MASTER THESIS

The role of α-synuclein in the gastrointestinal tract in Parkinson's disease

Author
Imke van Bunningen
s1680382

Faculty of Science and Technology (TNW)
Master Biomedical Engineering

Applied Stem Cell Technology (AST)

Examination Committee
Dr. K. Broersen (chair)
Prof. dr. M.M.A.E. Claessens
L. S. Koch, MSc.

March 13 , 2023

UNIVERSITY OF TWENTE.

Summary

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide(Dorsey et al., 2018). The past decays a wealth of evidence has witnessed that the gastrointestinal system (GIT) and the Gut-Brain-Axis (GBA) are at least to some extent involved in PD pathology (Anis et al., 2022; Rao & Gershon, 2016a; Rietdijk et al., 2017; Schaeffer et al., 2020). Lewy Bodies (LBs) are a prominent biomolecular feature of PD(Kalia & Lang, 2015). These LBs are neuronal protein aggregates, termed amyloids, mainly composed of the α-synuclein protein (Fricova et al., 2020; Schaeffer et al., 2020). In healthy conditions α-synuclein occurs in an unfolded conformation in the cytosol or in association with cellular membranes(Emamzadeh, 2016). It is thought that once misfolded the protein can act as template and force other native proteins to fold in a similar pathogenic structure. In this way the protein can incite a chain-reaction of protein misfolding and aggregation into LBs(Jucker & Walker, 2013). Protein structures that may act as potent templates to induce aggregation are named seeds(Jucker & Walker, 2013). Lewy pathology (LP) in PD is not restricted to the brain but can also be found in the spinal cord and some parts of the peripheral nervous system including the enteric nervous system (ENS)(Braak et al., 2003). Whether the presence of LP and α-synuclein in the ENS is clinically relevant in the course of PD is a topic of ongoing research. This project aims to determine the effects of α-synuclein exposure at the intestinal epithelial barrier. To this end, the effect of direct amyloid exposure on intestinal epithelial barrier health was tested by stimulating a monolayer of Caco-2 cells, which are often used as intestinal model cells, with α-synuclein monomers, seeds or fibrils. Monomers had no significant effect on cell viability. On the contrary, in contrast to expectations, low concentrations of seeds combined with monomers or fibrils alone (50 – 1000 nM) increased cell viability. However, these observations need to be confirmed in additional experiments.

Before the cell viability experiments, it was sought to set-up a simple PD model which would enable to determine the effects of neuronal α-synuclein secretion at intestinal epithelial barrier functioning. To this end, neuroblastoma SH-SY5Y were differentiated by addition of retinoic acid (RA) into a more mature neuronal phenotype. SH-SY5Y cells are an immortalized cell line frequently used to study neurodegenerative mechanisms. Analysis of neuronal marker β-III-tubulin revealed significant neuritic process formation after RA-mediated differentiation. Finally, the use of a simple set-up of LP and the intestinal epithelial barrier was investigated. This Transwell-based set-up consisted of confluent Caco-2 monolayers in the apical compartment and SH-SY5Y overexpressing α-synuclein wild-type (wt) in the basolateral compartment. Some first experiments were performed to test potential model pitfalls. From these it could be concluded that the applied set-up required optimization before use in PD research.

Aims & objectives

The aim of this project was to study the effect of different α-synuclein structures at intestinal epithelial barrier health. To this end, the work was divided in several sub-objectives:

1. Determine the effect of α-synuclein at Caco-2 cell viability.
2. Differentiate SH-SY5Y into a more mature neuronal phenotype.
3. Efficiently transfect SH-SY5Y to introduce α-synuclein overexpression.
4. Combine Caco-2 and SH-SY5Y overexpressing α-synuclein in a Transwell system.

Content

Introduction

General

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease(Dorsey et al., 2018). At its core, PD is characterized by the loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc). The resultant dopamine deficiency in the basal ganglia then leads to the development of the typical motor symptoms associated with PD (Kalia & Lang, 2015). These symptoms may include tremor, muscular rigidity, bradykinesia, postural instability and freeze of gait(Kalia & Lang, 2015). Importantly, PD pathology seems not restricted to the central nervous system (CNS) but may implicate the peripheral nervous system (PNS) as well (Kalia & Lang, 2015; Lebouvier et al., 2010; Rao & Gershon, 2016a; Shannon et al., 2012a). This is among others evidenced by the long list of non-motor symptoms that manifest in PD. Possible non-motor features that occur are diverse ranging from sleep disorders and cognitive impairment to olfactory and autonomic dysfunction(Postuma et al., 2012; Sveinbjornsdottir, 2016). The symptoms commonly manifest at an early stage of PD progression and frequently precede the onset of motor impairments (Kalia & Lang, 2015; Sveinbjornsdottir, 2016). PD etiology is rather complex and despite many research efforts to date the etiology of PD genesis remains largely unknown (Kalia & Lang, 2015a).

Lewy pathology and Prion Paradigm

Lewy Bodies (LBs) are a prominent biomolecular feature of PD(Kalia & Lang, 2015). These LBs are neuronal protein aggregates mainly composed of the α-synuclein protein (Fricova et al., 2020; Schaeffer et al., 2020). In cortical regions the presence of LB is associated with neuronal dysfunction and cell death (Annerino et al., 2012; Corbillé et al., 2014). α-Synuclein is predominantly expressed in the brain where it represents 1% of total protein(Fricova et al., 2020; *Gene [SNCA Synuclein Alpha [Homo Sapiens (Human)]* , 2022). Besides its abundance in brain regions, α-synuclein may also be expressed in some other tissues including the skin, olfactory glands and the gastrointestinal tract(*Gene [SNCA Synuclein Alpha [Homo Sapiens (Human)]* , 2022). Naturally the protein occurs in an unfolded conformation in the cytosol or in association with cellular membranes(Emamzadeh, 2016). The exact cause inducing α-synuclein aggregation remains elusive although α-synuclein is thought to have prion like properties: in a misfolded state the protein can act as template and force other native proteins to fold in a similar pathogenic structure. In this way the protein can incite a chain-reaction of misfolding and aggregation of other proteins into toxic assemblies. The formed pathogenic structures may range from small oligomers to large masses of amyloids(Jucker & Walker, 2013). Structures that may act as template for the growth of fibrillar assemblies are called 'seeds'(Cremades et al., 2012). Seeds coarsely exist in two formats: β-sheet rich oligomers and small fibrillar strands or protofibrils(Cremades et al., 2012).

The GBA in PD

The past decades a wealth of evidence has witnessed that the gastrointestinal system (GIT) and the Gut-Brain-Axis (GBA) are at least to some extent involved in PD pathogenesis (Anis et al., 2022; Rao & Gershon, 2016a; Rietdijk et al., 2017; Schaeffer et al., 2020). The GBA is a bidirectional communication pathway between the GIT and the brain(Chakrabarti et al., 2022a; Rao & Gershon, 2016a). This Gut-Brain interaction occurs via several routes including the nervus vagus, enteric nervous system (ENS), gut microbiota, enteroendocrine signalling and the immune system (Chakrabarti et al., 2022a; Foster et al., 2017).

Lewy pathology (LP) in PD is not restricted to the brain but can also be found in the spinal cord and peripheral nervous system(Braak et al., 2003). In line with this, multiple studies have confirmed the presence of Lewy body pathology in the enteric nervous system (ENS) of PD patients(Lebouvier et al., 2010; Shannon et al., 2012b). Gastrointestinal dysfunction such as constipation and dysphagia are prominent non-motor symptoms in PD (Pfeiffer, 2018). However, whether the presence of LP and α-synuclein in the ENS is clinically relevant in the course of PD is a topic of ongoing research.

α-synuclein and the intestinal epithelial barrier

In the GIT, the intestinal epithelial barrier facilitates nutrient absorption while offering protection against pathogenic invasion(Turner, 2009; Vancamelbeke & Vermeire, 2017). Proper barrier function is essential for life. Chronic intestinal inflammation and decreased barrier integrity are associated with multiple diseases including Crohn's disease, obesity, asthma and depression(Turner, 2009; Vancamelbeke & Vermeire, 2017)The physiological function of α-synuclein in the GIT remains poorly understood. Results from several reports point towards a role as a mediator of innate immune responses(Barbut et al., 2019; Chandra et al., 2017; Gorecki et al., 2021; Prigent et al., 2019; Stolzenberg et al., 2017). Based on this, it is suggested that neuronal α-synuclein might function as pro-inflammatory mediator in intestinal inflammation(Barbut et al., 2019; Gorecki et al., 2021). Intestinal inflammation and an increased intestinal permeability are conditions repeatedly observed in PD(Chen et al., 2019; Schwiertz et al., 2018). Pro-inflammatory responses can be detected in the gut already in early diseases stages(Devos et al., 2013). Chronic inflammation is associated with intestinal barrier damage (Thoo et al., 2019) thereby increasing the exposure to endotoxins and the translocation of commensal bacteria and luminal content(Vancamelbeke & Vermeire, 2017). In line with this, an evaluation of the intestinal permeability in PD patients revealed that PD patients exhibited loss of barrier integrity and that this intestinal hyperpermeability significantly correlated with increased intestinal mucosa staining for E. coli bacteria, oxidative stress, and α-synuclein staining (Forsyth et al., 2011; Shannon et al., 2012b). Nevertheless, more research is required to unravel the role of intestinal α-synuclein in PD. Therefore, one part of this project investigates the effect of direct α-synuclein stimulation at the intestinal epithelial barrier.

PD models

To date the mainstream of PD treatment is symptomatic and the absence of a disease-modifying therapy is a great unmet need(Kalia & Lang, 2015). Unravelling the underlying pathological mechanism and corresponding pharmaceutical targets is of outmost importance to improve PD treatment. Animal studies are still the golden standard before testing novel therapeutics in human. Nevertheless, use of animal models comes with ethical concerns. Further, inter-specie differences at genetic and proteomic level limit the translatability of these models to humans. (Chia et al., 2020) On top of that, despite promising results in pre-clinical studies, drug candidates frequently fail to prove efficiency in clinical trials(Olanow et al., 2008). Therefore, there is a high demand for inventive *in vitro* models with greater physiological relevance(Chakrabarti et al., 2022b; Moysidou & Owens, 2021a; Slanzi et al., 2020a).

As discussed, over the past decades PD has emerged as a complex disease in which both the CNS and PNS are involved(Kalia & Lang, 2015). Especially, the GBA has emerged as a critical player involved in PD pathology(Anis et al., 2022; Rao & Gershon, 2016a; Rietdijk et al., 2017). This raises the need for more complete models that capture more relevant tissues of the GBA. So far, the effect of α-synuclein at the ENS and the intestinal epithelium in PD has not been widely studied. To clarify the relevance of α-synuclein and the GIT in PD, *in vitro* models capturing some of the main interaction aspects are highly necessary(Chakrabarti et al., 2022a; Moysidou & Owens, 2021b). To date several 3D PD *in vitro* models have been developed ranging from organoids to organs-on-chip

systems(Moysidou & Owens, 2021a; Slanzi et al., 2020a). Each model has its own assets and limitations(Slanzi et al., 2020a). Nevertheless, many challenges remain to be addressed for generation of an *in vitro* GBA model that captures all aspects of PD disease (Slanzi et al., 2020a). Depending on the research question relatively simple models can still provide an useful experimental platform. Therefore, the other part of the project is devoted to the establishment of a simple *in vitro* model mimicking the interplay between α-synuclein and the GIT. Therefore, first it is aimed to test a differentiation protocol for neuronal cells. The second aim is to set-up a model of α-synuclein, the intestinal epithelial barrier and neuronal cells.

Cell lines and SH-SY5Y cells

Many *in vitro* models of PD are based on immortalized cell lines grown in 2D culture(Slanzi et al., 2020b). The SH-SY5Y neuroblastoma cell line is one of the cell lines frequently used in PD research (Peng et al., 2021) and will be discussed in more detail. Other cell types that are often used for PD research are for example Lund mesencephalic cells (LUMES), human neuroglioma (H4) and human pheochromocytoma (PC12) cells(Lopes, Bristot, et al., 2017; Lotharius et al., 2005; Slanzi et al., 2020b). The SH-SY5Y neuroblastoma cell line is well-established model to study neuronal processes in vitro(Peng et al., 2021). These cells can be used either in their native undifferentiated or in their differentiated form (Kovalevich & Langford, 2013a; Peng et al., 2021; Shipley et al., 2016a). Morphologically differentiated SH-SY5Y extend long, branched processes and may in some cases have polarized as well. On the contrary, non-differentiated cells are non-polarized, with very few, short processes. Further, differentiated cells express various markers of mature neurons including growth-associated protein (GAP-43), neuronal nuclei (NeuN),synaptophysin (SYN),synaptic vesicle protein II (SV2), neuron specific enolase (NSE) and synaptic associated protein-97 (SAP-97) and an absence of glial fibrillary acidic protein (GFAP) expression. All in all, differentiated SH-SY5Y possess a more mature neuronal phenotype. Therefore differentiated SH-SY5Y are preferred to obtain most accurate results for translation and comparison with *in vivo* models. (Kovalevich & Langford, 2013a; Shipley et al., 2016a)

A number of different methods exist to differentiate SH-SY5Y. This usually includes addition of a differentiation agent along with serum deprivation. A commonly used agent is retinoic acid (RA). Other examples include the use of phorbol esters, dibutyryl cyclic AMP and growth factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Further combination of RA treatment with addition of growth factors such as brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF) has been shown to support neuronal differentiation and maintenance of the mature neuronal phenotype. Further, prior evidence suggests that different differentiation agents may induce different neuronal subtypes such as cholinergic, adrenergic and dopaminergic neurons (Kovalevich & Langford, 2013a). A fundamental part of PD pathogenesis is the neurodegeneration of dopaminergic neurons in cortical regions(Kalia & Lang, 2015). If dopaminergic differentiation desired addition of only RA is not sufficient(Presgraves et al., 2003). However, previous studies found conflicting results with regard to the question which neuronal subtypes are mainly involved in PD in the ENS(Chalazonitis & Rao, 2018). PD pathology in the gut is the main focus of this project. For that reason, RA was initially not combined with another agent.
It is important to realize that the used differentiation method can influence experimental outcomes (Krishtal et al., 2019) and that this could bias comparison between the results from different studies (Kovalevich & Langford, 2013a). For that reason, it is of great importance to develop and use a standardized differentiation procedure and to characterize the treatment-induced cell features before continuing with further experiments(Shipley et al., 2016a). Therefore, a part of this project is devoted to characterize the neuronal phenotype of SH-SY5Y induced by an RA mediated-differentiation procedure. To this end, the extent of differentiation in SH-SY5Y cells will be

characterized after exposing them to 6 days treatment with 10 µM RA. Once well-validated, these cells may be implemented in a Transwell based model of the GBA as well.

TW based models of the intestinal epithelial barrier

The most extensively studied *in vitro* models of the intestinal epithelial barrier are Transwell-based models (Darling et al., 2020). Transwells are cell culturing systems composed of a basolateral and apical compartment in which cell monolayers are separated by a semipermeable filter(Peng et al., 2021). Caco-2 cells are frequently used to modulate adsorptive intestinal epithelial cells (Darling et al., 2020; Tor Lea, 2015). The Caco-2 cell line was originally derived from a human colon carcinoma and has been the most widely used cellular model of the intestinal barrier for years(Darling et al., 2020). These cells grow into a monolayer of cells with many typical properties of mature enterocytes(Lea, 2015). Once polarized Caco-2 have formed tight junctions between adjacent cells and express a large number of enzyme and transporter proteins of the normal human intestine. Cultivation of the cells on filter supports as present in Transwell systems improves their morphological and functional differentiation (Tor Lea, 2015). Transwell-based co-cultures of intestinal epithelial and neuronal cells have been created before. Puzan et al. created a model of Murine duodenal intestinal stem cells cultured above subepithelial myofibroblasts or above complete enteric cultures, containing primary enteric neurons, enteric glia, and myofibroblasts(Puzan et al., 2018).In another approach Satsu et al. established a Transwell-based model of PC12 and Caco-2 cells(Satsu et al., 2014). Both these models were focussed on the influence of the presence (ENS) neurons on the epithelial barrier health (Puzan et al., 2018; Satsu et al., 2014).

