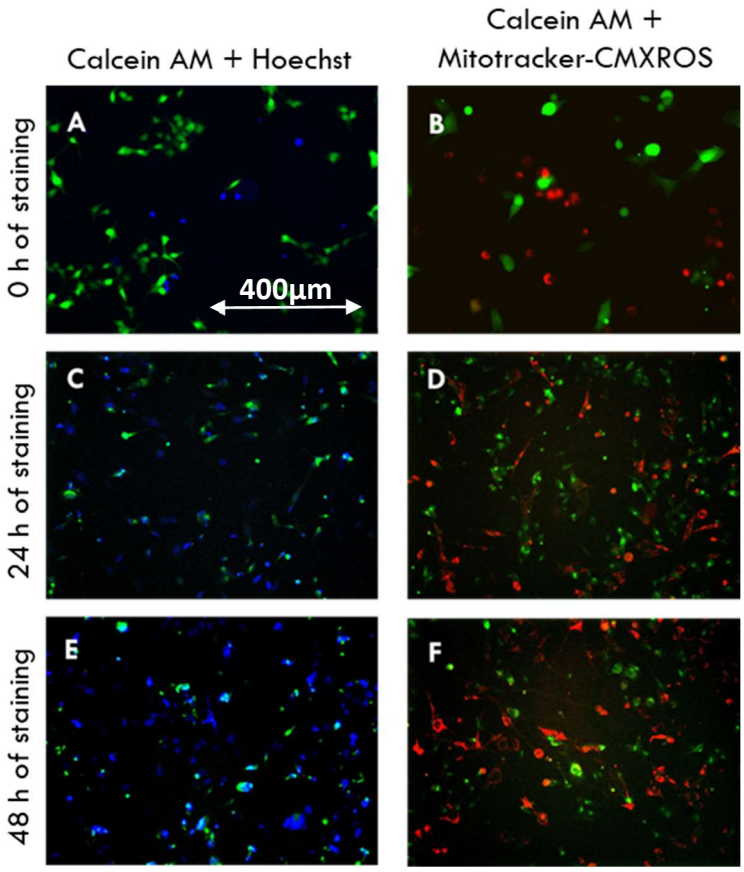

Figure 36. Staining a co-culture of HMC3 and SH-SY5Y-SNCA. HMC3 cells were stained in blue (Hoechst) and SH-SY5Y-SNCA in green (Calecin AM) at 0, 24 and 48 hours in (A,C,E) respectively. HMC3 cells were stained in red (Mitotracker-CMXROS) and SH-SY5Y-SNCA in green (Calcein AM) at 0, 24 and 48 hours in (B,D,F) respectively.

It could be observed that HMC3 and SH-SY5Y-SNCA cell lines can be distinguished in co-culture for at least 48 hours and cell-dyes are not exchanged. If cell-dyes were released to culture medium, cells stained in two colours at the same time would be observed. Once this has been checked, it is possible to carry out successive experiments.

4.9.2. Co-culturing transfected SH-SY5Y-SNCA and HMC3 cells

The purpose of co-culturing SH-SY5Y-SNCA and HMC3 cell lines is to study possible interactions between them. As microglia cells are in charge of immunological functions in the central nervous system, we analysed if there were reactions upon some treatments. One idea was to co-culture HMC3 with SH-SY5Y cells overexpressing α-

synuclen in order to obtain information about cell behaviour. Although SH-SY5Y-SNCA cells overexpress α-synuclein constitutively, they were transfected with plasmid pJAC332 (EGFP-αSyn) to overexpress the mentioned protein.

HMC3 and SH-SY5Y-SNCA cell lines were co-cultured as described in Materials and methods (Method 2). Cell lines were seeded at $2.5x10^4$ cells/well in 24 well plates and transfection was carried out using Lipofectamine 3000 in a plasmid DNA:Lipofectamine ratio of 1.5:1. Pictures were taken using the fluorescence microscope after 48/72 hours of transfection and the results are shown in figure 37.

