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OPTIMIZING CO-CULTURE CONDITIONS FOR HUMAN IPSC-NEURONS AND CACO-2 CELLS

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ABSTRACT

Magdalena Hyttinen: Optimizing co-culture conditions for iPSC-neurons and CaCo-2 cells
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Background and aims: The connection between brain and gut has been acknowledged for centuries. It is now known that gastrointestinal tract has straight link to the central nervous system (CNS) which further links it to the whole body. It is called gut-brain axis (GBA). GBA is a bidirectional pathway between the enteric nervous system and the CNS. It involves direct and indirect links between cognitive and emotional brain systems and peripheral intestinal functions. Abnormal GBA activity can lead to broad spectrum of pathologies since it affects the whole body. Even though the research on the GBA has increased, the understanding of the connection and its details is still limited. The discovery of induced pluripotent stem cells (iPSCs) has enabled the development of novel cell systems. Cell model with physical connection between gut cells and brain cells does not exist yet. The aim of this thesis was to optimize co-culture conditions of iPSC-neurons and CaCo-2 cells. These conditions included the co-culture media, cell co-culture proportions and cell culture time. Viable 3D co-culture could expand the understanding of GBA and allow studying the connection further.

Materials and methods: Two different cell lines were used in the thesis: hiPSC-line 04511.WTS which was differentiated into hiPSC neurons and CaCo-2 (cancer coli-2) cells. Four different experiments were performed. They included separate cultures of CaCo-2 and neuronal cells and co-cultures to find suitable culturing conditions including culture period, culturing media and cell proportions for both cell types. Cell surface and plating order of the cells was altered. Two of the experiments included only 2D cultures and other two had also 3D cultures. 2D cultures and co-cultures were cultured on plastic 48-wellplates. For 3D co-cultures 3D3C-chip, compartmentalized PDMS-based microfluidic device was used. 3D3C-chip consists of two parts, medium chamber and cell culturing chamber which are connected with microtunnels. Verification of the functionality and cell type specific protein expressions was conducted with immunofluorescence microscopy. For CaCo-2 the marker was ZO-1 and for neurons MAP2 and βIII-tubulin.

Results and discussion: Based on the phasecontrast microscopy imaging and indirect immunofluorescence staining results, it was shown in this thesis that CaCo-2 cells can be cultured on PLO and LN521 coated surfaces including 3D3C-chip. However, the results of this thesis indicate that the optimization of the co-culture was not successful, and no evidence was found for physical interaction between the cell types using the imaging techniques. After 14 days of the co-culture the morphology of the neuronal cells was abnormal and the number of the cells in the culture was low in both 2D and 3D co-cultures. Despite its exploratory nature, this thesis gives preliminary insight into the optimization process of the co-culture for intestinal epithelial cells and neuronal cells.

Keywords: co-culture, CaCo-2 cells, iPSC-neurons, gut-brain axis

TIIVISTELMÄ

Magdalena Hyttinen: Yhteiskasvatuksen olosuhteiden optimointi indusoiduista lähes kaikkikykyisistä kantasoluista erilaistetuille hermosoluille ja CaCo-2 soluille
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Tausta ja tavoitteet: Aivojen ja suoliston välinen yhteys on tunnettu jo vuosisatojen ajan. Nykyään tiedetään, että ruoansulatusjärjestelmästä on suora yhteys keskushermostoon ja sitä kautta koko kehoon. Tätä yhteyttä kutsutaan suoli-aivo-akseliksi. Suoli-aivo-akseli on kaksisuuntainen viestintäreitti enteerisen hermoston ja keskushermoston välillä. Siihen kuuluvat suorat ja epäsuorat yhteydet kognitiivisten ja emotionaalisten aivojärjestelmien ja suoliston toimintojen välillä. Suoli-aivo-akseli osallistuu koko kehon hemostasian ylläpitämiseen, joten häiriöt sen toiminnassa voivat vaikuttaa monien eri sairauksien puhkeamiseen. Viimeaikaisesta kiinnostuksesta ja tutkimuksen lisääntymisestä huolimatta suoli-aivo-akselin kaikkia toimintamekanismeja ei vielä ymmärretä täysin. Ihmisen indisoitujen lähes kaikkikykyisten kantasolujen käyttö on mahdollistanut uusien solumallien kehittämisen, mutta solumallia, jossa suolen epiteelin solut ovat suorassa kosketuksessa hermosolujen kanssa, ei ole vielä kehitetty. Tämän tutkimuksen tavoitteena oli optimoida yhteiskasvatuksen olosuhteet indusoiduista lähes kaikkikykyisistä kantasoluista (iPS-solut) erilaistetuille hermosoluille ja CaCo-2 suolisoluille. Kolmiulotteinen solumalli laajentaisi ymmärrystä suoli-aivo-akselista ja mahdollistaisi uusia tutkimussuuntia.

Metodit: Tutkimuksessa käytettiin kahta eri solulinjaa: hermosoluja, jotka erilaistettiin iPS-soluista ja CaCo-2 suolisoluja. Tutkimus jakautui neljään eri osaan, joissa hyödynnettiin sekä 2D, että 3D soluviljelmiä yhteiskasvatuksen olosuhteiden optimoimiseksi. Optimoitavana olivat kasvatuksen kesto, kasvatusmedium ja solujen määrä. Näiden lisäksi kasvatusalustaa ja kasvatuksen ajoitusta eri solutyyppien kohdalla muunneltiin tarvittaessa. 3D-soluviljelmissä käytettiin 3D3C-siruja, jotka ovat kahdesta osasta koostuvia mikrofluidistisia laitteita. Kasvatusmediumkammioita ja soluviljelykammioita erottavat mikrotunnelit. Solujen elinkyky ja solutyypeille spesifisten proteiinien eritys varmistettiin immunofluoresenssivärjäyksillä.

Tulokset: Faasikontrastimikroskopoinnin ja immunofluoresenssivärjäysten perusteella osoittautui, että CaCo-2 suolisoluja voidaan kasvattaa polyornitiini ja ihmisen laminiini 521 proteiinialustalla. Lisäksi todettiin, että CaCo-2 suolisoluja voidaan onnistuneesti kasvattaa 3D3C-siruilla. Tästä huolimatta, yhteiskasvatuksessa ei ollut havaittavissa käytetyillä kuvantamismenetelmillä hermo- ja suolisolujen välistä yhteyttä. Kahden viikon yhteiskasvatuksen jälkeen 2D- ja 3D-soluviljelmissä oli havaittavissa molempia solutyyppejä, mutta hermosolujen morfologia oli epänormaali ja niiden määrä oli vähäinen. Tutkimuksen kokeellisuudesta huolimatta se taustoittaa tulevaa tutkimusta suolen epiteelin ja hermosolujen yhteiskasvatukselle ja suoli-aivo-akselin solutason tutkimiselle.

Avainsanat: yhteiskasvatus, CaCo-2 solut, hermosolut, suoli-aivo-akseli

PREFACE

This thesis was conducted in the Celiac Disease research Centre and NeuroGroup, Faculty of Medicine and Health Technology at Tampere University, Finland. I want to thank my supervisors Kati Juuti-Uusitalo and Susanna Narkilahti for supervising this project. I also want to thank researchers and laboratory personnel of both research groups. Special thanks go to technician Hanna Mäkelä for her advice and help with the experimental part of my thesis. Lastly, I want to thank my family and friends for the support while working on my thesis during the COVID-19 pandemic time.