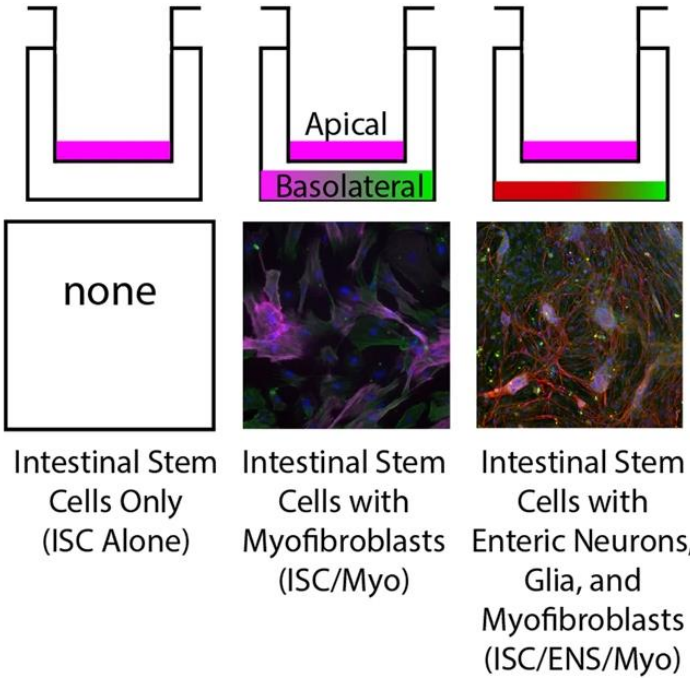


Figure 1 A previously created set-up of both intestinal and neuronal cells in a Transwell system. Murine duodenal intestinal stem cells (ISC) were harvested and grown as organoids for one 7 days prior to dissociation and monolayer seeding: either alone (ISC alone), above subepithelial myofibroblasts (ISC/Myo), or above complete enteric cultures, containing enteric neurons, enteric glia, and myofibroblasts (ISC/ENS/Myo). (Puzan et al., 2018)

Materials and Method

General approach

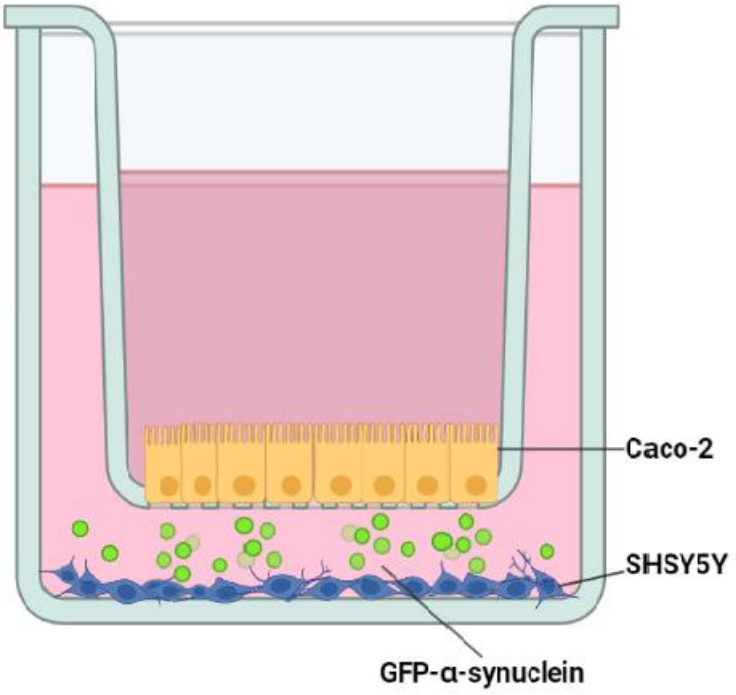


Figure 2 Schematic of the set-up used to modulate the intestinal epithelial barrier, neuronal cells and α-synuclein secretion. Caco-2 cells served as a proxy for the adsorptive cells of the intestinal epithelial barrier. SHSY5Y cells functioned as a reduced mimi of enteric neurons. To incorporate the presence of α-synuclein, the SHSY5Y cells were transiently transfected with a plasmid of α-synuclein conjugated to GFP (marker). The figure has been created using BioRender.com .

One part of this project focusses on the creation of a very simple PD model. Precisely it was sought to set-up a system that would enable to determine the effects of neuronal α-synuclein secretion at intestinal epithelial barrier functioning. First a standardized RA-based differentiation protocol for SH-SY5Y was tested. Subsequently, neuronal and intestinal epithelial cells were combined in a Transwell system. To modulate PD pathological processes SH-SY5Y cells transfected with an α-synuclein plasmid represented enteric neurons excreting α-synuclein(Peng et al., 2021). Caco-2 cells served as proxy for adsorptive intestinal epithelial cells (Darling et al., 2020; Tor Lea, 2015). Transwell-based co-cultures of intestinal epithelial and neuronal cells have been created before. The desired model of this project differed from these set-ups as it incorporates α-synuclein secretion to mimic PD LB pathology.

Cell culturing and differentiation

SH-SY5Y cells were cultured in Dulbecco's Minimum Eagle Media F-12 (DMEM, Gibco, Cat. no. 31331028) supplemented with GlutaMAX supplement and 10% Fetal Bovine Serum (FBS, Gibco, Cat. no. 10500064), 1 % penicillin/streptomycin (pen/strep, Gibco, Cat. no. 15070063) and 1 % non-essential amino acids (NEAA, Gibco, Cat. no. 11140050). Trypsin-EDTA (0,05%, Gibco, Cat. no. 2500005) was used for cell passaging. Cells were incubated at 37 ºC, 95% humidity, and 5% CO_2 and used between P15-32. For cell differentiation, culture medium was replaced one day after seeding the cell in well plates or coverslips by starvation medium consisting of DMEM/F12 supplemented with 10 µM retinoic acid (RA, Sigma, Cat. no. 2625), GlutaMAX, 1 % FBS, 1% pen/strep and 1% NEAA. Cell medium was refreshed every 1-2 days.

For co-cultures, non-differentiated SH-SY5Y cells were used. Caco-2 cells were cultured in DMEM completed by addition of 20% FBS, 1% pen/strep and 1% NEAA. The Caco-2 cells were used between P32-55 and cell passaging was done using Trypsin-EDTA with a concentration of 0.25%.

Immunofluorescence

Differentiated and undifferentiated SH-SY5Y cells were both characterized for the expression of a variety of differentiation markers: β-3-Tubulin, MAP-2 and Synapsin 1/2. To this end, cells were seeded on 24-well coverslips (Menzel-Gläser, Cat. no. MENZCB00190RA120, diameter: 19 mm).

Undifferentiated SH-SY5Y proliferate rapidly whereas differentiating SH-SY5Y have a decreased proliferation rate(Kovalevich & Langford, 2013a). Cells were seeded at a density of 75,000 and 5000 cells/well for differentiated and non-differentiated SH-SY5Y cells, respectively. Seeding densities were chosen to obtain a confluent cell layer at the start of the experiment and were based on experiences during previous experiments. Coverslips were first sterilized by incubation in 70% ethanol for 15 minutes at RT, followed by 3 times washing with DPBS (Gibco, Cat. no. 141 90094). The coverslips were coated with Laminin I (Biotechne R&D systems, Cat. no. 3400-010-02) 1:500 in DPBS to improve cell surface attachment. At day 6 of differentiation, cells were fixated with 4% PFA in PBS for 10-15 minutes at RT after which they were washed 3 times with PBS. Cells were then incubated for 1-2 hours at RT with blocking buffer consisting of 3% Bovine Serum Albumin (BSA, Sigma, Cat. no. A9418-50G) and 0.1% Triton-X (Sigma, Cat. no. T8787) in PBS.

Primary antibodies were diluted in the blocking buffer, added and cells were incubated overnight at 4ºC. The next day, cells were washed 4 times for 5 minutes in PBS (Gibco, Cat. no. 10010023). Subsequently, cells were incubated for 45 minutes at RT with secondary antibodies in blocking buffer. Cell nuclei were counterstained with DAPI (ThermoFisher Scientific, Cat. no. D1306). The used antibodies are summarized in table 1. After incubation, cells were washed again 4 times with PBS. The cells were imaged with the Fluorescent EVOS Microscope (Invitrogen EVOS FL Imaging System, ThermoFisher Scientific).

Table 1 Overview used antibodies immunohistochemistry

Antibody	Supplier	Category number	Used dilution
β-3-Tubulin (2G10)	Santa Cruz	sc-80005	1:500
Synapsin 1,2	SySy	106 004	1:500
MAP-2	Abcam	ab32454	1:500
Alexa Fluor 488 goat-anti-mouse	Invitrogen	A11001	1:500
Alexa Fluor 647 goat-anti-guinea pig	Invitrogen	A21244	1:500
Alexa Fluor 647 goat-anti-rabbit	Invitrogen	A21450	1:500

Cell Transfection

For Transwell studies SH-SY5Y cells were transiently transfected using Lipofectamine 2000 (Lipo 2000, Invitrogen, 11668030) in OptiMEM (OM, Gibco, 11058021) with plasmids expressing wild type (wt) α-synuclein conjugated with GFP (pEGFP-N1_ wt_aSYN(Y136TAC)). To this end, SH-SY5Y cells were seeded at 50,000 cells/well in Lamin-coated (1:500 in PBS) 24-well plates (Bio-one Greiner, Cat. no. 662160). One day before start of the co-culture, SH-SY5Y cells were washed once with PBS followed by addition of 950 µL OM. Then complex mixtures of DNA plasmid with Lipo 2000 were prepared by diluting the DNA plasmids and Lipo 2000 in OM in separate Eppendorf tubes. Both solutions were mixed well. Subsequently, the solutions were combined to yield a final concentration of 500 ng DNA plasmid per well. The complexes were incubated 5 minutes at RT and then 50 µL was dropwise added to each well. Cells were incubated for one more day before use.

The transfection procedure was based on an existing Lipo 2000 based cell transfection protocol which was optimized prior to Transwell experiments (Carla Annink, 2020; Technologies, 2000). During these experiments cells were seeded at variable seeding concentrations between 12,500 and 75,000 cells/ml in Lamin-coated (1:500 in PBS) 24-well plates. The optimal initial cell seeding density for cell transfection was found to amount 50,000 up to 75,000 cells/well for differentiated cells and 5,000 cells/well non-differentiated cells. Besides the wild type α-synuclein plasmid, the H2B conjugated with GFP (pEGFP-N1_H2B) plasmid was included to serve as a positive control. Further, the duration of plasmid expression was analysed. Plasmid expression was monitored up to 3 days post transfection and successful transfection was validated by fluorescence imaging using the Fluorescent EVOS Microscope (Invitrogen EVOS FL Imaging System, ThermoFisher Scientific). Protein production was clearly detected after 24 hours. For that reason, this duration was taken as a starting point for further experiments. The used plasmids were a kind gift of Kirsten Leijenhorst and obtained as described previously (Kirsten van Leijenhorst-Groener, 2021).

Co-culturing

To set-up the neuronal/intestinal epithelial co-culture, Caco-2 cells were seeded at 8000 cells/well in 24-well Transwell inserts (Bio-one Greiner, Cat. no. 662630, PET membrane, pore size 3 µm) in duplicate. Subsequently, the cells were cultured for 21 days at 37 ºC, 95% humidity, and 5% CO_2 to allow cell maturation. At day 14 of Caco-2 culturing SH-SY5Y cells were seeded at an eventual density of 27.000 cells/well in separate 24-well plates (Bio-one Greiner, Cat. no. 662160) and transfected with the wt α-synuclein plasmid after one day as described (see Materials & Method, cell transfection). One day before Transwell set-up the Caco-2 cells were subjected to medium with 10 % of FBS instead of 20%. At day 22 of Caco-2 culturing, Transwell inserts were transferred to the SH-SY5Y cell culture plates. As negative control, Caco-2 were cultured without SH-SY5Y cells in the basolateral compartment as well. Co-culture was maintained in 10% FBS medium until use for TEER measurements and RNA isolation.

TEER measurements

The transepithelial resistance (TEER) was determined daily up to three days after Transwell set-up using an EVOM transepithelial voltmeter (World Precision Instruments) connected to its compatible STX4 electrode (World Precision Instruments). Before use, electrode chopsticks were sterilized in 70% ethanol followed by DPBS washing (Gibco, Cat. no. 14190094). An insert with medium only served as blank and all TEER measurements were carried out in triplicate. To obtain the final TEER value in ohm*cm^2 , the final TEER value was calculated using the following formula:

$$\big((TEER_s) - blank\big) * 0{,}33\ cm^2 \qquad\qquad TEER_s = average\ TEER\ sample$$

qPCR

mRNA was isolated using the RNeasy Mini Kit (Qiagen, Cat. no. 74104&74106) according to the manufacturer's protocol except for cell lysis which was performed without addition of β-mercaptoethanol. To isolate Caco-2 cell lysate from Transwell inserts, a 200 µL pipet filter tip was used to scrape off the cells from the membrane surface. Potential sample contamination and the RNA concentration were determined with a Nanodrop 1000 spectrophotometer (NanoDrop, Unity Lab Services, Thermo Fisher Scientific). An 260/230 ratio between 1.8 and 2.2 was considered as pure RNA. cDNA samples were produced from 0,5 - 1 µg RNA as input, using an iScriptTM cDNA synthesis kit (Bio-Rad, Cat. no. 170-8891) according to the manufacturer's protocol. Gene expression was analysed with qPCR using a SensiMix SYBR® & Fluorescein Kit (Meridian bioscience Bioline, Cat. no. QT 615-05) in a 96-well plate (Bio-rad, Cat. no. HSP9645). The sequences of the used primers (Sigma) are listed in table 2. The thermal cycling protocol consisted of a hot start at 95 ºC for 10 minutes

followed by 40 cycles of 95 ºC of melting for 15 seconds, 62 ºC annealing for 15 seconds and 15 seconds of extension at 72 ºC. The procedure was finalized by a gradual temperature increment to obtain the melt curve. Thermocycling was performed with the Bio-Rad CFX96 thermocycler.

Table 2 Overview primer sequences

Gene	**sequence**	**Cat. no.**
TJP-1 (fwd.)	5'-TGGTGTCCTACCTAATTCAACTCA-3'	8816436497-000010
TJP-1 (rev.)	5'-CGCCAGCTACAAATATTCCAACA-3'	8816436497-000020
CLDN-1 (fwd.)	5'-CTGTCATTGGGGGTGCGATA-3'	8816436497-000030
CLDN-1 (rev.)	5'-CTGGCATTGACTGGGGTCAT-3'	8816436497-000040
NFκB (fwd.)	5'-GCAGCACTACTTCTTGACCACC-3'	8818409219-110/0
NFκB (rev.)	5'-TCTGCTCCTGAGCATTGACGTC-3'	881840219-120/0
GAPDH (fwd.)	5'-GTCTCCTCTGACTTCAACAGCG-3'	881849219-190/0
GAPDH (rev.)	5'-ACCACCCTGTTGCTGTAGCCAA-3'	8818409219-200/0

α-synuclein preparation

Previously, α-synuclein wt monomers had been aliquoted at 250 µM and stored at -20 ˚C till use. Aliquots were thaw and the protein concentration was measured using A280 with Nanodrop 1000 spectrophotometer (NanoDrop, Unity Lab Services, Thermo Fisher Scientific). Measurements were taken at 276 nm and 10 mM Tris PH 7.4 (Merck, Cat.no. 1.08382.0500) served as a blank. Subsequently, the protein concentration was calculated applying the law of Lambert beert.

$$E = \varepsilon * c * l$$

$$\varepsilon = molar\ extinction\ coëfficient\ \alpha - synuclein\ wt = 5600 \frac{L}{mol * cm}$$

$$c = concentration\ (\frac{mol}{L}) \qquad E = extinction\ (no\ unit) \qquad l = cuvet\ lenght = 1\ cm$$

Subsequently, a desired volume of protein was prepared at 100 µM in α-synuclein buffer which consisted of Tris 10 mM, NaCl 10mM and EDTA 0.1mM at pH 7.4 (All from Merck, Cat.no. 1.08382.0500, 1.06404.1000 and 1.08418.0250 respectively). Monomers were temporarily stored at – 4 ˚C and used the same day for cell stimulation. For fibril and seeds preparation, the protein sample was split in two parts and transferred to 2ml Low-binding round bottom tubes (Eppendorf). Then, the solutions were incubated for 8 days at 37˚C using the Thermomixer (Eppendorf) with an orbital shaking of 750 rpm.

Optionally, amyloid formation was monitored by frequently measuring the fluorescence spectrum of thioflavin T (ThT) with a Cary Eclipse fluorescence spectrophotometer (Varian). To this end, an aliquot of 5µL was taken and diluted in 2mL of ThT 5µM Glycine 50mM buffer with PH 8.2. The following settings were applied during measurements:

- Excitation wavelength: 458nm
- Emission wavelength: 475-600nm

- Excitation slit: 10nm
- Emission slit: 10nm
- Speed measure: medium
- PMT: medium

Incubation was stopped at the end of aggregation kinetics which appeared as plateau phase in the S-curve of the ThT spectrum. Preparations were performed multiple times and it turned out that steady state was achieved after 8 days. For that reason, an incubation period of 8 days was maintained during successive fibril preparations.

After incubation the samples were centrifuged at 21000 x g at RT for 1h using the IEC Micromax microcentrifuge (Thermo Fisher Scientific). To this end, duplo samples were pooled, mixed and split again to balance the centrifuge.

Subsequently, the supernatant was removed and A280 Nanodrop measurements were performed to obtain the residual monomer concentration (RMC). The resultant fibril concentration was calculated by subtraction of the RCM from the initial monomer concentration of 100 µM.
From these, fibril solutions were prepared by resuspending the pellet (fibrils) in a volume of α-synuclein buffer to obtain a final fibrils concentration of 100 µM (monomers equivalent). After preparation fibrils remained stable for approximately one year at RT.

$$pellet\ fibril\ concentration = 100 - RCM$$

To obtain seeds, fibrils were fragmented using the bath sonicator (Branson) for 4 min at regular power. This procedure yielded seeds of approximately 700 nm in length. Seeds were used within a day after fibril fragmentation.

All monomers, seeds and fibrils were prepared and kindly provided by Jonathan Vaneyck.

Cell viability

Cell viability was tested using the Cell Titer Glo assay (Promega, Cat. no. G9241) which is based on measuring the intracellular ATP concentration reflecting cell metabolic activity(Promega Corporation, n.d.). Cells were seeded in a 96-well plate (Greiner Bio-one, Cat. no. 655098) at a density of 33,000 cells/cm^2 (100 000 cells/ml) and exposed to increasing concentrations (100 nM – 25 µM) of concentrations of α-synuclein monomers or fibrils (low: 100 – 1000 nM and high: 1 - 25 µM) for 72 hours. Alternatively, cells were exposed to seeds or seeds together with monomers for 72 hours (50 – 500 nM of both structures). Proto-fibrils were used as seed. Cell viability was tested as well after exposure to cytokines TNF-α (10, 20 and 50 ng/ml) or IL-1β (10 and 25 ng/ml) and lipopolysaccharides or endotoxins (LPS, 10 µg/ml). Staurosporin (10 µM, Millipore Sigma, Cat. no. 569367) served as positive control. After 3 days of incubation, cell stimulation medium was replaced by 100 µL new medium and an equal volume of Titer Glo reagent was added to each well. Subsequently, the plate was shaken for 2 minutes at/using an orbital shaker (400 rpm, KS250 Basic, Ika Labortechnik) and allowed to react at RT for an additional 10 minutes. Luminescence was measured using the Victor 3 1420 Multilabel HtS Counter (PerkinElmer).