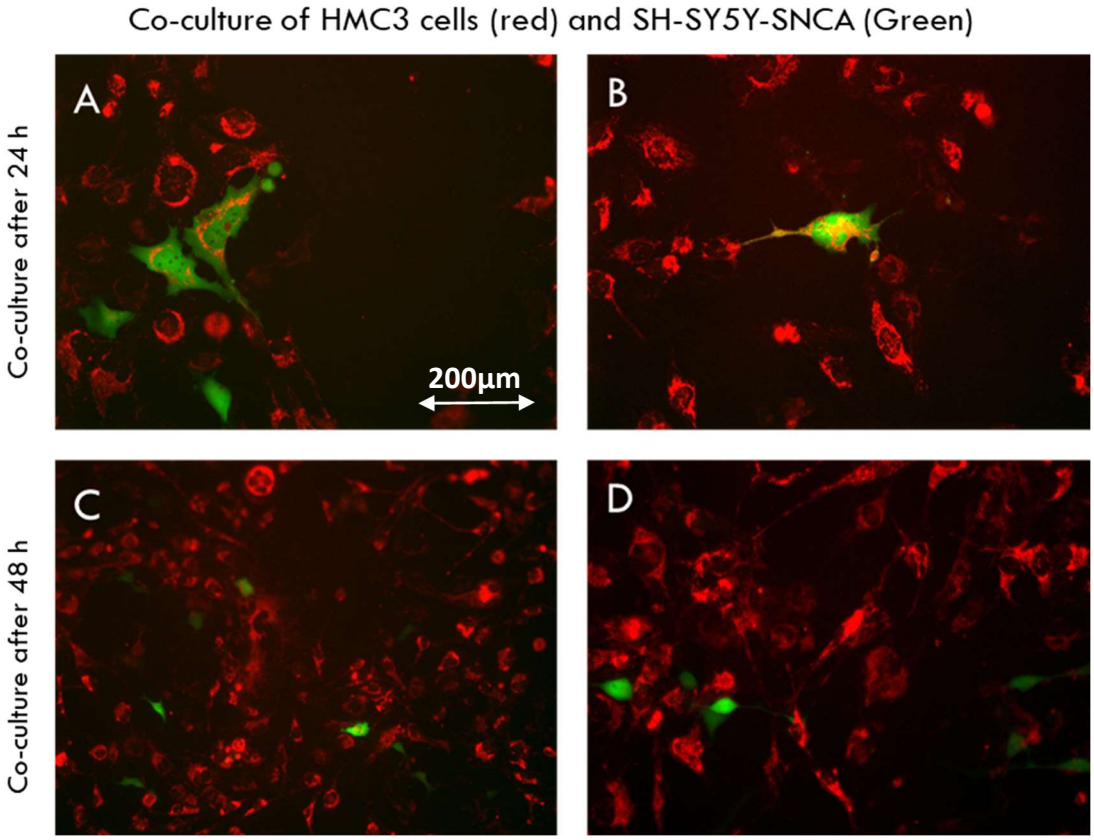

Figure 37. Co-culture of HMC3 (red) and transfected SH-SY5Y-SNCA (green) cells. A and B were taken 48 hours after transfection (24 hours in co-cultured) whereas C and D after 72 hours of transfection (48 hours in co-culture).

Looking at the pictures, it is quite complicated to say if there is an interaction between HMC3 and SH-SY5Y-SNCA cells. Besides it, transfection efficiency of SH-SY5Y-SNCA cells is very low, making it difficult to clarify if microglia cells are affecting SH-SY5Y-SNCA

cells. It would be necessary to carry out an in vivo assay to find out how the cells behave overtime.