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Magdalena Hyttinen

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LIST OF SYMBOLS AND ABBREVIATIONS

(h)IPSC	(human) induced pluripotent stem cells
ANS	autonomic nervous system
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CaCo-2	Cancer coli-2 cells
CBC	crypt base columnar cell
CDX2	homeobox protein CDX2
CNS	central nervous system
Db-cAMP	dibutyryl-cyclic-AMP
EGC	enteroglial cell
EGF	epidermal growth factor
ENCDC	enteric neural crest derived cell
ENS	enteric nervous system
FBS	fetal bovine serum
GBA	gut-brain axis
GDNF	glial cell line derived neurotrophic factor
HPA	hypothalamic pituitary adrenal
ICC	interstitial cells of Cajal
IEC	intestinal epithelial cell
ISC	intestinal stem cell
LN521	laminin 521
MAP2	microtubule-associated protein 2
NC	neural crest
NDS	normal donkey serum
NEAA	non-essential amino acid
NF-H	anti-neurofilament H
NMM	neuronal maturation medium
Notch	neurogenic locus notch homolog protein
P/S	penicillin-streptomycin
PLO	poly-L-ornithine
PNS	peripheral nervous system
R-spondin	roof plate-specific spondin
WNT	wingless-Int-1
ZO-1	zonula occludens-1
βIII-tubulin	class III β-tubulin

1. INTRODUCTION

The connection between brain and gut has been acknowledged since the antiquity. Especially popular topic it became in nineteenth century, in the era of rapidly accelerating scientific discovery and invention. Back then the mechanisms and pathways were unknown but the interaction between these organs was so clear that several theories of this connection arise. Typical for these theories was that they acknowledged the connection, but it was considered to be one-sided. Doctors used to blame the gut how it affects the mind and psychologists blamed the mind for its effects on the guts. (Miller, 2018)

Understanding of this complex connection has evolved. Research on the field has been rapidly increasing in the past decades, and the pathways between brain and gut has become clearer. It is now known that gastrointestinal tract has straight link to the central nervous system (CNS) which further links it to the whole body. It is called gut-brain axis (GBA). (Carabotti ym., 2015; Khlevner ym., 2018; Podolsky ym., 2015) The link is not only from the gut to the brain, but the GBA is a bidirectional pathway between the enteric nervous system (ENS) and the CNS. (Malagelada, 2020) The pathway is formed early in the development and persist throughout life. GBA controls several functions important for homeostasis in both CNS and ENS. These are sensation and motility in intestine, and control of behavior, cognition, and mental health in the brain. Earlier these functions were thought to happen separately from each other. (Khlevner ym., 2018)

Abnormal GBA activity can lead to broad spectrum of pathologies throughout the digestive system and neurological pathways since it affects the whole body (Khlevner ym., 2018). Even though the research on the GBA has increased, the understanding of the connection and its details is still limited. Models to study GBA has heavily relied on the animal models which have several weaknesses starting from the inability to fully represent the human systems. Just recently the development in the field of 3D cell biology, materials, natural science, discovery of the induced pluripotent stem cells (iPSCs) and bioengineering has enabled the use of human cells and organoids instead of animals in GBA *in vitro* studies. Despite the efforts on the research, the complete model representing the axis is still missing. Different organoid-models and cell-models exist but they rarely have physical connection between the gut and brain cells. (Moysidou & Owens, 2021)

2. LITERATURE REVIEW

2.1 Gastrointestinal tract

Human gastrointestinal tract (GI tract) is tube-like system. Structure of the GI tract with most important organs is presented in the figure 1. It is composed of several different organs which digest food, absorb nutrients and remove waste materials after extracting the energy. GI tract can be divided to upper and lower parts. Mouth, esophagus, stomach and small intestine make up the upper part of the GI tract. Lower part consists of colon, rectum, and anus. GI tract has multiple organs that assist the main functions such as salivary glands, pancreas, liver, and gallbladder. All these together make up the digestive system. (Greenwood-Van Meerveld, 2017; Latorre ym., 2016)

Digesting nutrients is the most important function of the GI tract. This is accomplished via complex tasks including secretion of digestive enzymes and absorption of nutrients. The process starts in the mouth where food is chewed with help of saliva. From the mouth, the esophagus moves the food bolus to the stomach with esophageal peristalsis. Digestive enzymes and gastric acids turn the food bolus into chyme when it reaches the stomach and starts the absorption. Most of the nutrients including proteins, fats, and carbohydrates are broken down and absorbed in the small intestine leaving only the waste material left. This material is moved to the large intestines and removed from the body. (Greenwood-Van Meerveld, 2017)

GI tract has a functional anatomy which consists of four layers to help digestive processes. These layers are presented in the figure 1. The most inner layer is the mucosal layer with absorptive and secretory epithelial cells. The submucosal layer contains nerves, lymphatics, and connective tissue. Longitudinal and circular smooth muscles are organised in the smooth muscle layer under the submucosal layer. The most outer layer is the serosal layer. Effective digestion is coordinated by neuronal signals. CNS and ENS are together controlling the digestive processes through complex signalling systems. (Greenwood-Van Meerveld, 2017; Liao ym., 2009)

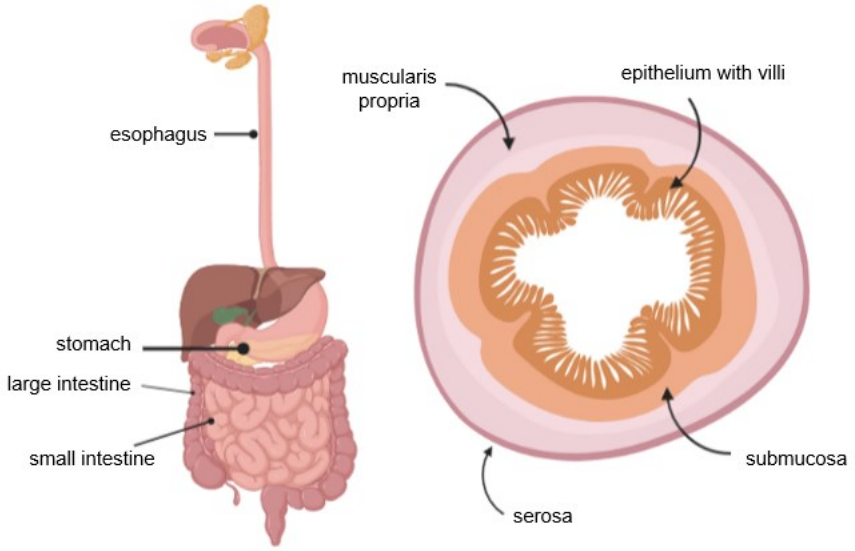

Figure 1. *Structure of the GI tract with main organs and four different layers of the intestinal wall. Picture created with Biorender.*

2.1.1 Structure and function of small intestine

Small intestine is a hollow structure in upper part of the GI tract with specialized function. It absorbs most of the nutrients from the food bolus coming from the stomach. Anatomically small intestine has three parts: duodenum, jejunum, and ileum. Duodenum separates the stomach and small intestine while being clearly its' own part with different structure. Jejunum and ileum are the proximal and distal parts of the small intestine, although their individual lengths are difficult to measure. Most of the absorption happens in the proximal part of the intestine which is seen in increased number and thickness of folds. (Podolsky ym., 2015; Thomson & Freeman, 2005)

The intestinal wall has four distinct layers throughout the GI tract. The most outer layer is the serosa which is formed of the mesothelial cells extending from the peritoneum. Muscularis propria creates the muscle layer of the intestine which is responsible of the peristaltic movements of the intestine. It consists of outer longitudinal and inner circular muscle layers which are separated by ganglion cells of the myenteric plexus (Auerbach's plexus). Under the muscularis propria is the submucosa made of connective tissue with several different cell types. These include lymphocytes, plasma cells, mast cells, eosinophils, macrophages and fibroblasts. Submucosa has numerous ganglion cells and nerve fibres in Meissner's plexus with vascular and lymphatic structures. The most inner layers of the intestinal wall, mucosa and lamina propria, are the most important parts of

the absorption function of the intestine. They are separated by layer of muscle cells, muscularis mucosae. Lamina propria has similar structure to submucosal layer while the mucosa is mostly consisted of epithelial cell layer. This epithelial cell layer is divided into villus and crypt (crypts of Lieberkühn) regions. Villi extends into the lumen of small intestine with finger-like projections that are covered with epithelial cells accompanied with goblet cells and intraepithelial lymphocytes. Epithelial cells are specialized for digestion and absorption. Epithelium is maintained and replenished by stem cells that reside in the crypts with less well-differentiated epithelial cells accompanied with Paneth cells and enteroendocrine cells. Differentiation of the stem cells occurs while the cells migrate from the crypt towards the villus. This process takes four to six days in total. (Podolsky ym., 2015; Thomson & Freeman, 2005; Thomson & Shaffer, 2012)