Statistical Analysis

Unless otherwise stated, all data is represented as mean ± SEM. Experiments were carried out in duplo or triplo. Statistical relevance was determined with GraphPad Prism 8.0.1 (GraphPad Software, La Jolla, CA) by applying one-way ANNOVA followed by Dunnet's post-hoc test. P-values < 0,05 were deemed statistically significant.

Results

α-synuclein molecular structures differentially effect Caco-2 cell viability

To date, only a few studies have investigated the effect of direct amyloid exposure at the epithelial barrier (Pellegrini et al., 2022; Puig et al., 2015). Excessive cell death leads to a decrease in epithelial barrier health and integrity(Delgado et al., 2016; Subramanian et al., 2020). Here the effect of α-synuclein stimulation on cell viability was tested using a Cell Titer Glo 2.0 assay. Intestinal Caco-2 cells were exposed for 72 hours to increasing concentrations of α-synuclein monomers, fibrils, seeds or monomers combined with seeds. Alpha-synuclein has been detected in enteroendocrine cells and enteric neurons in the mice and human intestine(Chandra et al., 2017). Nevertheless, data on the precise endogenous expression levels of α-synuclein by intestinal epithelial cells or enteric neurons in the ENS is lacking. The concentrations used are therefore based on previous research(Pellegrini et al., 2022; Puig et al., 2015) and amounted 100, 250, 500, 1000 nM for monomers, fibrils and seeds alone. In addition to that cells were stimulated with 1,5, 10, 25 µM monomers or fibrils. Further, cells were exposed to 50, 125, 250 or 500 nM seeds and monomers. Cell viability after exposure to cytokines interleukin-1 beta and tumor necrosis factor alpha (IL-1β and TNF-α) or lipopolysaccharides (LPS), was confirmed as well.

Monomeric α-synuclein is generally considered as functional and non-toxic (Roberts & Brown, 2015). In line with this, even the highest concentration of α-synuclein monomers did not affect cell viability (figure 1). The Cell Titer Glo 2.0 assay is based on cellular ATP production. Interestingly, lower concentrations of α-synuclein fibrils (100, 250, 500 nM) or monomers in combination with seeds (50-500 nM) increased the metabolic activity in Caco-2 cells compared to untreated cells (figure 2 & 3). Although it should be mentioned that the obtained results of 500 nM fibrils are controversial (figure 2A versus 2B). Seeds alone or higher concentrations of fibrils did not affect cell viability (figure 2 & 3).

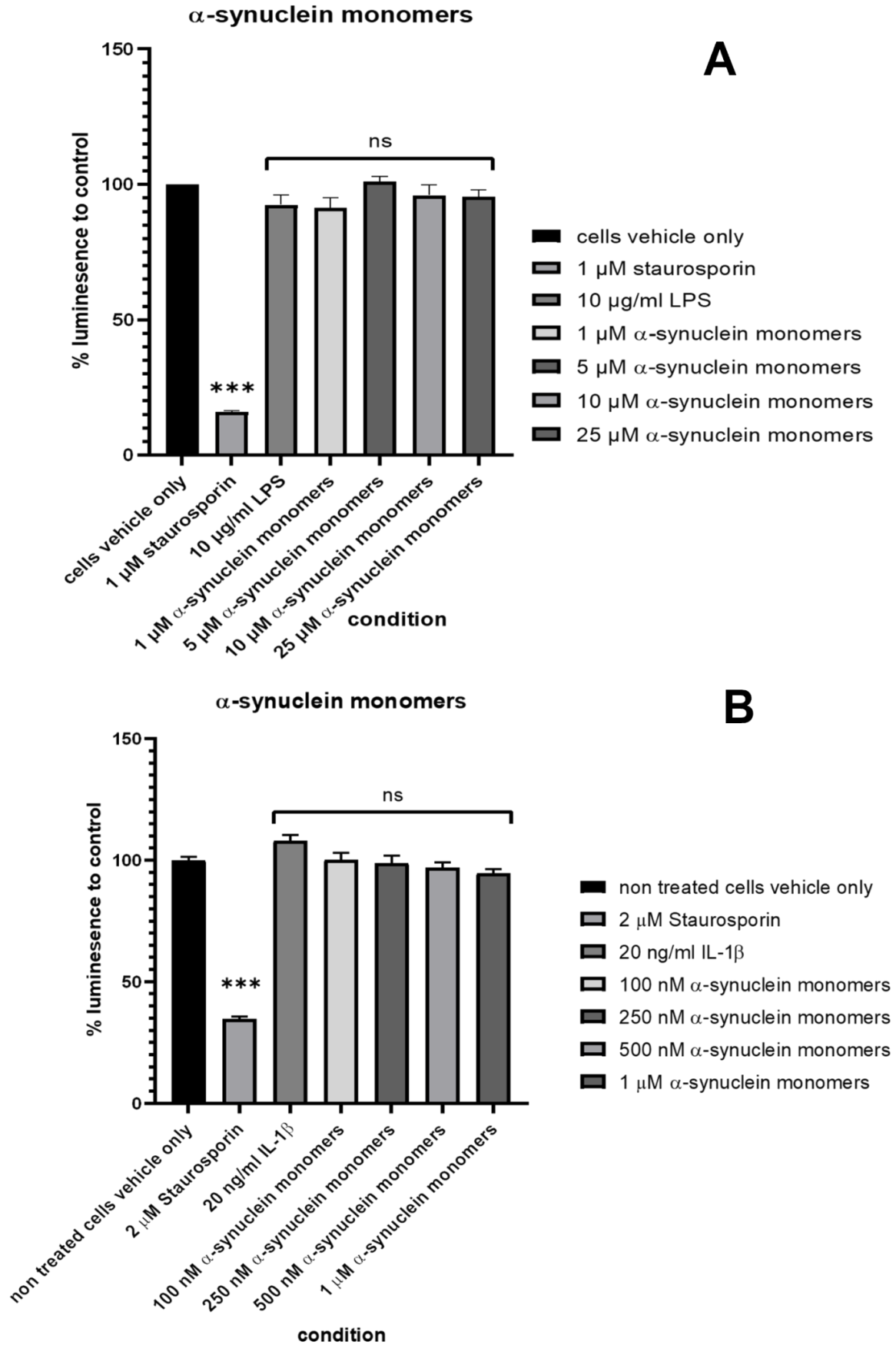


*Figure 3 Cell viability of Caco-2 after 72hrs exposure to high 1,5,10,25 µM (A) or low 100,250,500, 1000 nM (B) concentrations of α-synuclein monomers. Cell viability is expressed as % to non-treated cells. No effect on cell viability was observed for α-synuclein monomers (*** P<0,001 ;** P<0,01;* P< 0,05).*

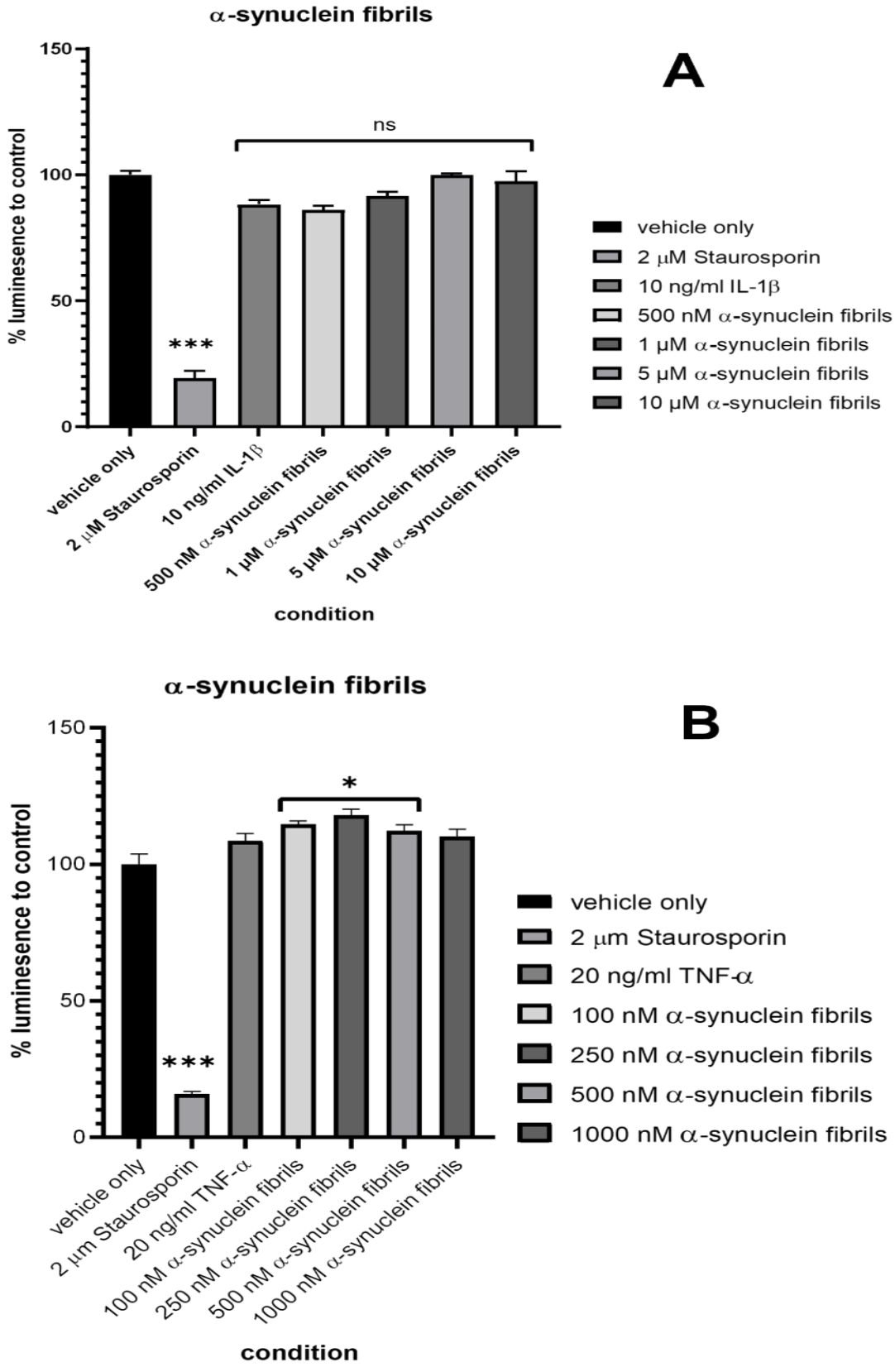


*Figure 4 Cell viability of Caco-2 cells after 72hrs exposure to high 1,5,10,25 μM (A) or low 100,250,500, 1000 nM (B) concentrations of α-synuclein fibrils. Cell viability is expressed as % to non-treated cells. No effect on cell viability was observed at high concentrations. On the contrary, low concentrations of fibrils tended to increase cell metabolic activity. (*** P<0,001;** P<0,01;* P< 0,05).*

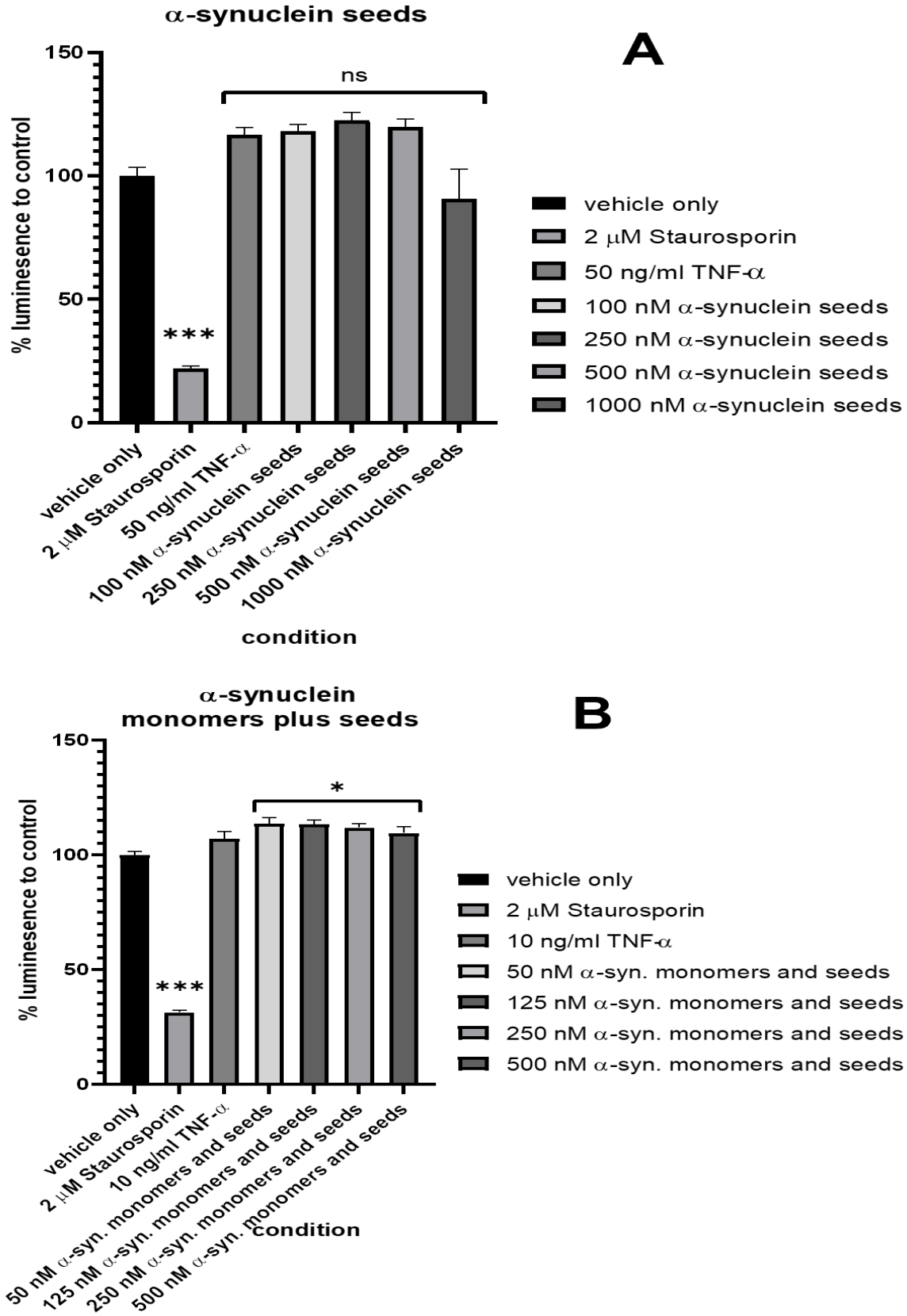
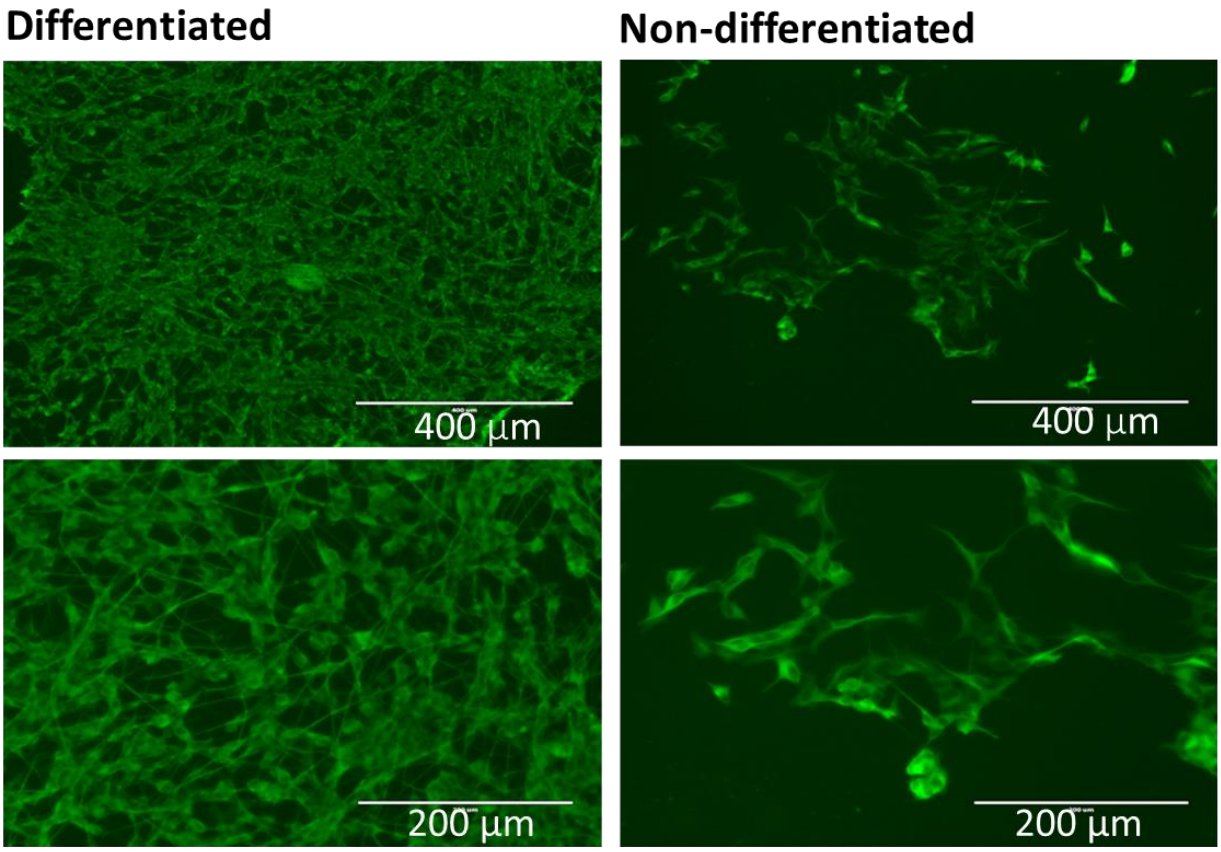


*Figure 5 Cell viability of Caco-2 after 72hrs exposure to 100,250,500, 1000 nM α-synuclein seeds (A) or seeds combined with monomers at equimolar concentrations (B) . Cell viability is expressed as % to non-treated cells. No effect on cell viability was observed for α-synuclein seeds alone. On the contrary, seeds combined with monomers seemed to increase cell metabolic activity. (*** P<0,001 ;** P<0,01;* P< 0,05).*

Formation of neuronal processes after RA treatment

One goal in this project was to test a standardized differentiation procedure for SH-SY5Y. For that reason, SH-SY5Y were differentiated by treatment with 10 µM retinoic acid in starvation medium for 6 days. After, treated and non-treated SH-SY5Y were characterized for the presence of different neuronal differentiation markers namely β-III-tubulin, microtubule associated protein 2 (MAP-2) and Synapsin 1/2. This to analyse the extent of neuronal differentiation induced by the applied protocol.