4.9.3. Co-culturing transfected SH-SY5Y5-SNCA and activated HMC3 cells

In this case, following the same idea explained above, to study possible interactions in the co-culture, transfected SH-SY5Y-SNCA cells were co-cultured with activated HMC3 cells. HMC3 cells were activated using IFN-γ and IL-1β at concentrations of 10 ng/mL for 48 hours. Then, transfected SH-SY5Y-SNCA cells were added on the top of HMC3 cells and after 24 and 48 hours of co-culture, pictures were taken and the results are shown in figure 38.

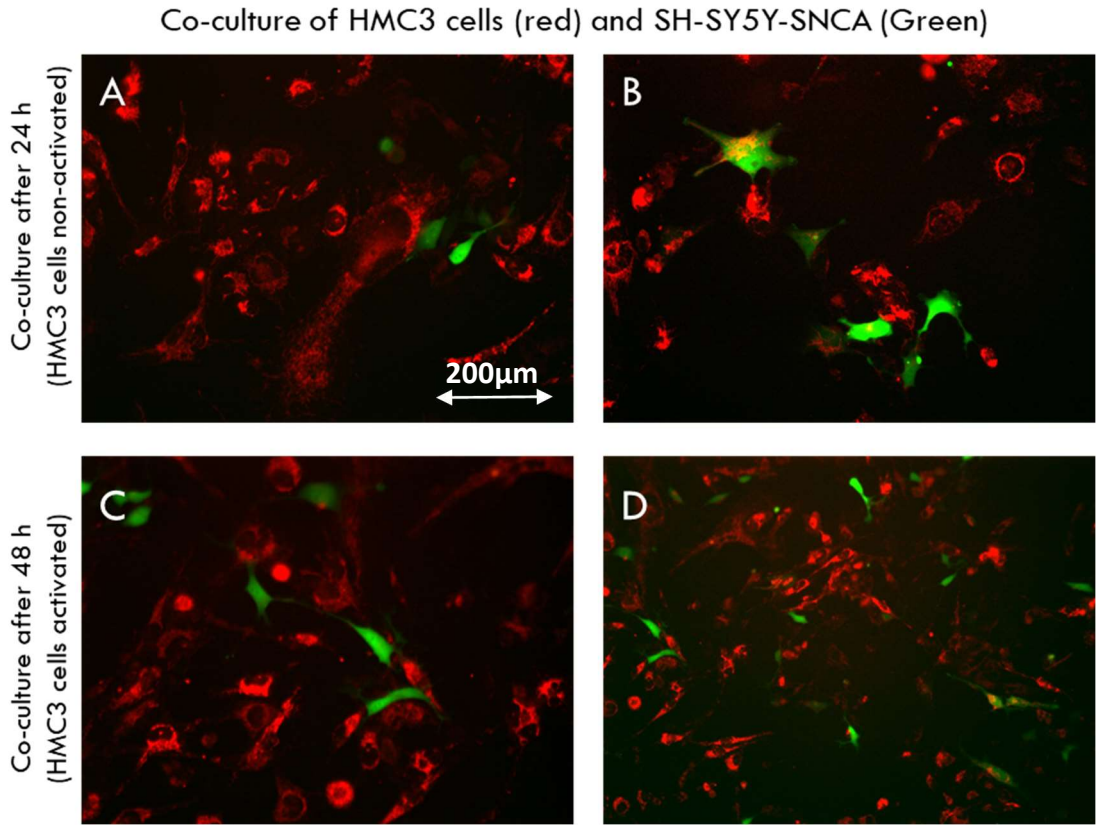

Figure 38. Co-culture of HMC3 and transfected SH-SY5Y-SNCA cells (A,B). A and B were taken 48 hours after transfection (24 hours in co-culture). Co-culture of activated HMC3 and transfected SH-SY5Y-SNCA cells (C,D). C and D were taken after 72 hours of transfection (48 hours in co-culture).

In this case, there are no differences between co-culturing transfected SH-SY5Y-SNCA cells with HMC3 activated and non-activated cells. We expected to observe some α-Syn aggregates inside SH-SY5Y-SNCA cells or an increase in mortality of SH-SY5Y-SNCA cells but it is quite difficult to observe an immunological response from HMC3 cells in the co-culture by fluorescence microscopy under these conditions.