2.1.2 Epithelium of small intestine

Epithelium makes up the most inner layer of the small intestine. Its main function is digestion and absorption of nutrients. To accomplish that function, epithelial cells specialize to specific tasks, and they are metabolically highly active. (de Santa Barbara ym., 2003) Intestinal epithelial cells (IECs) create a physical and biochemical barrier between the intestines and external environment (Peterson & Artis, 2014). Several different cell types can be identified in the epithelium of the small intestine, and they are presented in the figure 2. All the cell types in the epithelium are produced by the intestinal stem cells (ISCs), also known as crypt base columnar cells (CBC cells) which reside in the crypt. The stem cells are pluripotent with a high proliferative rate. They have embryonic cell-like features, and they don’t migrate from the crypt base as other cell types. (Podolsky ym., 2015; Thomson & Freeman, 2005) Stem cells are characterized by expression of the R-spondin (Rspo) receptor Lgr5 (Capdevila ym., 2021). One crypt holds around four to six stem cells. These stem cells produce the progenitor cells which stay undifferentiated in the crypt. Once these cells start to move towards the villus, they differentiate to five main cell types of the small intestine. The five main cell types are enterocytes, enteroendocrine cells, chemosensory tuft cells, Paneth cells, and goblet cells. (de Santa Barbara ym., 2003) In addition to the five main cell types, there are several other cell types in the small intestine. Pathogens are delivered to the lymphocytes, macrophages and other immune system components by Microfold cells (M-cells). M-cells are epithelial cells which reside in the lymphoid follicles. (Thomson & Freeman, 2005). Based on the differentiation state of the cells, the cellular position changes along the crypt-villous unit (in the RAD axis). The least differentiated cells are closer to the base of the crypt and more differentiated cells are on the top. Paneth cells are exception since they are at the

bottom of the crypts with the stem cells. The oldest cells in the tip of the villus die by apoptosis and shed to the intestinal lumen. The lifespan of the cells is usually only 5-6 days. This is important since the epithelial cells are exposed to high amount of mechanical stress and microbes. (de Santa Barbara ym., 2003)

Enterocytes are the most common intestinal epithelial cells. Up to 80% of intestinal epithelial cells are enterocytes. They are columnar cells connected to each other with lateral junctions creating impermeable layer. (de Santa Barbara ym., 2003) On top of that layer, they have apical microvilli which increases the absorptive surface of the intestine. Due to the microvilli, enterocytes are the main absorptive cells of the intestine. They have hydrolytic and absorptive functions along with secretion of digestive enzymes such as glycosidases, peptidases, and lipases. Mucus protects the epithelial layer of enterocytes. It is secreted by the Goblet cells which have special mucous granules in their cytoplasm. Goblet cells make up to 5% of the epithelial cells and they are scattered along the crypt-villus-axis. Their mucus protects all the cells contacting the intestinal lumen. GI motility is coordinated not only by neuronal signals but also with hormones. Enteroendocrine cells are epithelial cells which produce hormones to assist digestion. Enterocytes, goblet cells and enteroendocrine cells all have short lifespan since they are in the upper parts of the villi. In contrast to that, Paneth cells which are in the base of the crypt have a longer turnover time of around 20 days. Mature Paneth cells are columnar epithelial cells with apical cytoplasmic granules. Function of the Paneth cells is the antimicrobial defence of the intestine, however they have a role in maintaining the stem cell niche in the crypt due to secreting nonessential self-renewal factors. (de Santa Barbara ym., 2003)

Crypt-base columnar intestinal stem cells maintain the intestinal epithelium. They can self-renew and multilineage differentiation allows differentiation into absorptive and secretory cell lineages. Crypt-adjacent subepithelial mesenchymal cells are a heterogenous cell population which secrete the Wnt and Rspo transcription factors that are essential for the self-renewal. Stem cell niche holds several supportive factors which help to maintain the stem cell state and alter the self-renewal signals based on the differentiation state of the other cell types. (Capdevila ym., 2021) The most important transcription factors are Notch, Wnt, and EGF signals. These master regulator transcription factors with other signals dictate the selection of the specific cell type that a progenitor becomes. Transit-amplifying (TA) cells are thought to be at the interface between self-renewal and differentiation. They are the first step stem cells take to become differentiated and they proliferate rapidly and can renew for several divisions until they undergo multilineage differentiation. (Capdevila ym., 2021)

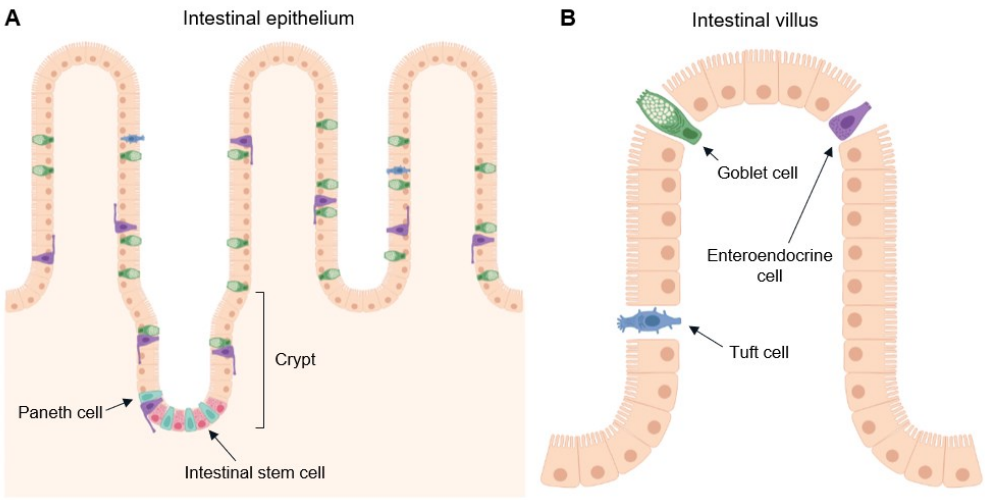

Figure 2. *Structure of the small intestine with main cell types presented. A. Intestinal epithelium B. Intestinal villus. Picture created with Biorender.*

2.2 Enteric nervous system

2.2.1 Structure and function of enteric nervous system

Neural system of the GI tract is responsible for the movement of the luminal contents along the tract, secretion of digestive enzymes, and absorption of luminal contents. (Greenwood-Van Meerveld, 2017). Normal functioning of the gut is controlled array of events and it requires neuronal monitoring and guidance. Movement of the ingested material needs to happen at a rate that allows all of the parts of the GI tract to accomplish their own task, smooth muscle contractions have to be coordinated to process the food bolus, right amount of electrolytes, enzymes and mucus is needed for absorption of nutrients and immunological defense is constantly adapted. (Rao & Gershon, 2016)

These functions are mainly coordinated by the GI tract's own subdivision of the autonomic nervous system (ANS), enteric nervous system. ENS enables routine mechanisms of digestion to happen without the involvement of the CNS (Greenwood-Van Meerveld, 2017). ENS is the largest component of the autonomic nervous system and part of peripheral nervous system (PNS) (Rao & Gershon, 2016).

The autonomic nervous system has three parts: sympathetic, parasympathetic and enteric nervous systems. ENS differs from the sympathetic and parasympathetic system by the lack of direct innervation from the CNS. ENS controls smooth muscle and mucosal functions and consist of some of the transmitters and neuromodulators found in the CNS. Sensory neurons, motor neurons and interneurons make up the basic structure of the

ENS. Neurons respond to local stimuli and integrate information to coordinate motor output. (Greenwood-Van Meerveld, 2017; Rao & Gershon, 2016) Even though ENS can mediate gut environment independently, two-way communication normally occurs with CNS (Rao & Gershon, 2016).

The ENS is derived from the neural crest (NC). NC is a cell population with high migratory and proliferation ability. It originates at the junction of the neural plate and the adjacent ectoderm. Starting in embryonic week 4, enteric NC-derived cells (ENCDCs) invade the foregut and travel all the way to the bowel where they migrate and form the two layers of ganglia that later develop to the myenteric and submucosal plexuses. (Lake & Heuckeroth, 2013)

The ENS has around 200-600 million neurons. Most of these neurons are located in thousands of small ganglia. Most neuron-riches are myenteric and submucosal plexuses. (Furness, 2008) Myenteric plexus coordinates the muscle movements and the submucosal plexus controls the secretion and absorption. (Spencer & Hu, 2020). Due to their specific tasks, myenteric plexus creates neuronal network from the upper esophagus to the internal anal sphincter to control the muscle movements of the whole GI tract. Submucosal plexus coordinates the secretion and absorption in the small and large intestines. ENS further connects to the CNS via vagus and pelvic nerves and sympathetic pathways. (Furness, 2008; Furness ym., 2014; Zhang & Que, 2020) Each plexus has population of neurons with different roles, projections and neurochemical coding. Internodal strands connect ganglia in each plexus together. Internodal strands can have axons over 13cm in length. (Spencer & Hu, 2020)

Different neuron types in the ENS are identified by their functions, proportions, connections and cell body morphologies. As stated earlier, most of the intrinsic neurons reside in the myenteric and submucosal plexuses. (Furness, 2000; Podolsky ym., 2015) Primary afferent neurons in the plexuses are sensitive to chemical and mechanical stimuli. Interneurons and motor neurons act of the different effector cells including smooth muscle, pacemaker cells, blood vessels, mucosal glands, and epithelia. (Costa, 2000; Podolsky ym., 2015) Enteric glia are a population of peripheral neuroglia. They are found throughout the digestive tract associated with the cell bodies and processes of enteric neurons. (Seguella & Gulbransen, 2021) They regulate homeostasis in the ENS, and they modulate intestinal reflexes through communicating between other neurons. They are one of the most dynamic signaling components of the ENS (Seguella & Gulbransen, 2021)