Differentiated and non-differentiated SH-SY5Y could be clearly distinguished by the staining with β-III-tubulin (figure 4). This staining revealed a morphology characteristic for differentiated SH-SY5Y with extended branched processes in RA treated cells compared to only short and diffuse extensions for non-treated cells. Signal of the MAP-2 staining was found both in differentiated and non-differentiated cells (figure 5). From optical analysis, it appeared that the extent of expression was not altered by RA treatment. Quantitative analysis however was limited by the low number of non-differentiated cells to analyse. Synapsin 1/2 served as neuronal synaptic marker (Mirza & Zahid, 2018). For that reason, the immunohistochemical staining of synapsin 1/2 is expected to be visible at or in the approximant of neural synaptic junctions. Nevertheless, no reliable data was obtained during this project of Synapsin 1/2 staining (data not shown).



1

Figure 6 Expression of neuronal marker β-III-tubulin in RA-treated (left) and non-differentiated (right) SHSY5Y at 10x (up) and 20x (bottom) magnification. Representative images after 6 days of differentiation. Images were adapted for brightness at an equal level. Differentiated SHSY5Y exhibit characteristic neuronal morphology with extended branches.

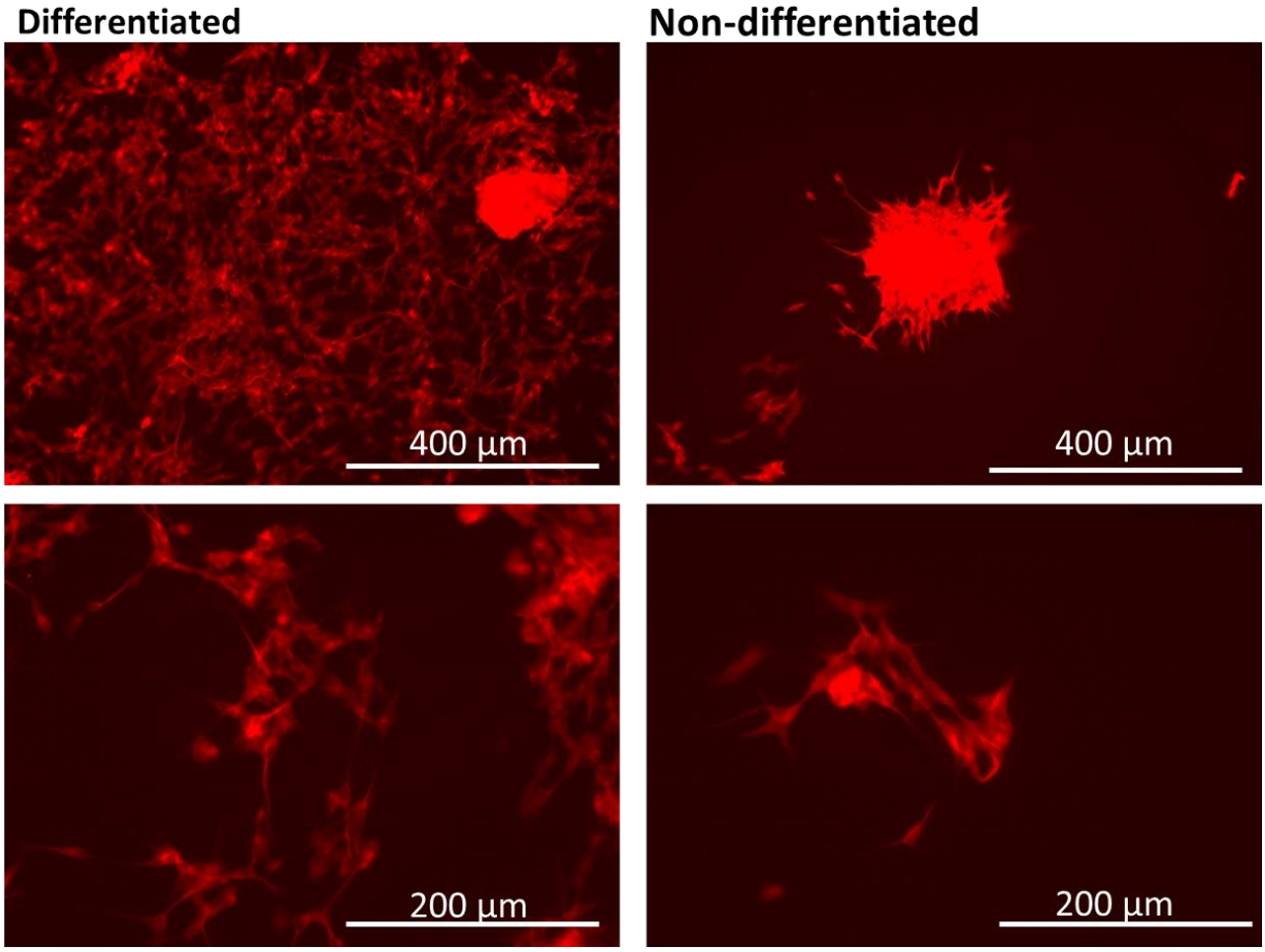


Figure 7 : Expression of neuronal marker MAP-2 in RA-treated (left) and non-differentiated (right) SH-SY5Y at 10x (up) and 20x (bottom) magnification. Representative images after 6 days of differentiation. Images were adapted for brightness at an equal level. Both treated and non-treated cells exhibit MAP-2 expression.

Co-culture of Caco-2 with SH-SY5Y seemingly decreased epithelial barrier integrity

In this project a simple system was set-up to study the effect of neuronal cells and α-synuclein on intestinal epithelial barrier functioning. To illuminate potential model pitfalls, some first experiments were performed. To this end, Caco-2 were cultured till confluency in Transwell inserts after which they were co-cultured with either SH-SY5Y overexpressing α-synuclein, SH-SY5Y wild-type (wt) or without cells in the basolateral compartment. Stable expression of α-synuclein by transfected SH-SY5Y for at least 3 days had been confirmed previously (see appendix figure 2). Gene expression of Claudin-1 and tight junction protein 1 (CLDN-1 and TJP-1) was determined after one day. These results indicated that the gene expression of both TJP-1 and CLDN-1 was not changed after 24hrs of co-culturing (see figure 7).

The initial TEER value of Caco-2 monolayers was determined one day before combining the cells with SH-SY5Y. To monitor epithelial barrier integrity, TEER measurements were performed up to 72hrs after combining the two cell lines. Measurements were performed daily and compared to the initial TEER values. Up to 48 hrs, TEER values did not reveal any significant difference between the different treatment groups (figure 6). On the contrary, 72 hours after combining the two cell lines in a Transwell system the TEER values of Caco-2 combined with SH-SY5Y had dropped remarkedly in comparison to those of the Caco-2 without any cells in the basolateral compartment. Subsequent microscopical analysis rose the suspicion that many neuronal cells had adopted a necrotic or apoptotic morphology (see figure 8). Cell morphology was compared to the morphology of SH-SY5Y cells one day before combining the cells with Caco-2 cells (figure 9). At 72hrs many SH-SY5Y cells had detached from the cell culture surface and some cells exhibited cell surface blebs of different structures, a feature of apoptosis(Kalinichenko & Matveeva, 2008). Besides floating cell clumps much more loose cell debris was observed in the culture medium as well. However, the image quality and magnification of the saved images were too low and did not allow to confidently distinguish potential cell features of neurodegeneration upon closer analysis (figure 8).

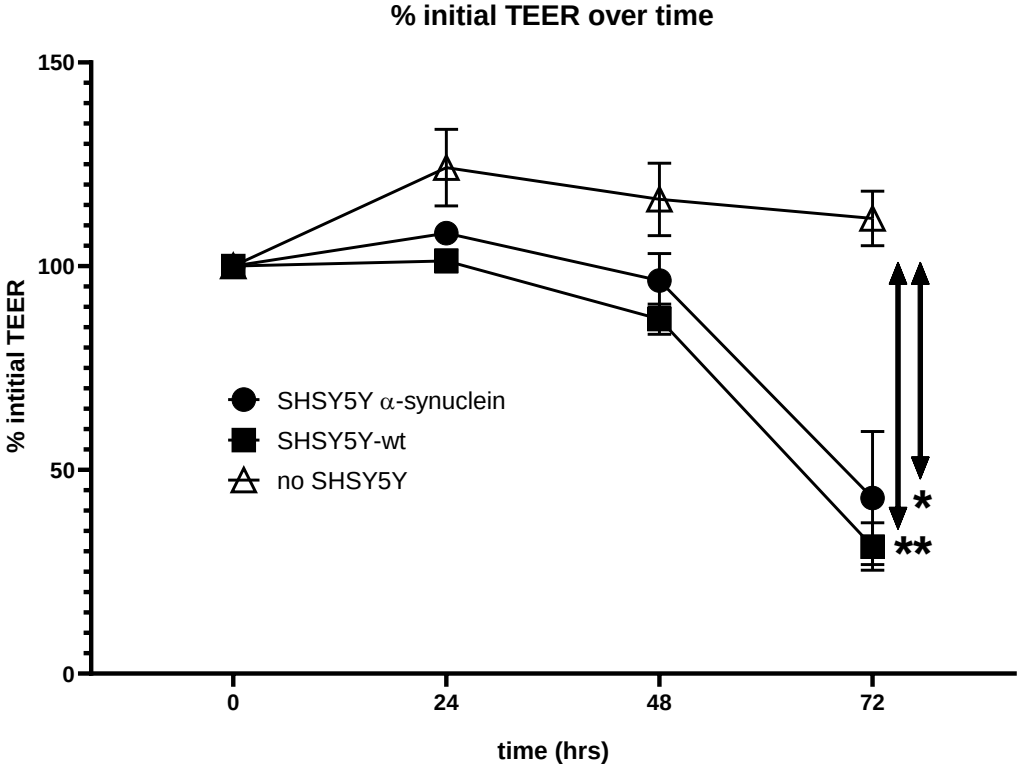


*Figure 8 TEER values of Caco-2/ SH-SY5Y transwells up to 3 days of co-culture with SH-SY5Y overexpressing α-synuclein or SH-SY5Y wt. TEER values are compared to initial TEER values. These are the measurements of Caco-2 alone one day before setting-up the Transwell system with both cell lines. Data analysis was performed with one-way ANNOVA followed by Dunnet's post-hoc test. Values are expressed as % of initial TEER value for each condition. Data are presented as mean ± SEM * (p<0,05); ** (p<0,01) ; *** (p< 0,001). The results indicated that TEER values remained stable up to 48hrs of co-culture after which they remarkedly dropped between 48 and 72hrs.*

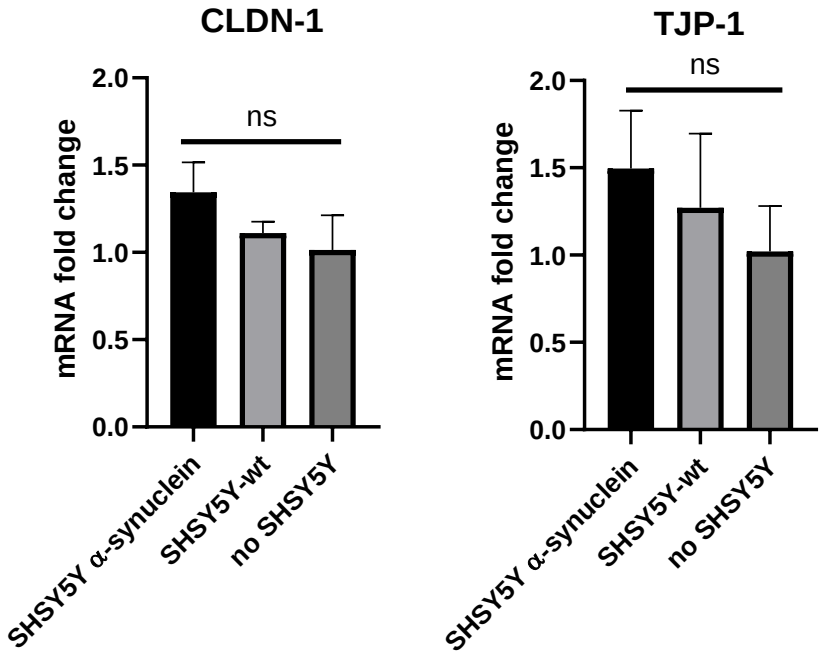


Figure 10 mRNA levels of CLDN-1 and TJP-1 after 24 hrs in co-cultures as compared to Caco-2 monoculture, normalized to GAPDH using the 2-ΔΔCt method determined with qPCR.

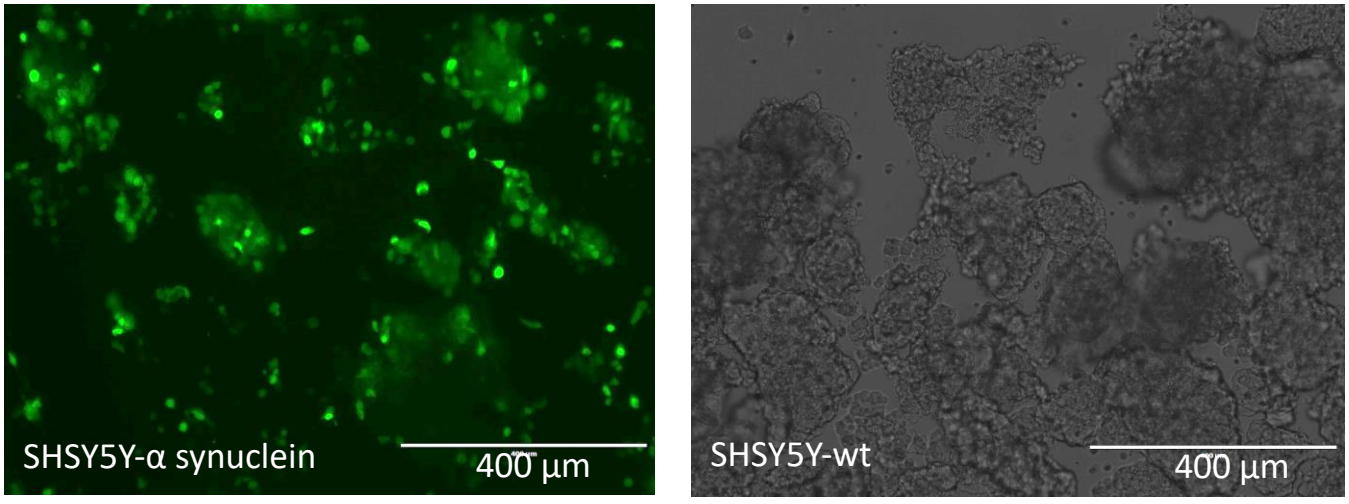


Figure 9 SHSY5Y overexpressing α-synuclein and SHSY5Y wt after 72 hrs of co-culturing with Caco-2 in Transwell systems at 10x magnification. The green fluorescence resulted from transfecting the SHSY5Y with a plasmid of α-synuclein conjugated to GFP and indicated α-synuclein expression. Parts of the cell layer appeared to have detached from the culture surface and cells seemingly exhibited signs of cell death as based on closer cell morphology analysis.