5. Discussion

As it was said in the introduction, Parkinson's disease affects millions of people worldwide and there is no effective treatment that can cure the disease, there are only some symptomatic treatments which are able to delay its effects. Therefore, it is necessary to invest money in new research lines to solve this social problem.

From a scientific point of view, an improvement of models simulating the brain microenvironment as accurate as possible is needed to develop neuroprotective drugs or methods to avoid neurodegeneration in the future. This has been the major focus of this work, in which we have attempted to develop a new simple cell-based model, as a first approach towards more complex models, using two different cell types found in the central nervous system: neurons and microglia. To do this, we have selected a commonly used neural cell line, SH-SY5Y and a microglia cell line, HMC3. To our knowledge, this combination of cell types has not been used so far to partially mimic the constitution of the brain. Microglia cells were chosen because of their immunological role in the brain, being responsible for neuroinflammation, which has been described as a major factor in the development of neurodegenerative diseases.

As it has been already mentioned, since the HMC3 cell line reception in the lab was delayed for more than three months, we started this work carrying out some assays with Caco-2 cells, which are being used in the lab as a cellular model of the small gut to explore the reported transmission of misfolded synuclein from the gut to the brain. Some of the assays that were set up using this cell line were later used in the other part of the work with HMC3 and SH-SY5Y cells.

Firstly, we have established that Mitotracker-CMXROS (mitochondria stain), Hoechst 33342 (nuclear stain), and Calcein AM (cytoplasm stain) cell dyes can be employed for staining Caco-2 cells at working concentrations of 25nM, 250ng/mL and 1µM, respectively. Viability results show that Caco-2 cells continue growing without major

problems, although slightly slower compared to unstained cells, and staining remains over time at least for the first 6 days. Therefore, Mitotracker-CMXROS, Hoechst, and Calcein AM cell dyes would be a good option if we want to observe mitochondria inside the cell, nuclei or the whole cell in Caco-2 cells. Furthermore, they can be used to distinguish different cell types in co-cultures, independently on the cellular compartment they label. The same approach was used with HMC3 cells, determining that Mitotracker-CMXROS and Hoechst can be used with these cells at the same concentrations used for Caco-2 cells without problems in terms of viability. Nevertheless, Calcein AM at 1µM promotes death in HMC3 cells, concluding that it is not a good option to stain this cell line for long term assays.

Secondly, we have also initiated the culture of Caco-2 cells in transwells in order to study, in the near future, the possible transmission of synuclein from gut cells to neural cells in an in vitro model. This has been the first time these devices have been used at our laboratory.

Thirdly, we have carried out assays oriented towards the induction of an inflammatory response in HMC3 microglia cells, first using known inductors of this reponse and then carrying out similar assays with in vitro synthesized neuromelanin. A combination of proinflammatory cytokines, IL-1β and IFN-γ, at a concentration of 10 ng/mL each can activate microglia cells, as it has been demonstrated by ELISA tests and previously described18. These cytokynes can induce the secretion of around 3000 pg/mL of IL-6 from 1x105 HMC3 cells, more than twenty times compared to uninduced. Although able to induce inflammation in HMC3 cells, these cytokines do not appear to affect viability and metabolic activity of SH-SY5Y and HMC3 cells for at least the first 72 hours, as demonstrated using resazurin assays, so they can be used in co-culture experiments with both cell types. In conclussion, IL-1β and IFN-γ can be safely used to induce a proinflammatory response in microglia co-cultured with SH-SY5Y cells without affecting the viability of any of both cell types by themselves.