Similar to the heart, gut has non-neuronal pacemaker cells which generate rhythmic electrical rhythmicity in the smooth muscle cells. These are the Interstitial Cells of Cajal (ICC). There is some controversy if these cells are part of the ENS or part of the gut. (Seguella & Gulbransen, 2021; Spencer & Hu, 2020)

2.2.2 Interaction of enteric nervous system and intestinal epithelium

GI tract has extrinsic and intrinsic nervous systems. ANS makes up the extrinsic part while the ENS creates the intrinsic part. Various intestinal cells such as epithelial cells have neurotransmitter receptors related to these nervous systems. Myenteric ganglia, smooth muscle, and mucosa form synaptic connections with sympathetic and parasympathetic nerve fibers of the ANS. Base of the crypt have nerve endings from the sympathetic nervous system while the epithelial cells are directly synapsed. These synapses enable the regulation of mucosal regeneration and inflammation of the epithelium. Permeability of the epithelium is one of the most important functions to control. (Duan ym., 2021)

Sympathetic and parasympathetic nerve fibers connect CNS to the ENS. ENS functions independently although synaptic connections are created with intestinal ganglion cells. Enteroglial cells (EGCs) are supporting cells of intestinal neurons. They secrete neuron-related factors which form a communication network in the intestinal wall. EGCs have direct nerve fiber connections with sympathetic nerves. (Duan ym., 2021)

2.3 Gut-brain axis

2.3.1 Structure and function of gut-brain axis

GBA is bidirectional communication pathway between the central and the enteric nervous system. It links emotional and cognitive centers of the brain to the peripheral intestinal functions. The simplified basic structure of the GBA is presented in the figure 3. The role of the GBA is to help maintain the homeostasis by monitoring and integrating gut functions and linking them to emotional and cognitive centers of the brain. This is accomplished by regulating the immune activation, intestinal permeability, enteric reflex, and entero-endocrine signalling. Different neuro-immuno-endocrine mediators are involved in this communication. Communication pathway of GBA includes the CNS, ANS, ENS and the hypothalamic pituitary adrenal (HPA) axis. HPA axis controls the responses

to different kinds of stressors. (Carabotti ym., 2015) Microbiome of the gut is often included as a part of the GBA, but it is not considered in this thesis.

The most important pathway from the CNS to the ENS is the vagus nerve although the link remains incompletely understood. Solitary tract and higher emotion-regulating networks in the brain are connected to the GI tract with the vagus nerve and its branches. Several other neuronal pathways are part of the communication along with the other communication mechanisms. (Margolis ym., 2021)

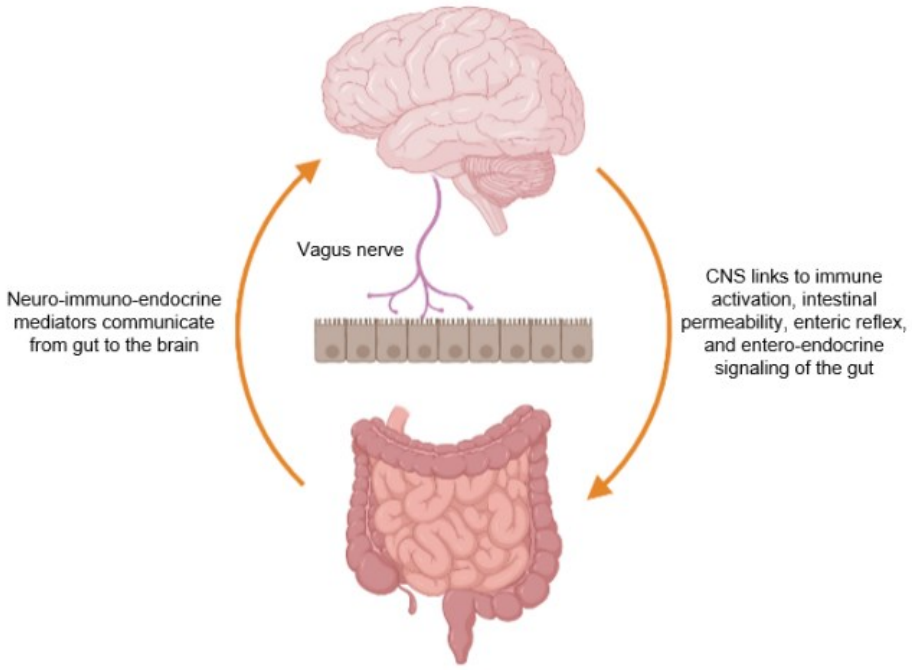

Figure 3. *Simplified structure of the gut-brain axis. The most important communication pathway is the vagus nerve although, the communication is complex system including several mechanisms. Picture created with Biorender.*

2.4 Models of gut-brain axis

GBA has been actively studied during the last decade. The study of the GBA relied on the animal models for a long period of time. Most commonly used animals have been rodents and studies have been limited to either gut or brain. Now new techniques on cell biology, computational biology and genetics have enabled new ways to model the connection including 2D and 3D cell models and chip devices. This has given opportunity to for example, identify real time changes in neurological activity and build correlation with changes in brain and gut function. This helps to explain how these complex systems interact with each other. (Zyoud ym., 2019)

Limited amount of research has been done on gut-brain axis using cell models due to lack of suitable cell types and advanced media formulations. (Satsu ym., 1999) performed a series of experiments with CaCo-2 human cells and neuronal PC12 rat cells. They examined the interaction between intestinal epithelial cells and peripheral neurons with co-culture of these two cell types. During the time gut-brain axis was not the main research line even though their research links strongly to it. As in other studies from the same decade, this one focus more on the gut side of the gut-brain axis and the link to the brain remains unacknowledged. Cells were cultured separately with CaCo-2 cells on inserts on wellplate and PC12 cells under the inserts on bottom of the well. Co-culture was continued for up to 15 days which proved that co-culture model is suitable for studying interaction between intestinal epithelial cells and other cell types including neuronal cells. Shortfall of this study was that there were no human neurons used.

Since most of the previous studies have failed to represent the GBA utilizing human cells, new methods have been needed. The discovery of the iPSCs has given new opportunity to expand the knowledge on GBA since previously many cell types including human neurons have been challenging to isolate and culture, yet human based cells represent best the situation *in vivo*. Cell models have now further expanded to organoid models derived from adult tissue biopsies and iPSCs which exhibit in vivo-like architecture, regional specification and diverse cellular subtypes. These complex models have enabled study setups with several different gut epithelial cells and building electrical connection through neuronal cells. (Moysidou & Owens, 2021) Cell model based on human cells with physical interaction between gut epithelium and neurons is still missing.

3. RESEARCH AIMS

The aim of this study was to optimize the co-culture conditions for the CaCo-2 cells when cultured with hiPSC-neurons. These conditions include the co-culture media, cell proportions and cell culture time. Verification of the functionality and cell type specific protein expressions were conducted with immunofluorescence microscopy. For CaCo-2 marker is ZO-1 and for neurons MAP2 and βIII-tubulin.

4. MATERIALS AND METHODS

4.1 Cells and ethics

In this study, two different cell lines were used: hiPSC-line 04511.WTS which was differentiated into hiPSC neurons and CaCo-2 (cancer coli-2) cells. Neuronal differentiation which included neuronal induction, precursor expansion and maturation phases was performed as previously (Hyvärinen ym., 2019) has described. CaCo-2 cells are human colon carcinoma cells which differentiate spontaneously in 14-21 days obtaining apical brush border with microvilli and express enzyme activities typical of enterocytes (Lea, 2015).

The hiPSCs used in this study were acquired from voluntary subjects who had given written and informed consent. The project has supportive statement from Pirkanmaa Hospital District to use the named hPC lines in neuronal research (R20159).

4.2 Culture substrata

Before experiments CaCo-2 cells were cultured in T25- and T75-flasks until they reached confluence over 80%, typically in 4-5 days. Cells were passaged at suitable confluence and used for experiments at passages 18.

For 2D experiments CaCo-2 cells were cultured on 48-wellplates which were coated with Geltrex. Geltrex aliquots were thawed at +4°C and diluted 1:100 in cold Minimum Essential Medium (MEM). 150µl of coating solution was used per well. Coated plates were stored at +4 °C up to one week if not used immediately.

Before experiments differentiating hiPSC neurons were cultured on 6-wellplates and on day 32 of differentiation the cells were seeded for the experiments. During cell detachment on day 32, 10 µM ROCK inhibitor (Sigma) was added to the medium.