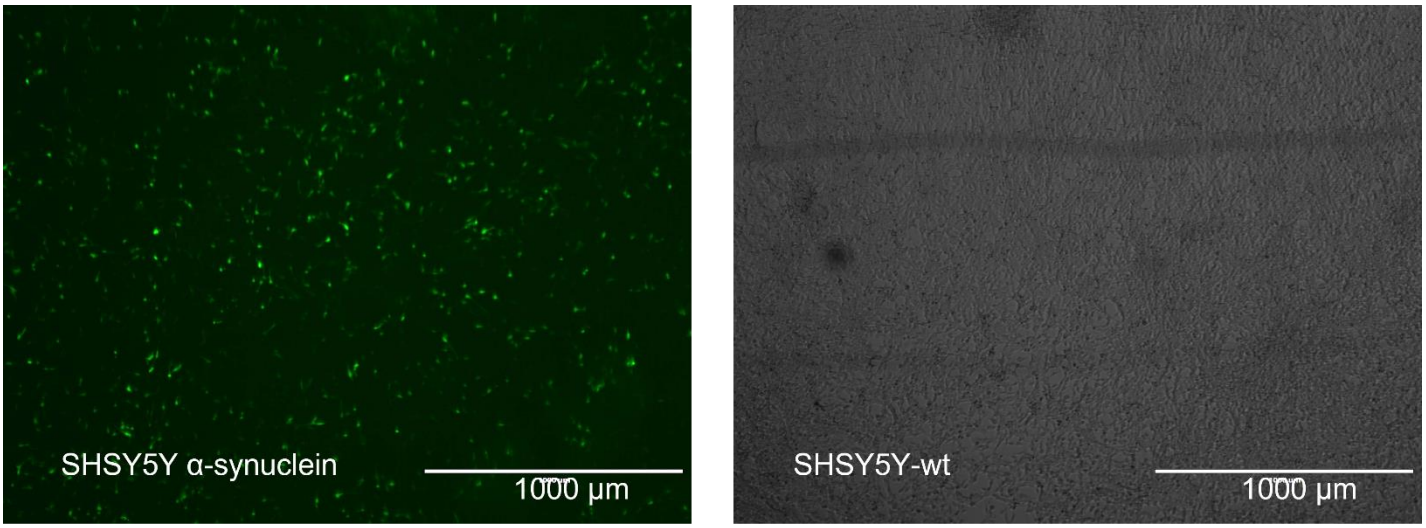


Figure 11 SHSY5Y overexpressing α-synuclein and SHSY5Y wt one day before starting the co-culturing with Caco-2 in Transwell systems at 4x magnification. Cells are attached to the culture surface and have nearly grown into a confluent monolayer. The green fluorescence resulted from transfecting the SHSY5Y with a plasmid of α-synuclein conjugated to GFP and indicated α-synuclein expression.

Discussion

Direct stimulation of Caco-2 with α-synuclein

First, it was aimed to study cell viability changes in Caco-2 cells upon exposure to α-synuclein monomers, fibrils and seeds. The Cell Titer glo 2.0 assay gives a qualitative indication of intracellular ATP concentrations. This is correlated to the extent of viable cells to allow comparison between different treatment conditions. We observed that α-synuclein monomers did not affect cell viability whereas fibrils and seeds together with monomers seemingly caused a slight increase in intracellular ATP content.

The term toxic α-synuclein oligomers is not well-defined and may include fibrillar as well as oligomeric structures of the protein. In neurons monomeric α-synuclein is generally considered as non-toxic. Oligomers on the contrary are considered as the main culprit. The toxicity of fibrillar structures is controversial (Roberts & Brown, 2015). Regarding the factors affecting the level of protein toxicity the amount of β-sheets secondary structures, which is enriched in oligomeric and fibrillar a-synuclein, is regarded as the most prominent factor (Roberts & Brown, 2015). Strong evidence exists for the toxicity of increased α-synuclein aggregation in neurons(Rietdijk et al., 2017; Roberts & Brown, 2015; Volpicelli-Daley et al., 2011). Volpicelli-Daley et. al. showed for example that the internalization of pre-formed α-synuclein fibrils induced α-synuclein aggregation in neurons thereby decreasing neuronal function and viability (Volpicelli-Daley et al., 2011). In another study, neuronal precursor cells exposed to neuron-derived α-synuclein showed signs of apoptosis, such as nuclear fragmentation and caspase 3 activation, both *in vitro* and *in vivo* (Desplats et al., 2009).

Despite the growing number of reports on α-synuclein-induced neurotoxicity, only a few investigate the direct effect of amyloid exposure on the epithelial barrier. Results from these studies vary from no to a significant effect of amyloid exposure on epithelial barrier health (Pellegrini et al., 2022; Puig et al., 2015). Different experimental set-up and amyloids used may explain this discrepancy.
In one report, incubation of non-differentiated Caco-2 cells with a high concentration (100 µM) of α-synuclein proto-fibrils, small fibrillar strands that can act as seed, strongly decreased cell viability(Horsley et al., 2022). This effect could be alleviated by addition of inhibitory peptides targeted at a protein region that is regarded as a key modulator involved in α-synuclein fibrilization(Horsley et al., 2022). In another report, direct incubation of epithelial cells with α-synuclein monomers (1 µM) did not compromise the protein expression of tight junction proteins TJP-1 and Occludin(Pellegrini et al., 2022). Similar to the results of Pellegrini et al., the results of this project indicated that incubation of Caco-2 cells with up to 25 µM α-synuclein monomers did not change intracellular ATP concentrations.

These findings conflict with results from previous research in neurons(Ganjam et al., 2019; Ludtmann et al., 2016, 2018). Genetic and biochemical data imply that mitochondrial disfunction is potentially a central feature in PD(Park et al., 2018). Under physiological conditions in neurons, monomeric α-synuclein interacts with ATP synthase thereby enhancing its efficiency(Ludtmann et al., 2016). On the contrary, stimulation with extracellular α-synuclein oligomers or α-synuclein overexpression induced mitochondrial dysfunction ultimately leading to cell death(Ganjam et al., 2019; Ludtmann et al., 2018). Considering this knowledge, the observations found here based on intestinal epithelial cells are quite remarkable. On one hand, neuronal and intestinal epithelial cells share some receptors induced by different structures of extracellular α-synuclein(Besnier et al., 2015; Ford et al., 2002; Gorecki et al., 2021; Surguchev et al., 2019). This favours the suggestion that α-synuclein would induce some similar pathways in both cell types. However, a critical note for this reasoning is that the receptors induced by α-synuclein that are known till now are often enriched in neuronal tissue and

less abundantly expressed in the GIT(Besnier et al., 2015; Ford et al., 2002; Gorecki et al., 2021; Surguchev et al., 2019). Moreover, increasing evidence in literature demonstrates that cellular effects induced by α-synuclein are strongly dependent on the precise protein structure and the cell type studied(Gorecki et al., 2021; Roberts et al., 2015). Based on this it is plausible that α-synuclein induced effects at cell metabolism differs between neurons and intestinal epithelial cells.
Moreover, there is evidence for the dysregulation of various cellular processes by α-synuclein fibrils and seeds(Roberts & Brown, 2015). Under physiological conditions elevated intracellular α-synuclein concentrations are compensated by an increased activity of the protein degradation pathway (Xilouri et al., 2013). Further, multiple studies highlight the potential involvement of α-synuclein intestinal inflammation in PD (Barbut et al., 2019; Chen et al., 2019; Gorecki et al., 2021). Protein degradation is an energy demanding process and inflammation is associated with substantial shifts in cell metabolisms(Kominsky et al., 2010; Myra Zerr, 2021). These examples illustrate that α-synuclein may induces multiple cellular processes that may indirectly affect cell energy status(Roberts et al., 2015). Nevertheless, the induced toxicity and downstream cellular effects of α-synuclein fibrils is still a highly debated topic(Roberts & Brown, 2015). Similarly, despite some lines of evidence for that co-stimulation with monomers and seeds of Aβ42 oligomers most potently affects cell health, this mode of action is not yet investigated for α-synuclein(Jan et al., 2011; Roberts et al., 2015).
All in all, it is difficult to decipher the precise mechanisms responsible for the observations done during this project. Moreover, the data gathered during this project are marginal and require further validation. This together with the absence of existing literature on this topic makes no more than assumptions can be made with regard to α-synuclein induced effects in the intestinal epithelial barrier on cytotoxicity and cell metabolism.

Analysis of mature neuronal marker expression induced by RA treatment

In this project it was aimed to characterize SH-SY5Y morphology induced by a standardized differentiation protocol. This to provide a cellular *in vitro* model for future studies of amyloid induced neurotoxicity and optionally implementation of the cells in a more complex TW set-up. To this end, SH-SY5Y were incubated for 6 days with 10 µM RA and cells were analysed for the expression of neuronal differentiational markers β-III-tubulin, MAP-2 and Synapsin 1/2.

The effect of the differentiation procedure was most pronounced when analysing β-III-tubulin staining: after RA treatment SH-SY5Y displayed a neuron-like morphology with neuritic extensions. This RA-induced difference in cytoskeleton network formation is similar to results found in previous studies(Filograna et al., 2015; Forster et al., 2016b). The microtubilin cytoskeleton plays a crucial role in neurogenesis, the maintenance of neuronal structure integrity, guidance of intracellular trafficking and neuronal plasticity(Jaworski et al., 2009; Katsetos et al., 2003; Lasser et al., 2018). β-III-tubulin is a component of the microtubule cytoskeleton network typically enriched in neuronal tissues(Katsetos et al., 2003). β-III-tubulin expression is differentially expressed in developing neurons(Katsetos et al., 2003). For that reason, β-III-tubulin is regarded as one of the earliest markers of neuronal differentiation(Katsetos et al., 2003).
The results of MAP-2 and Synapsin 1/2 were less exemplifying for neuronal maturation. Previous studies report variable results with regard to RA-induced changes in MAP-2 expression (Cheung et al., 2009; Encinas et al., 2000a; Pan et al., 2005). Although some studies showed an increase in MAP-2 expression within one week, others found no significant difference in the MAP-2 expression pattern between differentiated and undifferentiated cells (Cheung et al., 2009; Encinas et al., 2000a; Pan et al., 2005). Similarly, to the latter, in this study RA-mediated differentiation seemed not to change MAP-2 expression in SH-SY5Y. Quantitative analysis for marker expression may give a better insight

in the amount of MAP-2 expression. This option, however, was limited by the low amount of non-differentiated cells left for analysis: most of the non-differentiated cells had detached during washing steps. Cytoskeletal rearrangement is an integral process of neurogenesis(Compagnucci et al., 2016). Following neural commitment neuronal precursor cells form neuritic processes and later axons and dendrites(Compagnucci et al., 2016; Katsetos et al., 2003). The cytoskeleton plays a fundamental role in among others cell surface attachment(Compagnucci et al., 2016; Katsetos et al., 2003; Lasser et al., 2018). Non-differenced cells have a less expanded cytoskeleton network in comparison to differentiated cells(Kovalevich & Langford, 2013a). They are characterized by rapid proliferation, rounded cell bodies and tend to grow in clumps(Kovalevich & Langford, 2013a). Consequently, undifferentiated SH-SY5Y can form floating as well as attached cell populations. This may partly explain the striking difference in detachment between differentiated and non-differentiated cells during washing(Kovalevich & Langford, 2013a).
Synapsins are neuron-specific proteins located at the presynaptic nerve terminal(Mirza & Zahid, 2018). They associate with synaptic vesicles and are involved in many regulatory processes at neuronal synapses including synaptic vesicle docking, neuroplasticity and synapse formation (Mirza & Zahid, 2018). RA treatment has been shown to induce an increased expression of other neuronal synaptic markers such as SYP (Synaptophysin) and PDS-95 (postsynaptic density protein 95) (Unsicker et al., 2021). Nevertheless, staining attempts yielded no reliable results: staining appeared all over the cell body.

All in all, the obtained data suggested that the applied RA-treatment did not yield fully differentiated SH-SY5Y cells within 6 days. The creation of a more neuron-like morphology after 7 days of RA-treatment have been reported previously(Cheung et al., 2009; Jahn et al., 2017; Sarkanen et al., 2007). The gathered results re-confirmed this differentiation property of RA to induce neuritic outgrowth within 7 days. Still one week may not be sufficient to produce completely functionally differentiated neurons(Dravid et al., 2021; Encinas et al., 2000b; Sarkanen et al., 2007). In these studies, it is suggested that a minimum of 10 days is required to produce fully differentiated neurons(Dravid et al., 2021; Encinas et al., 2000b). Longer incubation periods of up to several weeks can be chosen as well(Shipley et al., 2016b). Not yet fully developed SH-SY5Y may serve as suitable model to investigate the outgrowth of neuronal processes(Kim & Yoo, 2016). Nevertheless, use of partly differentiated cells may limit their application in studies linked to neuronal functions and excitability. Moreover, if the differentiation state of SH-SY5Y is not well defined, their cell morphology may range from neuroblastoma features to neuronal progenitor cells or postmitotic neurons. This may cause variability and hence bias of results when the cells are applied in *in vitro* models.

Transwell-based set-up to model PD

Intestinal barrier health in the presence of neuronal cultures

Here it was aimed to develop a simple Transwell-based model mimicking features of the intestinal epithelial barrier and neuronal secretion of α-synuclein. The gathered data suggested that epithelial barrier integrity decreased over time in presence of neuronal cells. It was quite remarkable that the TEER values decreased in co-cultures with SH-SY5Y wt as well as with SH-SY5Y overexpressing α-synuclein. Emerging evidence suggests that interaction between the ENS and the intestinal epithelial barrier is an important factor in barrier maturation and maintenance(Walsh & Zemper, 2019). Nevertheless, reports on the influence of the presence of neuronal cells on functioning of intestinal epithelial cells are less widespread and results of these studies are often conflicting. This makes it difficult to deduce any potential causal explanation for the observed decrease in epithelial barrier

integrity in both intestinal/neuronal co-cultures from previous work. In one approach Puzan et al. used a Transwell-based system for the co-culture of murine intestinal stem cells with complete primary enteric cultures in the basolateral compartment(Puzan et al., 2018). The enteric cultures contained enteric neurons, enteric glia and myofibroblasts (Puzan et al., 2018). Their results indicated that co-culturing of intestinal epithelial cells with ENS and myofibroblast increased the intestinal barrier integrity(Puzan et al., 2018). Further, co-culturing upregulated the expression of MIP-2 (Macrophage Inflammatory Protein 2) TGF-β (Transforming Growth factor β) and IL-10 (Interleukin 10) in enteric neuronal cells. (Puzan et al., 2018). On the contrary, in another study a decrease in intestinal barrier TEER was observed in co-cultures of Caco-2 with neuronal cell line PC-12 (Hideo Satshu, 2003). Differences in experimental methods and approaches may explain these discrepancies and impedes comparison between different studies(Walsh & Zemper, 2019). Our findings in Caco-2/SH-SY5Y co-cultures were consistent with the results of Satshu et al.. Nevertheless, the results need to be confirmed in additional experiments. All in all, there is more research needed to study the effect of the presence of neuronal cells on Caco-2 monolayer functioning and viability.

Changes in SH-SY5Y morphology

In this project, optical analysis of SH-SY5Y cells 3 days after co-culturing revealed that the cell layer had partly detached from its surface and multiple cells had seemingly died. Some cells exhibited cell surface blebs of different structures, a feature of apoptosis(Kalinichenko & Matveeva, 2008). However, image quality was very low making it difficult to deduce any firm hypothesis from these and no other validating experiments were performed after. Consequently, the responsible mechanism for these findings remains unknown. Various explanations may exist, some are discussed.

First of all, as mentioned before undifferentiated SH-SY5Y are known to exist in two growth patterns: attached to the cell culture surface and floating in the cell culture medium(Kovalevich & Langford, 2013a; SH-SY5Y, ACTT, n.d.). This knowledge would provide a very straightforward explanation for the appearance of floating cells after 4 days of culturing. Some studies state vague assumptions that free-floating cells have a more significant role that commonly thought(Feles et al., 2022; Kovalevich & Langford, 2013a). Nevertheless, literature on the relevance of free-floating cells and their contribution to the total SH-SY5Y population is very limited(Feles et al., 2022). For that reason, most studies only utilize adherent cell populations and the floating cells are discarded during media changes(Kovalevich & Langford, 2013a).
There are quite some reports on the deteriorating impact of α-synuclein overexpression (including α-synuclein wt as well as mutated variants) in neuronal cell lines(Delenclos et al., 2019; Melnikova et al., 2020; Volpicelli-Daley et al., 2011). These effects tend to be more pronounced for the overexpression of mutated variants as compared to the wt protein(Melnikova et al., 2020; Tönges et al., 2014). Consequently, it might be suggested that the observed neuronal stress resulted from α-synuclein induced neurotoxicity. However, other studies demonstrate the absence of direct neurotoxicity upon overexpression of wt α-synuclein in neuronal cells(Noela Rodríguez-Losada, 2020; Stefanis et al., 2001; Vekrellis et al., 2009). Of these Vekrellis et al. created a conditionally inducible SH-SY5Y cell line for overexpression of α-synuclein(Vekrellis et al., 2009). They found that the induction of wt α-synuclein in differentiated SH-SY5Y led to gradually cell death with non-apoptotic features (Vekrellis et al., 2009). However, despite the prominent accumulation of oligomeric species the expression of α-synuclein as long as 10 days in non-differentiated cells was not accompanied with neuronal degeneration(Vekrellis et al., 2009). In this project SH-SY5Y were used in their non-differentiated state and, considering the work of Vekrellis et al., this making it less plausible that overexpression of wt α-synuclein would induce neuronal death. Further Rodríguez-Losada et al. created a stably transfected SH-SY5Y line expressing wt α-synuclein(Rodríguez-Losada, 2020). Analysis of and cell viability and proliferation rates revealed no significant signs of cell

death(Rodríguez-Losada, 2020). Only at low cell passage numbers when the cells were still recovering from thawing cell viability was slightly reduced in cells moderately expressing α-synuclein(Noela Rodríguez-Losada, 2020). Finally, features of neuronal cell death and cell detachment were observed both in in both the transfected and non-transfected SH-SY5Y cells 4 days after transfection. If α-synuclein overexpression would have induced neuronal neurotoxicity it would not explain the observed phenotype in SH-SY5Y wt cells. Together with previous findings these result favour the hypothesis that α-synuclein overexpression may not be the main cause of the apparent neuronal cell deterioration.