Fourthly, we have found out that synthesized neuromelanin is not a good option to induce a proinflammatory response in HMC3 cells under the conditions we have tested so far. Microglia cells treated with neuromelanin a concentration range from 1 µg/mL to 100 µg/mL do not increase significatively the secretion of the pro-inflammatory citokine IL-6. In this case, using neuromelanin at 87 µg/mL, the level of IL-6 is only increased around 20% compared to control, something insignificant compared to the activation induded by cytokines IL-1ß and IFN-γ. On the other hand, neuromelanin within that same concentration range induces SH-SY5Y cell death and reduces the metabolic activity of HMC3 cells around 80% - 90% after 24 and 72 hours respectively. Further experiments are necessary to determine a dose of neuromelanin that will no affect cells in such a drastic mode but rather induce cell stress that could be involved in synuclein misfolding and aggreations. Nevertheles, our assays have been useful in i) synthesizing neuromelanin in vitro in the lab and ii) determining that neuromelaning seriously affects the viability of our neuronal cell model and the metabolic activity of our microglia cell model. Our assays in this line have paved the way for further research in this direction in the laboratory.

Lastly, under the conditions used we have not detected differences in synuclein aggregation inside neuronal cells cultivated independently or in co-cultures with HMC3 cells by flourescence microscopy analysis. We were expecting to observe some α-Syn aggregates inside SH-SY5Y-SNCA cells, an increase in mortality of SH-SY5Y-SNCA cells or an immunological response from HMC3 cells in the co-culture. Nevertheless, this has been a classical problem in Parkinson´s disease using in vitro cellular models and, importantly, we have only had time to carry out some preliminary assays in this direction. Our established conditions for co-culturing SH-SY5Y and HMC3 cells lines can be further exploited in the lab to pursue this research line. For instance, we have not had time to carry out experiments in which cells are stressed with different treatments that can induce oxidative stress, like rotenone, arsenite or iron, which have been described to promote synuclein misfolding and aggregation. These experiments are currently going on in the lab.

In summary, this work has established a new cellular model, which will be further exploited in the lab, based on co-culture of microglia and neuronal cells using, for the first time to our knowledge, the microglia HMC3 cell line together with the neuronal SH-SY5Y cell line. Neuromelanin synthesis in vitro has also been acchieved in order to treat co-cultured cells in an attempt to reproduce the situation in the substantia nigra of the midbrain. Toxicity of neuromelanin to both cell types has been demonstrated.

6. Conclusions

- An experimental co-culture model involving HMC3 and SH-SY5Y cells has been established, optimizing staining conditions to distinguish both cell types and study their interactions by fluorescence microscopy over time.

- We have also set up the culture and differentiation of Caco-2 cells in transwells for future experiments related to the transmission of misfolded synuclein from gut cell to neurons.

- We have set up the enzyme-linked immunosorbent assay (ELISA) at the Institute for Biocomputation and Physics of Complex Systems (BIFI) for measuring pro-inflammatory responses specifically, but also for other similar assays required that level of sensibility.

- We have set up in the lab the induction of a proinflammatory response in HMC3 celsl using IL-1β and IFN-γ cytokines.

- We have been able to synthesize neuromelanin *in vitro* in the lab.

- We have demonstrated that synthesized neuromelanin does not induce a proinflammatory response in HMC3 cells under the conditions tested, but promotes death of SH-SY5Y cells and reduces considerately the metabolic activity of HMC3 cells.

- We have proved that α-synuclein monomers at a range of concentrations between 70nM and 0,3μM do not induce the activation in HMC3 microglia cells.

- Although we have not seen differences in synuclein aggregation inside SH-SY5Y cells co-cultured with HMC3 cells with respect to SH-SY5Y cultured individually under the conditions tested so far, our model allow further experiments in this direction. Cultures for longer times and inducers of oxidative stress are ongoing in the lab to determine which combination of factors is responsible for the induction of synuclein aggregation inside neurons in our *in vitro* model.