HiPSC neurons and CaCo-2 cells were co-cultured on Nunclon Delta Surface 48-wellplates and 3D3C-chips which were coated with 15µg/cm^2 poly-L-ornithine (PLO; Sigma) and 2µg/cm^2 human laminin 521 (LN521; Biolamina). PLO was diluted in 0.1M borate buffer (1:100 dilution) and added to the wells, 165µl per well for 48-wellplate. After 1 hour incubation at 37°C PLO was removed and wells were washed three times thoroughly with sterile water. Plates were air dried in the laminar hood and LN521 diluted to PBS++ (PBS with added calcium and magnesium) was added, 146µl per well for 48-wellplate.

After overnight incubation at +4°C the plates were ready to use. Coated plates were stored up to one week at +4°C if not used immediately for the experiments.

For 3D3C-chips 22x22mm coverslips were coated with 25µg/cm^2 PLO diluted in 0.1M borate buffer, 150µl per coverslip. Coverslips were stored in filtered ethanol, and they were air dried in the laminar hood before coating. PLO was pipetted in the middle of the coverslips and coverslips were incubated for 1.5 hours at 37°C. After incubation, coverslips were washed three times with sterile water and air dried in the laminar hood. LN521 coating was done for the chips after assembly of the chips with 30µg/ml LN521 in PBS++, 110µl per chip.

4.3 Culture media

CaCo-2 cells were cultured in Minimum Essential Media (MEM) which was supplemented with Penicillin/Streptomycin, non-essential amino acids (NEAA), Sodium pyruvate (Na Pyruvate), L-alanine-L-glutamine (Glutamax) and fetal bovine serum (FBS) (figure 4). Medium was replaced every other day. Preprepared medium was stored for up to two weeks.

CaCo-2 medium

Name	Concentration	Supplier
MEM	1x	Thermo Fisher
Penicillin/Streptomycin	1 %	Lonza
NEAA	1 %	Thermo Fisher
Na Pyruvate	1 %	Thermo Fisher
GlutaMAX	1 %	Thermo Fisher
FBS	19.2 %	Sigma

Figure 4. *Table of growth factors and medium supplements for culturing CaCo-2 cells.*

After day 32 the hiPSC neurons were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, GlutaMAX™) and Neurobasal medium, which was supplemented with N2, B27 with RA, GlutaMAX supplement, non-essential amino acids (NEA), 2-mercaptoethanol, insulin, penicillin/streptomycin, brain-derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF), dibutyryl-cyclic-AMP (db-cAMP) and ascorbic acid (figure 5). Medium was replaced every other day and BDNF, GDNF, db-cAMP and ascorbic acid were added to the medium during the change.

Medium excluding BDNF, GDNF, db-cAMP and ascorbic acid was stored up to one month.

Neuronal maturation medium (NMM)

Component	Concentration	Supplier
D-MEM/F-12 with Glutamax	0.5x	Thermo Fisher
Neurobasal	0.5x	Thermo Fisher
N2	0.5 %	Thermo Fisher
B27 with RA	1 %	Thermo Fisher
GlutaMAX-I Supplement	0.5 mM	Thermo Fisher
NEA	0.5 %	Thermo Fisher
2-mercaptoethanol	50 µM	Thermo Fisher
Insulin	2.5 µg/ml	Sigma
Penicillin / Streptomycin	0.1 %	Lonza
BDNF	20 ng/ml	R&D Systems
GDNF	10 ng/ml	R&D Systems
db-cAMP	500 µM	Sigma
Ascorbic acid	200 µM	Sigma

Figure 5. *Table of composition of neuronal maturation medium (NMM).*

4.4 Culture device

2D cultures and co-cultures were cultured on 48-wellplates. For 3D co-cultures 3D3C-chip, compartmentalized polydimethylsiloxane (PDMS) based microfluidic device was used. 3D3C-chip has two parts, medium chamber and cell culturing chamber under it attached to glass coverslip (figure 6). There are three compartments. Two compartments for neurons on the sides (length = 3, width = 4 mm) and in the middle compartment for co-culture (length = 5, width = 4 mm). 40 microtunnels (length = 250, width = 10, height = 3.5 µm) connect these compartments. (Häkli ym., 2022) Axons can stretch through the microtunnels to the co-culture compartment. The medium chamber above the cell culturing chamber is separated in three compartments. Both cell culturing and medium chambers are separate excluding the microtunnels which enable minimal media change. This allows to culture different cells in different media at the same time. 3D3C-chips were provided by Pasi Kallio's Micro- and Nanosystems Research Group.

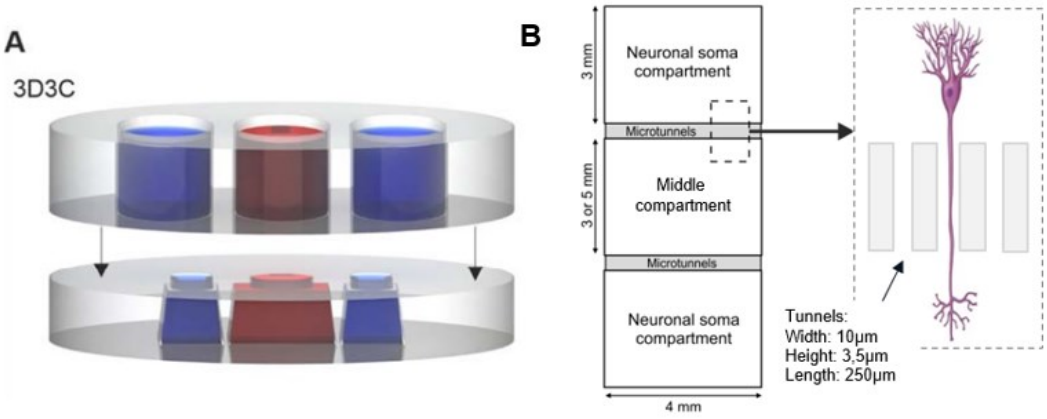

Figure 6. *A. A schematic 3D illustration of the structure of the 3D3C-chip. Chip contains two PDSM-based parts: cell culturing chamber (lower) and medium chamber (upper). (Häkli ym., 2022) B. Illustration of the structure of the micro-tunnels in the chip.*

4.5 Culture conditions

Four different experiments (figure 7) were performed including separate cultures of CaCo-2 and neuronal cells and co-cultures to find suitable culturing conditions including culture period, culturing media and cell proportions for both cells. Cell surface and plating order of the cells was altered. Two of the experiments included only 2D cultures and other two had also 3D cultures using the 3D3C-chip.

	Exp1	Exp2	Exp3	Exp4
Wellplate	X	X		
3D3C-chip			X	X
Cell number		X	X	
Coating	X			
Media	X	X		
Plating order		X	X	X
Culture period	7 days	7 days	14 days	10 days

Figure 7. *Experimental design of all four experiments with culturing conditions which were altered during the experiment. Culture period refers to the time period of the co-culture.*

Optimization of the media, protein coating of the cell surface and plating order of the cells were performed in the experiments 1 and 2. Culturing hiPSC-line 04511.WTS differentiated neuronal cells have been optimized for cell surface protein coating and cell proportions. Optimized culturing conditions for neuronal cells were tested on CaCo-2 cells in experiment 1. CaCo-2 cells were cultured on Geltrex and PLO and LN521 coated 48-wellplates in three different media including CaCo-2 MEM, neuronal maturation medium (NMM) and 1:1 mixture of these media as illustrated in figure 8. Neurons were cultured on PLO and LN521 coated wells although media testing was performed on them as well. Coatings were done as described earlier.