The created model in perspective

To date several PD models have been created each with its own assets and limitations(Slanzi et al., 2020a). It should be evident that, to capture the complexity of the GBA in PD in its entirely, the model described herein has its limitations.

First of all, it should be noted that mono-cultures do not completely capture the diverse composition of the GIT. In monoculture Caco-2 lack a mucus producing layer which results in a lower permeability as compared to the normal human intestine(Darling et al., 2020; Tor Lea, 2015). To address this issue several co-cultures of Caco-2 combined with another cell type have been designed ranging from immune cells(Kleiveland, 2015; Zoumpopoulou et al., 2009) and neurons (Holland-Cunz et al., 2004; Satsu et al., 2014) to fibroblasts (Puzan et al., 2018). Inclusion of mucus-secreting HT29-MTX cells in the model is the most common approach and has shown to better mimic in vivo permeability data(Tor Lea, 2015).
Further, monolayer grown SH-SY5Y are an evident oversimplification of the ENS. The diversity of the neuronal phenotypes in the ENS is diverse contains virtually every class of neurotransmitter found in the CNS(Furness et al., 2014). The SH-SY5Y cell line was initially characterized as a cell line with moderate activity of dopamine-β-hydroxylase and tyrosine activity(Biedler et al., 1978; Ross & Biedler, 1985). Still, undifferentiated SH-SY5Y do certainly not present a mature neuronal signature. Predominant features of their immature state are the limited formation of neuronal branches and the absence of mature neuronal markers such as synaptic vesicle protein II (SV2)(Forster et al., 2016a; Kovalevich & Langford, 2013b; Shipley et al., 2016a). Based on these characteristics it is plausible that undifferentiated SH-SY5Y exhibit hardly any synaptic activity. These features are all not consistent with neurons in vivo.
Use of differentiated SH-SY5Y would (partly) overcome these issues. Differentiated cells are characterized by the presence of various neuronal markers. In addition to that, they may form extended neuronal branches and, in some cases, form synapses as well(Kovalevich & Langford, 2013b). Importantly, experimental outcomes may differ for undifferentiated versus differentiated SH-SY5Y(Lopes, da Motta, et al., 2017; Vekrellis et al., 2009). An issue that is certainly relevant for studies on neurotoxicity and drug development. For example, the low levels of dopamine transporters expressed in undifferentiated SH-SY5Y make that they do not mimic neurotoxin induced cell death(Lopes, da Motta, et al., 2017). As illustrated the translatability to the in vivo situation without differentiation can be limited.
Differentiation of SH-SY5Y into neuron-like cells requires the presence of ECM and neurotropic factors(Kovalevich & Langford, 2013b). As described, during this project a first start was made to test a standardized RA-based differentiation protocol for SH-SY5Y. Nevertheless, the efficiency of the applied procedure remained unclear: the resultant neuronal morphology was not completely characterized in the course of this project. For that reason, to maintain reproducibility of the model during the initial phase of model development undifferentiated SH-SY5Y were used in the first model set-up.

Once expressed, α-synuclein can be released from cells in a variety of ways including exosome-mediated secretion or during apoptosis (Danzer et al., 2012; Moussaud et al., 2015). In line with this, several studies demonstrated the secretion of a-synuclein by neuronal cells after inducing overexpression(Danzer et al., 2012; Delenclos et al., 2017; Marques & Outeiro, 2012; Moussaud et al., 2015). Based on this, it was hypothesised that in the used set-up, Transwells with SH-SY5Y/Caco-2 co-cultures, the Caco-2 monolayer would be exposed to α-synuclein through the secretion of the SH-SY5Y cells in the basal compartment. In this work, SH-SY5Y were transfected with wt α-synuclein fused to GFP to allowing monitoring transfection with fluorescence microscopy. The obtained results indicated successful expression of α-synuclein in transfected cells for up to 3 days. However, the presence of α-synuclein in the culture medium or cells was not further validated. This makes that this hypothesized exposure mode remains questionable.

In summary, the created set-up evidently has some limitations. Further, with regard to the huge complexity of LB pathology in the ENS and the GIT, a co-culture of Caco-2 with SH-SY5Y overexpressing α-synuclein is quite a reductionistic mimic. There exist some other PD models that more faithfully recapitulate the in vivo situation in PD ((Moysidou & Owens, 2021a; Slanzi et al., 2020a). Nevertheless, despite the inherent restrictions of the model design, the simplicity of it may be regarded as a strength as well. Transwell systems are useful tools for linear kinetic studies during drug discovery(Slanzi et al., 2020a). Co-culturing a neuronal and intestinal cell line in a Transwell could provide first test platform to study the interplay between α-synuclein, neuronal cells and the intestinal epithelial barrier. Nevertheless, before use in other research the presented model requires further validation and optimization.

Recommendations

Determining cell viability after α-synuclein exposure

This study investigated the effect of diverse α-synuclein molecular structures at the intestinal epithelial barrier cell viability. Determination of the cell viability relied on one single assay, Cell Titer-Glo® 2.0 assay, and it is strongly recommended to validate the results gathered. First of all, additional experiments on cell viability are desired. Besides the Cell Titer-Glo® 2.0 assay other assays such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay or use of PrestoBlue® reagents may give a better view on overall cell metabolism. Alternatively, simple live/death staining such as simultaneously staining with Hoechst and Propidium Iodide dye could be applied. Measurements of intracellular ATP content may provide further validation for the results of this project. To this end various commercial kits are available such as the ViaLight TM plus kit (Lonza, Verviers, Belgium) based on luminescence or the colorimetric ATP Assay Kit (Abcam, ab83355).

Ultimately it is desired to assess the effect of diverse α-synuclein variants at epithelial barrier health. This was an initial goal of this project but could unfortunately not be finished.
Intestinal inflammation reduces intestinal epithelial barrier integrity and increases the expression of pro-inflammatory cytokines in intestinal epithelial cells(Andrews et al., 2018; Lechuga & Ivanov, 2017). Therefore, assessment of cytokine and TJ protein expression could give a more complete view on α-synuclein induced effects. Ideally, expression levels are determined at both RNA and protein level for example by means of qPCR and Western Blotting. During this project various agents were tested to serve as a positive control namely IL-1β, TNF-α and LPS. However, these assays yielded no conclusive candidate. Use of higher cytokine concentrations or a combination of multiple agents may provide the desired results and could be tested in further research(van de Walle et al., 2010).
Further, if more close analysis of mitochondrial function is desired diverse approaches can be taken. Measuring mitochondrial membrane potential by means of rhodamine dyes such as TMRE or TMRM may serve as a start.

Improve the analysis of mature neuronal markers

The presence of mature neuronal makers by SH-SY5Y cells was analysed to characterize the neuronal morphology induced upon RA-mediated differentiation. Not all markers yielded reliable results. aFirst of all, analysis of MAP-2 expression was limited because the majority of non-differentiated cells detached during the washing steps preceding cell fixation. This impeded staining quantification in non-differentiated versus differentiated sample. To avoid this, it is desired to optimize cell adhesion. The applied surface coating strongly influences cell adhesion and neurogenesis(Sun et al., 2012). In this project, Lamin and Poly-L-lysine were tested and no significant difference in cell attachment was observed. Alternatives commonly used for neuronal cultures include polyethyleneimine, Poly-D-Lysine and fibronectin(Sun et al., 2012). Of these, polymeric coatings may be better suitable for neuronal attachment(Sun et al., 2012). Increasing coating agent concentration is another strategy to increase cell attachment(Sun et al., 2012). To quantify neuronal marker expression, assays such as qPCR and Western Blot may provide a good approach.

Despite several attempts, the results of Synapsin 1/2 did not allow to analyse the formation of synaptic junctions. To get a better view on the extent of synapse formation optimization of the applied protocol is desired. Optionally, some more synaptic markers like synaptophysin and synaptic vesicle protein II could be used as well. When doing so, it should be realized that one week of RA-treatment may not be sufficient to achieve fully developed nerve terminals in SH-SY5Y(Dravid et al., 2021).

Finally, as described, extending the RA-treatment duration with several days may generate a more homogenous mature neuron population. To investigate this, it is recommended to compare neuronal maker expression at different time points for at least up to 2 weeks. This may give a better insight in the effect of treatment duration.

Optimization of the Transwell-based set-up

This project yielded a first set-up of a simple Transwell-based model of neuronal cells expressing α-synuclein and the intestinal epithelial barrier. Some explaining mechanisms for the first gathered results remain elusive: TEER values decreased after several days but this reduce in epithelial barrier resistance was not confirmed by any follow-up experiments. Moreover, many SH-SY5Y had detached from the cell culture surface and exhibited indications of cell death. This, however, could not be confirmed with the obtained results. When continuing the project, it recommend to test the cell viability of both neuronal and intestinal cells resulting from co-culturing. Further, possible negative effects of α-synuclein production on SH-SY5Y cell viability requires attention. To this end, a cell health indicator such as the PrestoBlue® reagent can serve as start. Further measurements of cell viability may be based on energy production such as for the Cell Titer-Glo® 2.0 assay. In addition to that, frequent cell microscopical monitoring during the co-culturing period may give a good first impression of cell state.

To mimic the presence of α-synuclein in the ENS during PD, SH-SY5Y were transiently transfected with wt α-synuclein. Although some first results on the transfection efficiency and duration were obtained, these are yet incomplete. For that reason, more research focussed on a better quantification and duration of α-synuclein expression is desired. For further validation of the transfection there are multiple options including approaches based on Western Blot or ELISA for protein detection or qPCR for the detection of α-synuclein mRNA.

More improvements may involve the use of differentiated SH-SY5Y. As explained, differentiated cells have a more mature neuronal phenotype which would favour the translatability of model. Another improvement may involve the co-culture of Caco-2 with HT29-MTX cells. This is, as mentioned before, a frequently used approach to improve the translatability of intestinal epithelial barrier models. Introduction of more different cell types may enhance the resemblance of the model to the in vivo cell composition of the ENS and the epithelial barrier(Darling et al., 2020; Furness et al., 2014). However, these steps are considered as too far-fetched at this stage of the development process.

Conclusion

Past decades have witnessed a wealth of evidence that the GIT is involved in PD disease(Anis et al., 2022; Rao & Gershon, 2016b; Rietdijk et al., 2017). Nevertheless, the role of α-synuclein in the GIT during PD is not elucidated yet(Pfeiffer, 2018; Rao & Gershon, 2016b; Rietdijk et al., 2017).
This project made a first start to investigate α-synuclein induced effects at the intestinal epithelial cell viability in PD. The obtained cell viability results are remarkable and additional experiments are required before any conclusions can be drawn.
Experimental models to explore the mechanisms associated with α-synuclein-mediated toxicity are essential to aid the development and validation of future pharmacological interventions. This highlights the need for the development of standardized techniques and protocols. Therefore, one aim of this project was to create a simple standardized set-up of the ENS-gut junction for PD research. First, an RA-based differentiation for SH-SY5Y protocol was tested. The results of β-III-tubulin staining revealed some first indications of neuronal differentiation. Nevertheless, the obtained results are scant to draw any more conclusions. All in all, better characterization of the induced SH-SY5Y cell morphology and optimization of the described protocol is required before using the protocol in further research. Continuing the project, a first step was made setting up a Transwell-based system to study α-synuclein induced effect in co-cultures of intestinal and neuronal cells. A first test session was performed to detect fledgling model pitfalls. Based on these tests it can be concluded that the presented Transwell system certainly requires optimization and validated before it can be applied in PD research. All in all, the involvement of the GBA and GIT in PD pathology remains a topic of ongoing research and more research is needed to unravel the role of α-synuclein in the GIT during PD.

References

Andrews, C., McLean, M. H., & Durum, S. K. (2018). Cytokine Tuning of Intestinal Epithelial Function. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.01270

Anis, E., Xie, A., Brundin, L., & Brundin, P. (2022). Digesting recent findings: gut alpha-synuclein, microbiome changes in Parkinson's disease. In *Trends in Endocrinology and Metabolism* (Vol. 33, Issue 2, pp. 147–157). Elsevier Inc. https://doi.org/10.1016/j.tem.2021.11.005

Annerino, D. M., Arshad, S., Taylor, G. M., Adler, C. H., Beach, T. G., & Greene, J. G. (2012). Parkinson's disease is not associated with gastrointestinal myenteric ganglion neuron loss. *Acta Neuropathologica*, *124*(5), 665–680. https://doi.org/10.1007/s00401-012-1040-2

Barbut, D., Stolzenberg, E., & Zasloff, M. (2019). Gastrointestinal Immunity and Alpha-Synuclein. *Journal of Parkinson's Disease*, *9*, S313–S322. https://doi.org/10.3233/JPD-191702

Besnier, L. S., Cardot, P., da Rocha, B., Simon, A., Loew, D., Klein, C., Riveau, B., Lacasa, M., Clair, C., Rousset, M., & Thenet, S. (2015). The cellular prion protein PrP ^{c} is a partner of the Wnt pathway in intestinal epithelial cells. *Molecular Biology of the Cell*, *26*(18), 3313–3328. https://doi.org/10.1091/mbc.e14-11-1534

Biedler, J. L., Roffler-Tarlov, S., Schachner, M., & Freedman, L. S. (1978). Multiple Neurotransmitter Synthesis by Human Neuroblastoma Cell Lines and Clones. *Cancer Research*, *38*(11_Part_1), 3751–3757.

Braak, H., Rüb, U., Gai, W. P., & Tredici, K. del. (2003). Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen. *J Neural Transm*, *110*, 517–536. https://doi.org/10.1007/s00702-002-0808-2

Carla Annink. (2020). *Transfection*.

Chakrabarti, A., Geurts, L., Hoyles, L., Iozzo, P., Kraneveld, A. D., la Fata, G., Miani, M., Patterson, E., Pot, B., Shortt, C., & Vauzour, D. (2022a). The microbiota–gut–brain axis: pathways to better brain health. Perspectives on what we know, what we need to investigate and how to put knowledge into practice. In *Cellular and Molecular Life Sciences* (Vol. 79, Issue 2). Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00018-021-04060-w

Chalazonitis, A., & Rao, M. (2018). Enteric nervous system manifestations of neurodegenerative disease. *Brain Research*, *1693*, 207–213. https://doi.org/10.1016/j.brainres.2018.01.011

Chandra, R., Hiniker, A., Kuo, Y.-M., Nussbaum, R. L., & Liddle, R. A. (2017). α-Synuclein in gut endocrine cells and its implications for Parkinson's disease. *JCI Insight*, *2*(12), e92295. https://doi.org/10.1172/jci.insight.92295

Chen, Q. Q., Haikal, C., Li, W., & Li, J. Y. (2019). Gut Inflammation in Association with Pathogenesis of Parkinson's Disease. In *Frontiers in Molecular Neuroscience* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fnmol.2019.00218

Cheung, Y. T., Lau, W. K. W., Yu, M. S., Lai, C. S. W., Yeung, S. C., So, K. F., & Chang, R. C. C. (2009). Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *NeuroToxicology*, *30*(1), 127–135. https://doi.org/10.1016/J.NEURO.2008.11.001

Chia, S. J., Tan, E. K., & Chao, Y. X. (2020). Historical perspective: Models of Parkinson's disease. In *International Journal of Molecular Sciences* (Vol. 21, Issue 7). MDPI AG. https://doi.org/10.3390/ijms21072464

Compagnucci, C., Piemonte, F., Sferra, A., Piermarini, E., & Bertini, E. (2016). The cytoskeletal arrangements necessary to neurogenesis. *Oncotarget*, *7*(15), 19414–19429. https://doi.org/10.18632/oncotarget.6838

Corbillé, A.-G., Coron, E., Neunlist, M., Derkinderen, P., & Lebouvier, T. (2014). Appraisal of the Dopaminergic and Noradrenergic Innervation of the Submucosal Plexus in PD. *Journal of Parkinson's Disease*, *4*, 571–576. https://doi.org/10.3233/JPD-140422

Corporation, P. (n.d.). *CellTiter-Glo ® 2.0 Assay Instructions for Use of Products G9241, G9242 and G9243*. www.promega.com

Cremades, N., Cohen, S. I. A., Deas, E., Abramov, A. Y., Chen, A. Y., Orte, A., Sandal, M., Clarke, R. W., Dunne, P., Aprile, F. A., Bertoncini, C. W., Wood, N. W., Knowles, T. P. J., Dobson, C. M., & Klenerman, D. (2012). Direct Observation of the Interconversion of Normal and Toxic Forms of α-Synuclein. *Cell*, *149*(5), 1048–1059. https://doi.org/10.1016/j.cell.2012.03.037

Danzer, K. M., Kranich, L. R., Ruf, W. P., Cagsal-Getkin, O., Winslow, A. R., Zhu, L., Vanderburg, C. R., & McLean, P. J. (2012). Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Molecular Neurodegeneration*, *7*(1). https://doi.org/10.1186/1750-1326-7-42

Darling, N. J., Mobbs, C. L., González-Hau, A. L., Freer, M., & Przyborski, S. (2020). Bioengineering Novel in vitro Co-culture Models That Represent the Human Intestinal Mucosa With Improved Caco-2 Structure and Barrier Function. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00992

Delenclos, M., Burgess, J. D., Lamprokostopoulou, A., Outeiro, T. F., Vekrellis, K., & McLean, P. J. (2019). Cellular models of alpha-synuclein toxicity and aggregation. *Journal of Neurochemistry*, *150*(5), 566–576. https://doi.org/10.1111/JNC.14806