7. Bibliography

1. Fahn S. *Parkinson's Disease: The Life Cycle of the Dopamine Neuron*. Vol 991.; 2003.
2. Yamamoto M. Depression in Parkinson's disease: Its prevalence, diagnosis, and neurochemical background. *J Neurol Suppl*. 2001;248(3):5-11. doi:10.1007/pl00022917
3. Brogley JE. DatQuant: The future of diagnosing Parkinson disease. *J Nucl Med Technol*. 2019;47(1):21-26. doi:10.2967/jnmt.118.222349
4. James Surmeier D, Obeso JA, Halliday GM. Parkinson's disease is not simply a prion disorder. *J Neurosci*. 2017;37(41):9799-9807. doi:10.1523/JNEUROSCI.1787-16.2017
5. Breydo L, Wu JW, Uversky VN. α-Synuclein misfolding and Parkinson's disease. *Biochim Biophys Acta - Mol Basis Dis*. 2012;1822(2):261-285. doi:10.1016/j.bbadis.2011.10.002
6. Giráldez-Pérez RM, Antolín-Vallespín M, Muñoz MD, Sánchez-Capelo A. Models of α-synuclein aggregation in Parkinson's disease. *Acta Neuropathol Commun*. 2014;2(1):1-17. doi:10.1186/s40478-014-0176-9
7. Vila M. Neuromelanin, aging, and neuronal vulnerability in Parkinson's disease. *Mov Disord*. 2019;34(10):1440-1451. doi:10.1002/mds.27776
8. Zhang W, Phillips K, Wielgus AR, et al. Neuromelanin activates microglia and induces degeneration of dopaminergic neurons: Implications for progression of parkinson's disease. *Neurotox Res*. 2011;19(1):63-72. doi:10.1007/s12640-009-9140-z
9. Sekiya H, Kowa H, Koga H, et al. Wide distribution of alpha-synuclein oligomers in multiple system atrophy brain detected by proximity ligation. *Acta Neuropathol*. 2019;137(3):455-466. doi:10.1007/s00401-019-01961-w
10. Parvatam S. Can skin cancers reduce the risk of getting Alzheimer ' s disease ? 2018;(May).
11. Krumova P, Meulmeester E, Garrido M, et al. Sumoylation inhibits α-synuclein aggregation and toxicity. *J Cell Biol*. 2011;194(1):49-60. doi:10.1083/jcb.201010117
12. Shahmoradian SH, Lewis AJ, Genoud C, et al. Lewy pathology in Parkinson's disease consists of crowded organelles and lipid membranes. *Nat Neurosci*. 2019;22(7):1099-1109. doi:10.1038/s41593-019-0423-2
13. Lashuel HA, Overk CR, Oueslati A, Masliah E. The many faces of α-synuclein: From structure and toxicity to therapeutic target. *Nat Rev Neurosci*. 2013;14(1):38-48. doi:10.1038/nrn3406
14. Gómez-Benito M, Granado N, García-Sanz P, Michel A, Dumoulin M, Moratalla R. Modeling Parkinson's Disease With the Alpha-Synuclein Protein. *Front Pharmacol*. 2020;11(April):1-15. doi:10.3389/fphar.2020.00356
15. Alam P, Bousset L, Melki R, Otzen DE. A-Synuclein Oligomers and Fibrils: a Spectrum of Species, a Spectrum of Toxicities. *J Neurochem*. 2019;150(5):522-534. doi:10.1111/jnc.14808
16. Wang R, Wang Y, Qu L, et al. Iron-induced oxidative stress contributes to α-synuclein phosphorylation and up-regulation via polo-like kinase 2 and casein kinase 2. *Neurochem Int*. 2019;125:127-135. doi:10.1016/j.neuint.2019.02.016
17. Fink J, Pathak H, Smith J, Achat-Mendes C, Haining RL. Development of a competition-binding assay to

determine binding affnity of molecules to neuromelanin via fluorescence spectroscopy. *Biomolecules*. 2019;9(5):1-13. doi:10.3390/biom9050175