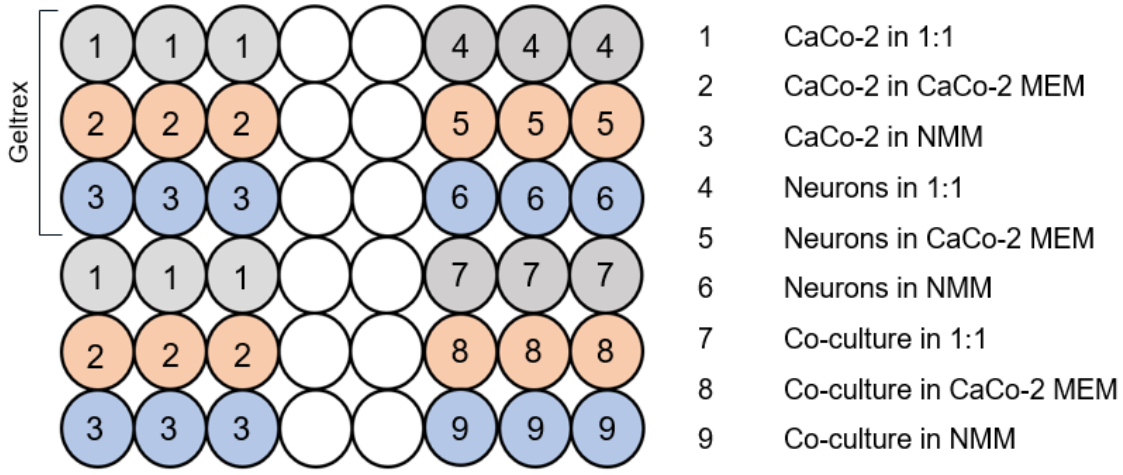

Figure 8. *Experimental design of the first experiment. Wells of 1, 2 and 3 in the first three rows were coated with Geltrex and all the other wells were coated with PLO and LN521. 9 different combinations of cells and media were tested. Co-culture period was 7 days.*

CaCo-2 cells used in the experiments 1 and 2 were on passage 18. CaCo-2 cells were rinsed twice with PBS and detached with Trypsin (Lonza). Trypsin was aspirated and cells were resuspended in CaCo-2 MEM. Cells were centrifuged, counted and resuspended to CaCo-2 MEM, NMM or 1:1 mixture of both media. Neurons were detached with Accutase (RT), counted and resuspended in NMM. In the first experiment, CaCo-2 cells and neurons were plated at the same time and both cells were plated 55 000 cells per well including co-cultures which had 110 000 cells in total. Cultures were continued for 7 days.

Plating order and the cell proportions were altered for the second experiment as shown in the figure 9. Coatings, cell detachment and plating were done identically to the first experiment except the neurons were plated to the wells 3 days and 7 days earlier than

CaCo-2 cells and number of CaCo-2 cells altered. Medium was changed every other day.

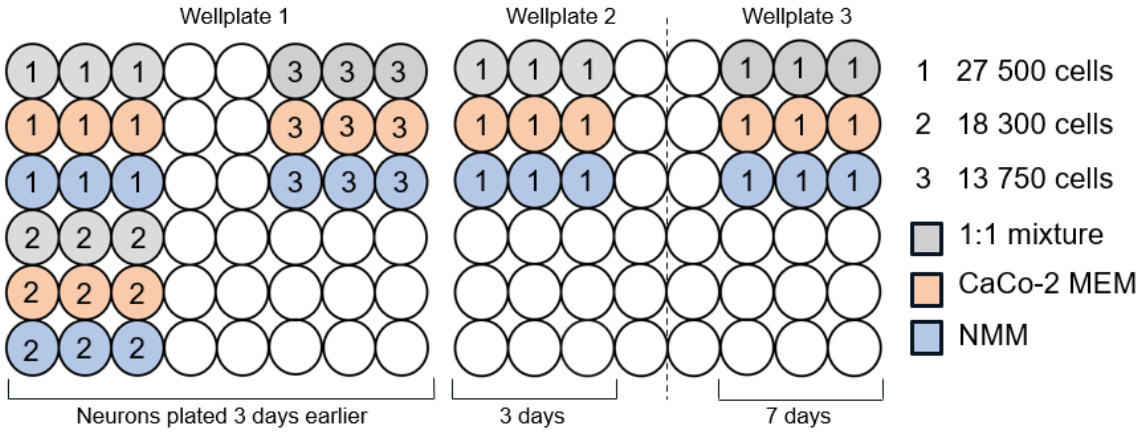

Figure 9. *Experimental design of second experiment. Number of CaCo-2 cells was altered, and neurons were plated 3 days and 7 days before CaCo-2 cells. Co-culture period was 7 days.*

3D co-cultures were cultured on 3D3C-chips with two different experiment designs (figure 10). 3D3C-chips were prepared for the experiments by dipping them in 70% ethanol for sterilization and air dried before manually assembling by attaching them on top of PLO-coated coverslips. 3D3C-chips and coverslips were coated with PLO and LN521 as described earlier. After overnight incubation at +4°C the chips were ready to use in the experiments. Neurons were plated one week before CaCo-2 cells either on both side compartments or on one side compartment of the chip. 40 000 neurons were seeded per one compartment and CaCo-2 cell number altered between 2500, 3180 and 5000. During the week, chips had only NMM, and CaCo-2 MEM was added to the middle compartment one day before CaCo-2 plating. Medium was changed every other day. Co-culture was continued for 14 days.

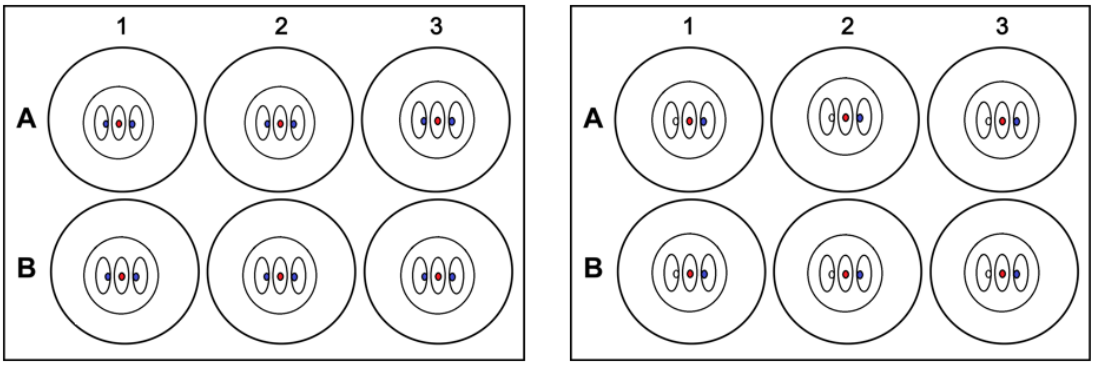

Figure 10. *The experimental design of the third experiment which utilized 3D3C-chip. Cell culturing chambers with CaCo-2 cells in red and cell culturing chambers with neurons in blue. Neurons were plated 7 days before CaCo-2 cells. Co-culture period was 14 days.*

The study design of the fourth experiment is shown in figure 11. 3D3C-chips were prepared considering the coating and assembling identically to the previous 3D3C-chip experiment. Neurons were plated on the chips in one side chamber of each chip in NMM one week before the start of the co-culture. Medium in middle chambers was changed to CaCo-2 MEM one day before plating the CaCo-2 cells. 40 000 neuronal cells were plated in one side compartment and 2500 CaCo-2 cells in the middle compartment. Detachment and plating of the cells was performed identically to previous experiment. Neurons and CaCo-2 cells were co-cultured on a chip for 10 days in total, after which indirect immunofluorescence staining was performed. Medium was changed every other day. On time-points 0, 2, 4 and 7 days two chips were fixed and stained.

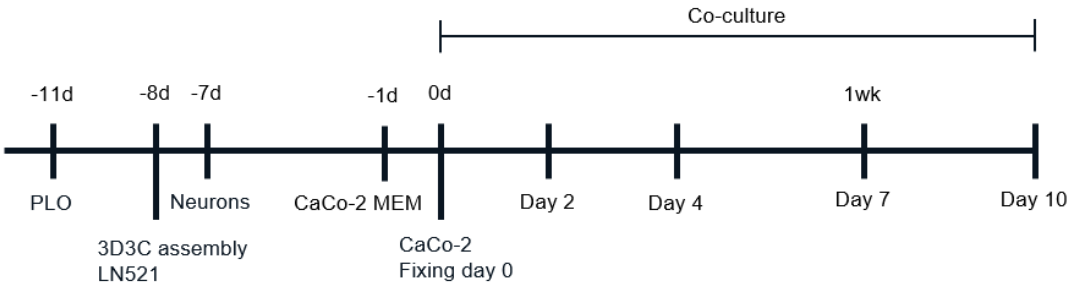

Figure 11. *The experimental design of the fourth experiment which utilized 3D3C-chip with neuron and CaCo-2 co-culture. Neurons and CaCo-2 cells were co-cultured in 3D3C-chips for 10 days in total.*

4.6 Indirect immunofluorescence

Both 2D and 3D cultures were stained utilizing the protocol previously described by (Hyvärinen ym., 2019). In 2D cultures cells were fixed in 4% paraformaldehyde (PFA; Thermo Fisher Scientific) for 15 minutes at room temperature and washed twice for 5 minutes with phosphate buffered saline (PBS; Medicage). Cells cultured in medium containing serum were washed twice for 5 minutes in PBS before fixing. If needed, cells were stored in PBS in +4°C after fixing before following staining protocol. PBS was removed and cells were blocked with 10% normal donkey serum (NDS), 0.1% Triton X-100 and 1% bovine serum albumin (BSA; Sigma) in PBS for 45 minutes at room temperature. Cells were washed once with primary antibody washing solution containing 1% NDS, 0.1% Triton X-100 and 1% BSA in PBS. Primary antibodies (figure 12) were diluted to the primary antibody washing solution mentioned earlier. Cells were exposed to the antibodies in +4°C on shaker overnight.