Delenclos, M., Trendafilova, T., Mahesh, D., Baine, A. M., Moussaud, S., Yan, I. K., Patel, T., & McLean, P. J. (2017). Investigation of Endocytic Pathways for the Internalization of Exosome-Associated Oligomeric Alpha-Synuclein. *Frontiers in Neuroscience*, *11*. https://www.frontiersin.org/articles/10.3389/fnins.2017.00172

Delgado, M. E., Grabinger, T., & Brunner, T. (2016). Cell death at the intestinal epithelial front line. *The FEBS Journal*, *283*(14), 2701–2719. https://doi.org/https://doi.org/10.1111/febs.13575

Desplats, P., Lee, H.-J., Bae, E.-J., Patrick, C., Rockenstein, E., Crews, L., Spencer, B., Masliah, E., & Lee, S.-J. (2009). Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(31), 13010–13015. https://doi.org/10.1073/pnas.0903691106

Devos, D., Lebouvier, T., Lardeux, B., Biraud, M., Rouaud, T., Pouclet, H., Coron, E., Bruley des Varannes, S., Naveilhan, P., Nguyen, J. M., Neunlist, M., & Derkinderen, P. (2013). Colonic inflammation in Parkinson's disease. *Neurobiology of Disease*, *50*(1), 42–48. https://doi.org/10.1016/J.NBD.2012.09.007

Dorsey, E. R., Elbaz, A., Nichols, E., Abd-Allah, F., Abdelalim, A., Adsuar, J. C., Ansha, M. G., Brayne, C., Choi, J.-Y. J., Collado-Mateo, D., Dahodwala, N., Do, H. P., Edessa, D., Endres, M.,

Fereshtehnejad, S.-M., Foreman, K. J., Gankpe, F. G., Gupta, R., Hankey, G. J., … Murray, C. J. L. (2018). Global, regional, and national burden of Parkinson's disease, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology*, *17*(11), 939–953. https://doi.org/10.1016/S1474-4422(18)30295-3

Dravid, A., Raos, B., Svirskis, D., & O'Carroll, S. J. (2021). Optimised techniques for high-throughput screening of differentiated SH-SY5Y cells and application for neurite outgrowth assays. *Scientific Reports*, *11*(1), 23935. https://doi.org/10.1038/s41598-021-03442-1

Emamzadeh, F. N. (2016). Alpha-synuclein structure, functions, and interactions. In *Journal of Research in Medical Sciences* (Vol. 21, Issue 2). Isfahan University of Medical Sciences(IUMS). https://doi.org/10.4103/1735-1995.181989

Encinas, M., Iglesias, M., Liu, Y., Wang, H., Muhaisen, A., Ceña, V., Gallego, C., & Comella, J. X. (2000a). Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated, Neurotrophic Factor-Dependent, Human Neuron-Like Cells. *Journal of Neurochemistry*, *75*(3), 991–1003. https://doi.org/https://doi.org/10.1046/j.1471-4159.2000.0750991.x

Feles, S., Overath, C., Reichardt, S., Diegeler, S., Schmitz, C., Kronenberg, J., Baumstark-Khan, C., Hemmersbach, R., Hellweg, C. E., & Liemersdorf, C. (2022). Streamlining Culture Conditions for the Neuroblastoma Cell Line SH-SY5Y: A Prerequisite for Functional Studies. *Methods and Protocols*, *5*(4). https://doi.org/10.3390/mps5040058

Filograna, R., Civiero, L., Ferrari, V., Codolo, G., Greggio, E., Bubacco, L., Beltramini, M., & Bisaglia, M. (2015). Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. *PloS One*, *10*(8), e0136769–e0136769. https://doi.org/10.1371/journal.pone.0136769

Ford, M. J., Burton, L. J., Morris, R. J., & Hall, S. M. (2002). Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience*, *113*(1), 177–192. https://doi.org/https://doi.org/10.1016/S0306-4522(02)00155-0

Forster, J. I., Köglsberger, S., Trefois, C., Boyd, O., Baumuratov, A. S., Buck, L., Balling, R., & Antony, P. M. A. (2016a). Characterization of Differentiated SH-SY5Y as Neuronal Screening Model Reveals Increased Oxidative Vulnerability. *SLAS Discovery*, *21*(5), 496–509. https://doi.org/https://doi.org/10.1177/1087057115625190

Forsyth, C. B., Shannon, K. M., Kordower, J. H., Voigt, R. M., Shaikh, M., Jaglin, J. A., Estes, J. D., Dodiya, H. B., & Keshavarzian, A. (2011). Increased intestinal permeability correlates with sigmoid mucosa alpha-synuclein staining and endotoxin exposure markers in early Parkinson's disease. *PloS One*, *6*(12), e28032–e28032. https://doi.org/10.1371/journal.pone.0028032

Foster, J. A., Rinaman, L., & Cryan, J. F. (2017). Stress & the gut-brain axis: Regulation by the microbiome. *Neurobiology of Stress*, *7*, 124–136. https://doi.org/10.1016/J.YNSTR.2017.03.001

Fricova, D., Harsanyiova, J., & Trancikova, A. K. (2020). Alpha-synuclein in the gastrointestinal tract as a potential biomarker for early detection of parkinson's disease. In *International Journal of Molecular Sciences* (Vol. 21, Issue 22, pp. 1–21). MDPI AG. https://doi.org/10.3390/ijms21228666

Furness, J. B., Callaghan, B. P., Rivera, L. R., & Cho, H.-J. (2014). The Enteric Nervous System and Gastrointestinal Innervation: Integrated Local and Central Control. In M. Lyte & J. F. Cryan

(Eds.), *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease* (pp. 39–71). Springer New York. https://doi.org/10.1007/978-1-4939-0897-4_3

Ganjam, G. K., Bolte, K., Matschke, L. A., Neitemeier, S., Dolga, A. M., Höllerhage, M., Höglinger, G. U., Adamczyk, A., Decher, N., Oertel, W. H., & Culmsee, C. (2019). Mitochondrial damage by α-synuclein causes cell death in human dopaminergic neurons. *Cell Death and Disease*, *10*(11). https://doi.org/10.1038/s41419-019-2091-2

Gene [SNCA synuclein alpha [Homo sapiens (human)] . (2022, May 15). National Library of Medicine (US), National Center for Biotechnology Information.

Gorecki, A. M., Anyaegbu, C. C., & Anderton, R. S. (2021). TLR2 and TLR4 in Parkinson's disease pathogenesis: the environment takes a toll on the gut. In *Translational Neurodegeneration* (Vol. 10, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s40035-021-00271-0

Hideo SATSU, † Tatsuya YOKOYAMA, Nobumasa OGAWA, Yoko FUJIWARA-HATANO, and Makoto SHIMIZU. (2003). EŠect of Neuronal PC12 Cells on the Functional Properties of Intestinal Epithelial Caco-2 Cells. *Bioscience, Biotechnology, and Biochemistry*, *67*(6), 1312–1318.

Holland-Cunz, S., Bainczyk, S., Hagl, C., Wink, E., Wedel, T., Back, W., & Schäfer, K. H. (2004). Three-dimensional co-culture model of enterocytes and primary enteric neuronal tissue. *Pediatric Surgery International*, *20*(4), 233–237. https://doi.org/10.1007/s00383-003-1117-z

Horsley, J. R., Jovcevski, B., Pukala, T. L., & Abell, A. D. (2022). Designer D-peptides targeting the N-terminal region of α-synuclein to prevent parkinsonian-associated fibrilization and cytotoxicity. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1870*(10). https://doi.org/10.1016/j.bbapap.2022.140826

Jahn, K., Wieltsch, C., Blumer, N., Mehlich, M., Pathak, H., Khan, A. Q., Hildebrandt, H., & Frieling, H. (2017). A cell culture model for investigation of synapse influenceability: epigenetics, expression and function of gene targets important for synapse formation and preservation in SH-SY5Y neuroblastoma cells differentiated by retinoic acid. *Journal of Neural Transmission*, *124*(11), 1341–1367. https://doi.org/10.1007/s00702-017-1769-9

Jan, A., Adolfsson, O., Allaman, I., Buccarello, A.-L., Magistretti, P. J., Pfeifer, A., Muhs, A., & Lashuel, H. A. (2011). Aβ42 Neurotoxicity Is Mediated by Ongoing Nucleated Polymerization Process Rather than by Discrete Aβ42 Species. *Journal of Biological Chemistry*, *286*(10), 8585–8596. https://doi.org/10.1074/jbc.M110.172411

Jaworski, J., Kapitein, L. C., Gouveia, S. M., Dortland, B. R., Wulf, P. S., Grigoriev, I., Camera, P., Spangler, S. A., di Stefano, P., Demmers, J., Krugers, H., Defilippi, P., Akhmanova, A., & Hoogenraad, C. C. (2009). Dynamic Microtubules Regulate Dendritic Spine Morphology and Synaptic Plasticity. *Neuron*, *61*(1), 85–100. https://doi.org/10.1016/j.neuron.2008.11.013

Jucker, M., & Walker, L. C. (2013). Self-propagation of pathogenic protein aggregates in neurodegenerative diseases. *Nature*, *501*(7465), 45–51. https://doi.org/10.1038/NATURE12481

Kalia, L. v., & Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896–912. https://doi.org/10.1016/S0140-6736(14)61393-3

Kalinichenko, S. G., & Matveeva, N. Yu. (2008). Morphological characteristics of apoptosis and its significance in neurogenesis. *Neuroscience and Behavioral Physiology*, *38*(4), 333–344. https://doi.org/10.1007/s11055-008-0046-7

Katsetos, C. D., Legido, A., Perentes, E., & Mörk, S. J. (2003). Class III β-Tubulin Isotype: A Key Cytoskeletal Protein at the Crossroads of Developmental Neurobiology and Tumor Neuropathology. *Journal of Child Neurology*, *18*(12), 851–866. https://doi.org/10.1177/088307380301801205

Kim, H. B., & Yoo, B. S. (2016). Propolis Inhibits Neurite Outgrowth in Differentiating SH-SY5Y Human Neuroblastoma Cells. *Toxicological Research*, *32*(3), 239–243. https://doi.org/10.5487/TR.2016.32.3.239

Kirsten van Leijenhorst-Groener. (2021). *Preparation of α-synuclein*.

Kleiveland, C. R. (2015). Co-culture Caco-2/Immune Cells. In C. P. L.-E. I. et al. , Verhoeckx K (Ed.), *The Impact of Food Bioactives on Health* (pp. 197–205). Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_18

Kominsky, D. J., Campbell, E. L., & Colgan, S. P. (2010). Metabolic Shifts in Immunity and Inflammation. *The Journal of Immunology*, *184*(8), 4062–4068. https://doi.org/10.4049/jimmunol.0903002

Kovalevich, J., & Langford, D. (2013a). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods in Molecular Biology (Clifton, N.J.)*, *1078*, 9–21. https://doi.org/10.1007/978-1-62703-640-5_2

Krishtal, J., Metsla, K., Bragina, O., Tõugu, V., & Palumaa, P. (2019). Toxicity of Amyloid-β Peptides Varies Depending on Differentiation Route of SH-SY5Y Cells. *Journal of Alzheimer's Disease*, *71*(3), 879–887. https://doi.org/10.3233/JAD-190705

Lasser, M., Tiber, J., & Lowery, L. A. (2018). The Role of the Microtubule Cytoskeleton in Neurodevelopmental Disorders. *Frontiers in Cellular Neuroscience*, *12*. https://doi.org/10.3389/fncel.2018.00165

Lea, T. (2015). Caco-2 cell line. *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*, 103–111. https://doi.org/10.1007/978-3-319-16104-4_10

Lebouvier, T., Neunlist, M., Bruley des Varannes, S., Coron, E., Drouard, A., N'Guyen, J.-M., Chaumette, T., Tasselli, M., Paillusson, S., Flamand, M., Galmiche, J.-P., Damier, P., & Derkinderen, P. (2010). Colonic biopsies to assess the neuropathology of Parkinson's disease and its relationship with symptoms. *PloS One*, *5*(9), e12728–e12728. https://doi.org/10.1371/journal.pone.0012728

Lechuga, S., & Ivanov, A. I. (2017). Disruption of the epithelial barrier during intestinal inflammation: Quest for new molecules and mechanisms. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1864*(7), 1183–1194. https://doi.org/https://doi.org/10.1016/j.bbamcr.2017.03.007

Lopes, F. M., Bristot, I. J., da Motta, L. L., Parsons, R. B., & Klamt, F. (2017). Mimicking Parkinson's Disease in a Dish: Merits and Pitfalls of the Most Commonly used Dopaminergic In Vitro Models. *NeuroMolecular Medicine*, *19*(2), 241–255. https://doi.org/10.1007/s12017-017-8454-x

Lopes, F. M., da Motta, L. L., de Bastiani, M. A., Pfaffenseller, B., Aguiar, B. W., de Souza, L. F., Zanatta, G., Vargas, D. M., Schönhofen, P., Londero, G. F., de Medeiros, L. M., Freire, V. N., Dafre, A. L., Castro, M. A. A., Parsons, R. B., & Klamt, F. (2017). RA Differentiation Enhances Dopaminergic Features, Changes Redox Parameters, and Increases Dopamine Transporter

Dependency in 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells. *Neurotoxicity Research*, *31*(4), 545–559. https://doi.org/10.1007/s12640-016-9699-0

Lotharius, J., Falsig, J., van Beek, J., Payne, S., Dringen, R., Brundin, P., & Leist, M. (2005). *Neurobiology of Disease Progressive Degeneration of Human Mesencephalic Neuron-Derived Cells Triggered by Dopamine-Dependent Oxidative Stress Is Dependent on the Mixed-Lineage Kinase Pathway*. https://doi.org/10.1523/JNEUROSCI.1746-05.2005

Ludtmann, M. H. R., Angelova, P. R., Horrocks, M. H., Choi, M. L., Rodrigues, M., Baev, A. Y., Berezhnov, A. v., Yao, Z., Little, D., Banushi, B., Al-Menhali, A. S., Ranasinghe, R. T., Whiten, D. R., Yapom, R., Dolt, K. S., Devine, M. J., Gissen, P., Kunath, T., Jaganjac, M., … Gandhi, S. (2018). α-synuclein oligomers interact with ATP synthase and open the permeability transition pore in Parkinson's disease. *Nature Communications*, *9*(1). https://doi.org/10.1038/s41467-018-04422-2

Ludtmann, M. H. R., Angelova, P. R., Ninkina, N. N., Gandhi, S., Buchman, V. L., & Abramov, A. Y. (2016). Monomeric alpha-synuclein exerts a physiological role on brain ATP synthase. *Journal of Neuroscience*, *36*(41), 10510–10521. https://doi.org/10.1523/JNEUROSCI.1659-16.2016

Marques, O., & Outeiro, T. F. (2012). Alpha-synuclein: from secretion to dysfunction and death. *Cell Death and Disease*, *3*. https://doi.org/10.1038/cddis.2012.94

Melnikova, A., Pozdyshev, D., Barinova, K., Kudryavtseva, S., & Muronetz, V. I. (2020). α-Synuclein Overexpression in SH-SY5Y Human Neuroblastoma Cells Leads to the Accumulation of Thioflavin S-positive Aggregates and Impairment of Glycolysis. *Biochemistry (Moscow)*, *85*(5), 604–613. https://doi.org/10.1134/S0006297920050090

Mirza, F. J., & Zahid, S. (2018). The Role of Synapsins in Neurological Disorders. *Neuroscience Bulletin*, *34*(2), 349–358. https://doi.org/10.1007/s12264-017-0201-7

Moussaud, S., Malany, S., Mehta, A., Vasile, S., Smith, L. H., & McLean, P. J. (2015). Targeting α-synuclein oligomers by protein-fragment complementation for drug discovery in synucleinopathies. *Expert Opinion on Therapeutic Targets*, *19*(5), 589–603. https://doi.org/10.1517/14728222.2015.1009448

Moysidou, C. M., & Owens, R. M. (2021a). Advances in modelling the human microbiome-gut-brain axis in vitro. In *Biochemical Society Transactions* (Vol. 49, Issue 1, pp. 187–201). Portland Press Ltd. https://doi.org/10.1042/BST20200338

Myra Zerr. (2021, March 1). *UBIQUITIN-PROTEASOME SYSTEM*. Https://Lifesensors.Com/Ubiquitinproteasomesystem/.

Noela Rodríguez-Losada, a, 1 J. de la R. b, 1 M. L. c R. W. d J. A. A. a, 2 J. S. C. b and S. J. B. (2020). Overexpression of alpha-synuclein promotes both cell proliferation and cell toxicity in human SH-SY5Y neuroblastoma cells. *Journal of Advanced Research*, *23*, 37–45.