18. Dello Russo C, Cappoli N, Coletta I, et al. The human microglial HMC3 cell line: Where do we stand? A systematic literature review. *J Neuroinflammation*. 2018;15(1):1-24. doi:10.1186/s12974-018-1288-0
19. Goers L, Freemont P, Polizzi KM. Co-culture systems and technologies: Taking synthetic biology to the next level. *J R Soc Interface*. 2014;11(96). doi:10.1098/rsif.2014.0065
20. Chemmarappally JM, Pegram HCN, Abeywickrama N, et al. A co-culture nanofibre scaffold model of neural cell degeneration in relevance to Parkinson's disease. *Sci Rep*. 2020;10(1):1-14. doi:10.1038/s41598-020-59310-x
21. Fanying. M, Lowell CA. Activation and Signal Transduction in the Absence of. *J Exp Med*. 1997;185(9).
22. Timmerman R, Burm SM, Bajramovic JJ. An overview of in vitro methods to study microglia. *Front Cell Neurosci*. 2018;12(August):1-12. doi:10.3389/fncel.2018.00242
23. Chiavari M, Ciotti GMP, Navarra P, Lisi L. Pro-inflammatory activation of a new immortalized human microglia cell line. *Brain Sci*. 2019;9(5). doi:10.3390/brainsci9050111
24. Xicoy H, Wieringa B, Martens GJM. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Mol Neurodegener*. 2017;12(1):1-11. doi:10.1186/s13024-017-0149-0
25. Hasegawa T, Matsuzaki M, Takeda A, et al. Accelerated α-synuclein aggregation after differentiation of SH-SY5Y neuroblastoma cells. *Brain Res*. 2004;1013(1):51-59. doi:10.1016/j.brainres.2004.04.018
26. Watson PMD, Kavanagh E, Allenby G, Vassey M. Bioengineered 3D Glial Cell Culture Systems and Applications for Neurodegeneration and Neuroinflammation. *SLAS Discov*. 2017;22(5):583-601. doi:10.1177/2472555217691450
27. Duval K, Grover H, Han LH, et al. Modeling physiological events in 2D vs. 3D cell culture. *Physiology*. 2017;32(4):266-277. doi:10.1152/physiol.00036.2016
28. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: Influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol*. 2005;21(1):1-26. doi:10.1007/s10565-005-0085-6
29. Ahn EH, Kang SS, Liu X, et al. Initiation of Parkinson's disease from gut to brain by δ-secretase. *Cell Res*. 2020;30(1):70-87. doi:10.1038/s41422-019-0241-9
30. Salvo DI. Human Retinal Vascular Cells Differ from Umbilical Cells in Synthetic Functions and The. (43345):183-191.
31. Kumar R, Harris-Hooker S, Kumar R, Sanford G. Erratum: Co-culture of retinal and endothelial cells results in the modulation of genes critical to retinal neovascularization (Vascular Cell (2011) 3 (27)). *Vasc Cell*. 2012;4:1-15. doi:10.1186/2045-824X-4-6
32. Viceconte N, Burguillos MA, Herrera AJ, Pablos RM De, Joseph B. Neuromelanin activates proinflammatory microglia through a caspase-8-dependent mechanism. Published online 2015:1-15. doi:10.1186/s12974-014-0228-x
33. Smith JA, Das A, Ray SK, Banik NL. Role of pro-inflammatory cytokines released from microglia in neurodegenerative diseases. *Brain Res Bull*. 2012;87(1):10-20. doi:10.1016/j.brainresbull.2011.10.004

34. Ferreira SA, Romero-Ramos M. Microglia response during Parkinson's disease: Alpha-synuclein intervention. *Front Cell Neurosci*. 2018;12(August):1-17. doi:10.3389/fncel.2018.00247

35. Hoenen C, Gustin A, Birck C, et al. Alpha-synuclein proteins promote pro-inflammatory cascades in microglia: Stronger effects of the a53t mutant. *PLoS One*. 2016;11(9):1-24. doi:10.1371/journal.pone.0162717