Cells were washed twice for 5 minutes with 1% BSA in PBS. Secondary antibodies (Figure x) were diluted to 1% BSA in PBS and cells were exposed for 1 hour in room temperature on shaker protected from the light. Two 5 minutes washes with PBS and one wash with PB were done before mounting. Cells were dried and mounted with Prolong Gold with DAPI (Thermo Fisher) using 9mm coverslips. Cells were stored in +4°C light protected until imaging.

In 3D cultures the cells were fixed in 4% PFA for 45 minutes at room temperature and after two washes with PBS blocked in 10% NDS, 0.1% Triton X-100 and 1% BSA in PBS for 45 minutes at room temperature. Cells were washed once with primary antibody washing solution containing 1% NDS, 0.1% Triton X-100 and 1% BSA in PBS. Stronger dilutions of primary antibodies were used (figure 12) for 3D cultures. Water was added to empty wells to prevent evaporation during the overnight incubation in +4°C.

Cells were washed once quickly and thereafter three times for 30 minutes with 1% BSA in PBS. Secondary antibodies (figure 12) were diluted to 1% BSA in PBS and cells were incubated for 1.5 hours in room temperature protected from the light. Previous washing protocol was repeated after the incubation with DAPI added to the PBS. Cells were mounted with Vectashield without DAPI, and no coverslips were used.

Primary Antibodies

Target	Target marker for	Host	Dilution for 2D	Dilution for 3D
MAP2	axons	chicken	1:4000	1:2000
βIII-tubulin	axons	chicken	1:200	1:100
NF-H	neurofilaments	mouse	1:1000	1:500
S100β	nuclei of neuronal cells	mouse	1:500	1:250
GFAP	astrocytes	chicken	1:4000	1:2000
ZO-1	tight junctions	rabbit	1:300	1:150
CDX2	nuclei of intestinal epithelial cells	rabbit	1:300	1:150

Secondary Antibodies

Target	Wavelength	Host	Dilution for 2D	Dilution for 3D
Chicken IgG	488	goat	1:400	1:200
Mouse IgG	488	donkey	1:400	1:200
Rabbit IgG	488	donkey	1:400	1:200
Mouse IgG	568	donkey	1:400	1:200

Figure 12. *Primary and secondary antibodies used for indirect immunofluorescence staining.*

All primary antibodies were tested on both cell types for specificity. Both 2D and 3D samples were imaged using Olympus IX-51 epifluorescence microscope (Olympus Corporation, Japan). Acquired greyscale images were colorized and contrast, brightness, and intensity were adjusted in Photoshop (Adobe Inc., USA). All adjustments were applied to the image as a whole and all samples with same staining have been processed identically.

5. RESULTS

5.1 2D culture

5.1.1 Culture substrata

CaCo-2 cells were cultured on Geltrex and PLO and LN521 coated 48-wellplates. When cultured on Geltrex, CaCo-2 cells spontaneously differentiate after 14-21 days and start to polarize and form a characteristic apical brush border with microvilli (Lea, 2015). Tight junctions between adjacent cells are visible. Both coating options lead to characteristic morphology of the cells revealing tight junctions during the 7 days culturing period as is seen in figure 13. Tight junctions create mosaic-like pattern which is seen in the pictures. There was no visually significant difference in the number of dead cells when cells were interpreted on phasecontrast microscope. This indicates that CaCo-2 cells can be cultured on both Geltrex and PLO and LN521 coating and experiments can be continued using the PLO and LN521 coating which is optimized coating option for the neuronal cells.

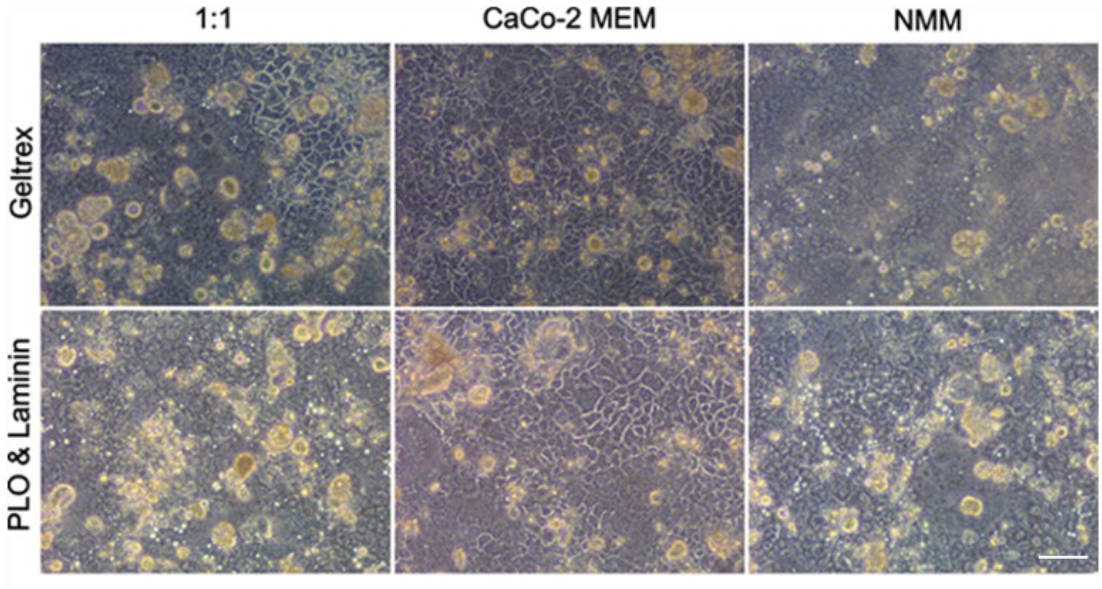

Figure 13. *Phasecontrast microscopy pictures of CaCo-2 cells cultured on Geltrex, and PLO & Laminin coated well plates in different media. Culture was continued for 7 days. Scalebar is 100µm.*

5.1.2 Culture media

Both cell types were cultured on 48-wellplate in three different media: CaCo-2 medium (CaCo-2 MEM) optimized for culturing CaCo-2 cells; neuronal maturation medium (NMM) optimized for culturing differentiated neuronal cells and 1:1 mixture of these media. In the figure 14 is shown phasecontrast microscopy pictures of cells and their co-culture in three different media. All the cells are cultured in PLO and LN521 coated wells.

NMM contains more growth factors than CaCo-2 medium which induces the cell growth. In the upper row of the figure x are the CaCo-2 cells in different media which shows the difference between CaCo-2 medium and NMM on CaCo-2 cells. CaCo-2 cells in 1:1 mixture medium and in NMM were dividing more rapidly due to which less tight junctions were visible in the culture compared to the CaCo-2 MEM. Monolayer of cells was renewing quickly and during medium changes there were more dead cells.

HiPSC neurons need growth factors to stay differentiated and continue differentiation. Differentiated hiPSC neurons appear with round soma and spiky, long and numerous axons which can be seen in figure 14 lower row where neurons are cultured in their optimized NMM. In the 1:1 mixture medium neurons are packed together and their somas appear round and axons short. Difference in the morphology is most drastic in the CaCo-2 MEM. Neuronal somas are bulky, and axons appear short. This indicates that CaCo-2 MEM contains too few neuronal growth factors to be used in further experiments. Using CaCo-2 MEM could lead to neuronal cells differentiating to other cell types during longer culture periods.