Olanow, C. W., Kieburtz, K., & Schapira, A. H. v. (2008). Why have we failed to achieve neuroprotection in Parkinson's disease? *Annals of Neurology*, *64*(S2), S101–S110. https://doi.org/https://doi.org/10.1002/ana.21461

Pan, J., Kao, Y.-L., Joshi, S., Jeetendran, S., DiPette, D., & Singh, U. S. (2005). Activation of Rac1 by phosphatidylinositol 3-kinase in vivo: role in activation of mitogen-activated protein kinase (MAPK) pathways and retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *Journal of*

Neurochemistry, *93*(3), 571–583. https://doi.org/https://doi.org/10.1111/j.1471-4159.2005.03106.x

Park, J. S., Davis, R. L., & Sue, C. M. (2018). Mitochondrial Dysfunction in Parkinson's Disease: New Mechanistic Insights and Therapeutic Perspectives. In *Current Neurology and Neuroscience Reports* (Vol. 18, Issue 5). Current Medicine Group LLC 1. https://doi.org/10.1007/s11910-018-0829-3

Pellegrini, C., D'Antongiovanni, V., Miraglia, F., Rota, L., Benvenuti, L., di Salvo, C., Testa, G., Capsoni, S., Carta, G., Antonioli, L., Cattaneo, A., Blandizzi, C., Colla, E., & Fornai, M. (2022). Enteric α-synuclein impairs intestinal epithelial barrier through caspase-1-inflammasome signaling in Parkinson's disease before brain pathology. *Npj Parkinson's Disease*, *8*(1). https://doi.org/10.1038/s41531-021-00263-x

Peng, Y., Chu, S., Yang, Y., Zhang, Z., Pang, Z., & Chen, N. (2021). Neuroinflammatory In Vitro Cell Culture Models and the Potential Applications for Neurological Disorders. In *Frontiers in Pharmacology* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fphar.2021.671734

Pfeiffer, R. F. (2018). Gastrointestinal Dysfunction in Parkinson's Disease. In *Current Treatment Options in Neurology* (Vol. 20, Issue 12). Current Science Inc. https://doi.org/10.1007/s11940-018-0539-9

Postuma, R. B., Aarsland, D., Barone, P., Burn, D. J., Hawkes, C. H., Oertel, W., & Ziemssen, T. (2012). Identifying prodromal Parkinson's disease: Pre-Motor disorders in Parkinson's disease. *Movement Disorders*, *27*(5), 617–626. https://doi.org/https://doi.org/10.1002/mds.24996

Presgraves, S. P., Ahmed, T., Borwege, S., & Joyce, J. N. (2003). Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. *Neurotoxicity Research*, *5*(8), 579–598. https://doi.org/10.1007/BF03033178

Prigent, A., Lionnet, A., Durieu, E., Chapelet, G., Bourreille, A., Neunlist, M., Rolli-Derkinderen, M., & Derkinderen, P. (2019). Enteric alpha-synuclein expression is increased in Crohn's disease. *Acta Neuropathologica*, *137*(2), 359–361. https://doi.org/10.1007/s00401-018-1943-7

Puig, K. L., Manocha, G. D., & Combs, C. K. (2015). Amyloid Precursor Protein Mediated Changes in Intestinal Epithelial Phenotype In Vitro. *PLOS ONE*, *10*(3), e0119534-. https://doi.org/10.1371/journal.pone.0119534

Puzan, M., Hosic, S., Ghio, C., & Koppes, A. (2018). Enteric Nervous System Regulation of Intestinal Stem Cell Differentiation and Epithelial Monolayer Function. *Scientific Reports*, *8*(1). https://doi.org/10.1038/S41598-018-24768-3

Rao, M., & Gershon, M. D. (2016a). The bowel and beyond: The enteric nervous system in neurological disorders. In *Nature Reviews Gastroenterology and Hepatology* (Vol. 13, Issue 9, pp. 517–528). Nature Publishing Group. https://doi.org/10.1038/nrgastro.2016.107

Rietdijk, C. D., Perez-Pardo, P., Garssen, J., van Wezel, R. J. A., & Kraneveld, A. D. (2017). Exploring Braak's hypothesis of parkinson's disease. *Frontiers in Neurology*, *8*(FEB). https://doi.org/10.3389/fneur.2017.00037

Roberts, H. L., & Brown, D. R. (2015). Seeking a mechanism for the toxicity of oligomeric α-synuclein. In *Biomolecules* (Vol. 5, Issue 2, pp. 282–305). MDPI AG. https://doi.org/10.3390/biom5020282

Ross, R. A., & Biedler, J. L. (1985). Presence and regulation of tyrosinase activity in human neuroblastoma cell variants in vitro. *Cancer Research*, *45*(4), 1628–1632.

Sarkanen, J.-R., Nykky, J., Siikanen, J., Selinummi, J., Ylikomi, T., & Jalonen, T. O. (2007). Cholesterol supports the retinoic acid-induced synaptic vesicle formation in differentiating human SH-SY5Y neuroblastoma cells. *Journal of Neurochemistry*, *102*(6), 1941–1952. https://doi.org/https://doi.org/10.1111/j.1471-4159.2007.04676.x

Satsu, H., Yokoyama, T., Ogawa, N., Fujiwara-Hatano, Y., & Shimizu, M. (2014). *Bioscience, Biotechnology, and Biochemistry Effect of Neuronal PC12 Cells on the Functional Properties of Intestinal Epithelial Caco-2 Cells*. https://doi.org/10.1271/bbb.67.1312

Schaeffer, E., Kluge, A., Böttner, M., Zunke, F., Cossais, F., Berg, D., & Arnold, P. (2020). Alpha Synuclein Connects the Gut-Brain Axis in Parkinson's Disease Patients – A View on Clinical Aspects, Cellular Pathology and Analytical Methodology. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.573696

Schwiertz, A., Spiegel, J., Dillmann, U., Grundmann, D., Bürmann, J., Faßbender, K., Schäfer, K. H., & Unger, M. M. (2018). Fecal markers of intestinal inflammation and intestinal permeability are elevated in Parkinson's disease. *Parkinsonism & Related Disorders*, *50*, 104–107. https://doi.org/10.1016/J.PARKRELDIS.2018.02.022

Shannon, K. M., Keshavarzian, A., Mutlu, E., Dodiya, H. B., Daian, D., Jaglin, J. A., & Kordower, J. H. (2012a). Alpha-synuclein in colonic submucosa in early untreated Parkinson's disease. *Movement Disorders*, *27*(6), 709–715. https://doi.org/https://doi.org/10.1002/mds.23838

Shipley, M. M., Mangold, C. A., & Szpara, M. L. (2016a). Differentiation of the SH-SY5Y Human Neuroblastoma Cell Line. *Journal of Visualized Experiments : JoVE*, *108*, 53193. https://doi.org/10.3791/53193

SH-SY5Y. (n.d.). www.atcc.org

Slanzi, A., Iannoto, G., Rossi, B., Zenaro, E., & Constantin, G. (2020a). In vitro Models of Neurodegenerative Diseases. In *Frontiers in Cell and Developmental Biology* (Vol. 8). Frontiers Media S.A. https://doi.org/10.3389/fcell.2020.00328

Stefanis, L., Larsen, K. E., Rideout, H. J., Sulzer, D., & Greene, L. A. (2001). Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(24), 9549–9560.

Stolzenberg, E., Berry, D., Yang, D., Lee, E. Y., Kroemer, A., Kaufman, S., Wong, G. C. L., Oppenheim, J. J., Sen, S., Fishbein, T., Bax, A., Harris, B., Barbut, D., & Zasloff, M. A. (2017). A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity. *Journal of Innate Immunity*, *9*(5), 456–463. https://doi.org/10.1159/000477990

Subramanian, S., Geng, H., & Tan, X.-D. (2020). Cell death of intestinal epithelial cells in intestinal diseases. *Sheng Li Xue Bao : [Acta Physiologica Sinica]*, *72*(3), 308–324. https://pubmed.ncbi.nlm.nih.gov/32572429

Sun, Y., Huang, Z., Liu, W., Yang, K., Sun, K., Xing, S., Wang, D., Zhang, W., & Jiang, X. (2012). Surface Coating as a Key Parameter in Engineering Neuronal Network Structures In Vitro. *Biointerphases*, *7*(1), 29. https://doi.org/10.1007/s13758-012-0029-7

Surguchev, A. A., Emamzadeh, F. N., & Surguchov, A. (2019). Cell responses to extracellular α-synuclein. In *Molecules* (Vol. 24, Issue 2). MDPI AG. https://doi.org/10.3390/molecules24020305

Sveinbjornsdottir, S. (2016). The clinical symptoms of Parkinson's disease. *Journal of Neurochemistry*, *139*(S1), 318–324. https://doi.org/https://doi.org/10.1111/jnc.13691

Technologies, L. (2000). *Lipofectamine ® 2000 Reagent Protocol Outline*. www.lifetechnologies.com/support.

Thoo, L., Noti, M., & Krebs, P. (2019). Keep calm: the intestinal barrier at the interface of peace and war. In *Cell Death and Disease* (Vol. 10, Issue 11). Springer Nature. https://doi.org/10.1038/s41419-019-2086-z

Tönges, L., Szegö, E. M., Hause, P., Saal, K.-A., Tatenhorst, L., Koch, J. C., D Hedouville, Z., Dambeck, V., Kügler, S., Dohm, C. P., Bähr, M., & Lingor, P. (2014). Alpha-synuclein mutations impair axonal regeneration in models of Parkinson's disease. *Frontiers in Aging Neuroscience*, *6*, 239. https://doi.org/10.3389/fnagi.2014.00239

Tor Lea. (2015). Caco-2 Cell Line. In C. P. L.-E. I. et al. , editors. C. (CH) Verhoeckx K (Ed.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models*. Springer.

Turner, J. R. (2009). Intestinal mucosal barrier function in health and disease. *Nature Reviews Immunology*, *9*(11), 799–809. https://doi.org/10.1038/nri2653

Unsicker, C., Cristian, F.-B., von Hahn, M., Eckstein, V., Rappold, G. A., & Berkel, S. (2021). SHANK2 mutations impair apoptosis, proliferation and neurite outgrowth during early neuronal differentiation in SH-SY5Y cells. *Scientific Reports*, *11*(1), 2128. https://doi.org/10.1038/s41598-021-81241-4

van de Walle, J., Hendrickx, A., Romier, B., Larondelle, Y., & Schneider, Y. J. (2010). Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in Vitro*, *24*(5), 1441–1449. https://doi.org/10.1016/J.TIV.2010.04.002

Vancamelbeke, M., & Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. In *Expert Review of Gastroenterology and Hepatology* (Vol. 11, Issue 9, pp. 821–834). Taylor and Francis Ltd. https://doi.org/10.1080/17474124.2017.1343143

Vekrellis, K., Xilouri, M., Emmanouilidou, E., & Stefanis, L. (2009). Inducible over-expression of wild type α-synuclein in human neuronal cells leads to caspase-dependent non-apoptotic death. *Journal of Neurochemistry*, *109*(5), 1348–1362. https://doi.org/https://doi.org/10.1111/j.1471-4159.2009.06054.x

Volpicelli-Daley, L. A., Luk, K. C., Patel, T. P., Tanik, S. A., Riddle, D. M., Stieber, A., Meaney, D. F., Trojanowski, J. Q., & Lee, V. M. Y. (2011). Exogenous α-Synuclein Fibrils Induce Lewy Body Pathology Leading to Synaptic Dysfunction and Neuron Death. *Neuron*, *72*(1), 57–71. https://doi.org/10.1016/J.NEURON.2011.08.033

Walsh, K. T., & Zemper, A. E. (2019). The Enteric Nervous System for Epithelial Researchers: Basic Anatomy, Techniques, and Interactions With the Epithelium. *Cellular and Molecular Gastroenterology and Hepatology*, *8*(3), 369–378. https://doi.org/10.1016/J.JCMGH.2019.05.003

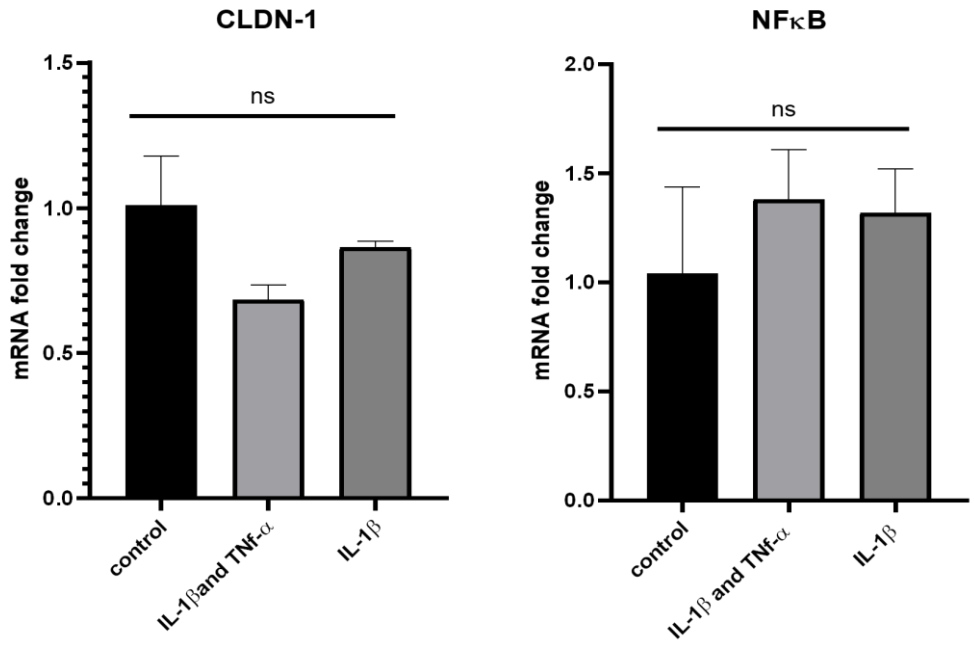
Xilouri, M., Brekk, O. R., & Stefanis, L. (2013). α-Synuclein and protein degradation systems: a reciprocal relationship. In *Molecular neurobiology* (Vol. 47, Issue 2, pp. 537–551). https://doi.org/10.1007/s12035-012-8341-2

Zoumpopoulou, G., Tsakalidou, E., Dewulf, J., Pot, B., & Grangette, C. (2009). Differential crosstalk between epithelial cells, dendritic cells and bacteria in a co-culture model. *International Journal of Food Microbiology*, *131*(1), 40–51. https://doi.org/10.1016/J.IJFOODMICRO.2008.12.037

Appendix

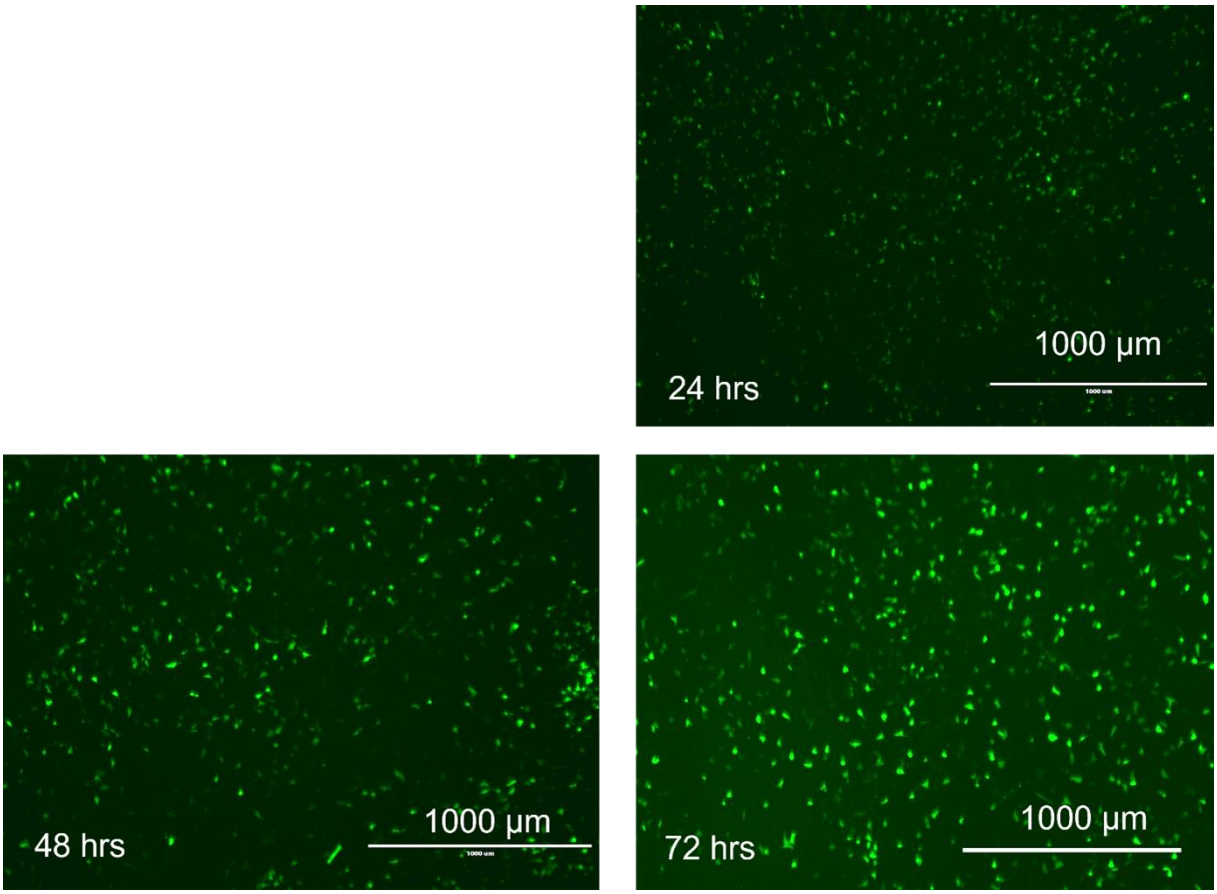
Positive control selection

Some first experiments were performed to select a suitable positive control for further experiments. For these assays, Caco-2 cells were seeded in 6-well plates and stimulated with different cytokines. This condition would ideally mimic both a reduced intestinal barrier integrity and elevate intestinal inflammation. As shown in figure 1 the results indicated that no of the tested conditions fulfilled this prerequisite.



Appendix figure 1 mRNA levels of CLDN-1 and NFκB after 72 hrs incubation with IL-1β alone or in combination with TNF-α (all 20 ng/ml) as compared to non-treated cells normalized to GAPDH using the 2-ΔΔCt method determined with qPCR.

Transfection validation α-synuclein plasmid



Appendix figure 2 Fluorescence images of non-differentiated SH-SY5Y transfected with α-synuclein conjugated with GFP showing increasing expression of the α-synuclein protein for at least up to 3 days.