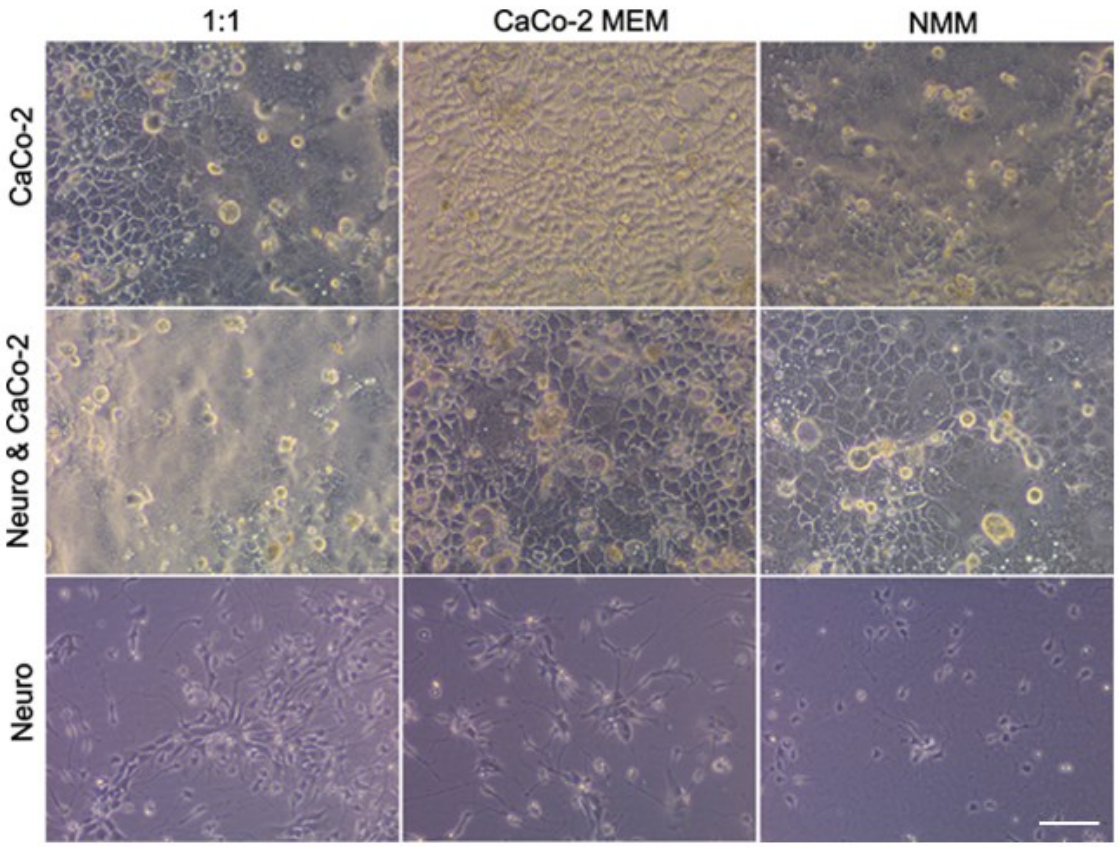

Figure 14. *Phasecontrast microscopy pictures of CaCo-2 cells, neuronal cells and co-culture in three different media; CaCo-2 MEM, NMM and 1:1 mixture of the media. Scalebar is 100µm.*

5.1.3 Culture period

In the first experiment CaCo-2 cells and neurons were plated at the same time on 48-wellplate wells and cultures were continued for 7 days at a time. Figure 15 shows the development of the cells during the 7 days culturing period. During the first days of the culture, neuronal cells were mostly detached from the surface when co-cultured with CaCo-2 cells and plated at the same time. Although the neuronal morphology remained normal during the first two days of the culture. After three days of the co-culture, neuronal cells started to form clusters of cells with no axonal growth. After 7 days of culture the clumps were completely detached during the medium change. When interpreted with phasecontrast microscope there were singular neuronal cells with very short axons with no contact to each other left.

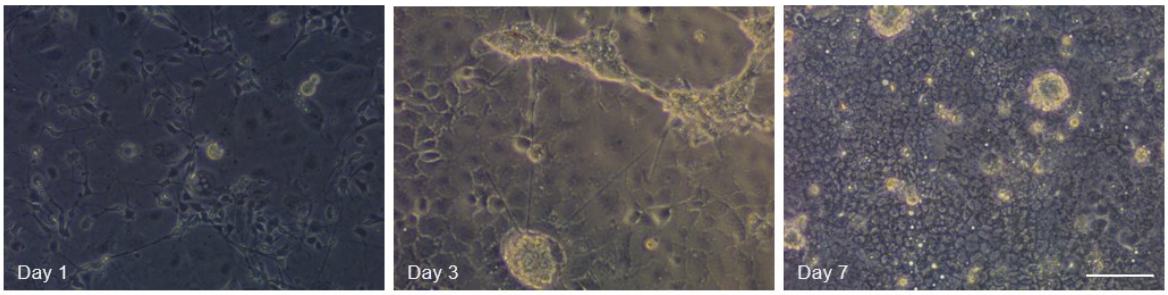

Figure 15. *Phasecontrast microscopy pictures representing the effect of culture period to the co-culture of CaCo-2 cells and neuronal cells on timepoints of day 1, day 3 and day 7. Scalebar is 100µm.*

Previous experiments with same neuronal cell line indicated that to avoid cell detachment and induce better axonal growth, neurons were plated before other cell types. CaCo-2 cells were plated 3 days or 7 days after neurons and total culturing period was either 7 days or 14 days for co-culture. All cultures had both cell types at the end of the culturing period, yet the number of neuronal cells was similar. Development of the cultures were similar to plating both cell types together. In the beginning there were more neuronal cells and the number of cells decreased when time passed.

Interaction between the cells and success of the neuronal plating was evaluated with indirect immunofluorescence staining shown in figure 16. Specificity of the markers were tested on both cell types. Intestinal marker ZO-1 (Zonula occludens-1, known as tight junction protein-1) for tight junctions of CaCo-2 cells and neuronal markers NF-H (Anti-neurofilament-H) and βIII-tubulin (Class III β-tubulin) for axons and MAP2 (Microtubule-associated protein 2) for neuronal cells were tested specific for their target proteins without excess background noise. Number of neuronal cells is slightly higher in experiment with neurons plated 7 days before plating of CaCo-2 cells when visually evaluated from the pictures.

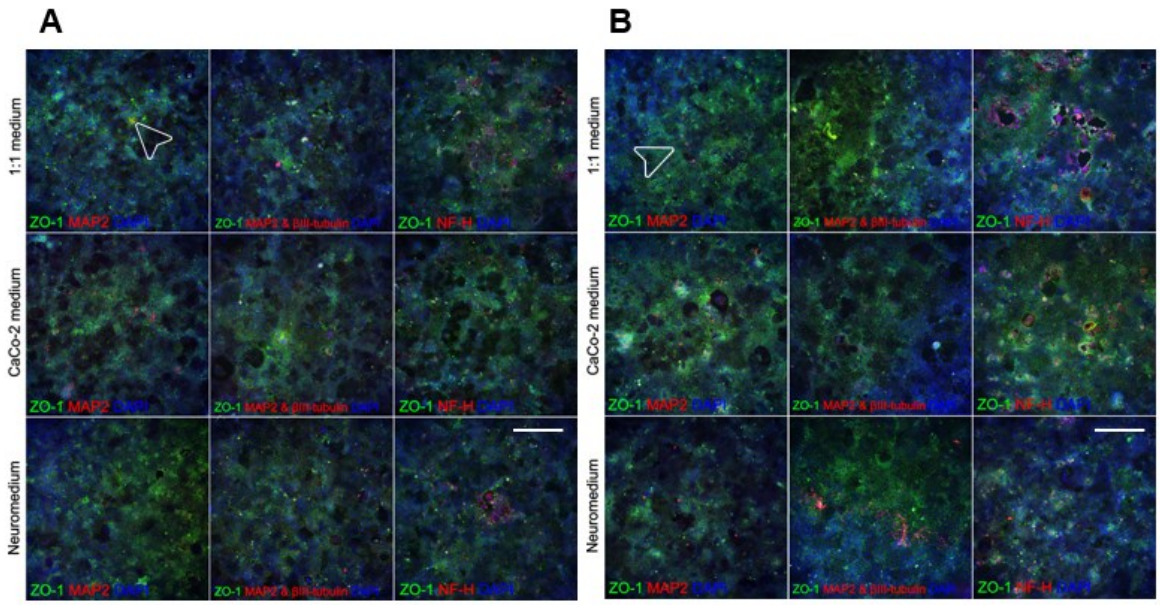

Figure 16. *Staining results of co-cultures with A. neurons plated 3 days before CaCo-2 cells, co-culture period 7 days and B. neurons plated 7 days before CaCo-2 days, co-culture period 14 days. Scalebar is 200 µm. Arrowheads pointing neuronal cells.*

5.1.4 Co-culture

Different cell proportions of CaCo-2 cells were evaluated in 2D co-cultures. Three different cell numbers were tested based on the number of neuronal cells (55000 per well): 27500, 18300 and 13750 CaCo-2 cells (figure 17). At the beginning of the co-culture different cell proportions of the CaCo-2 cells had minor differences. Co-cultures with less CaCo-2 cells had more neuronal cells and the neurons had longer axons. In higher cell proportions the CaCo-2 cells appeared smaller and more differentiated, and tight junctions more visible meanwhile in smaller cell proportions the cells were bigger and less organized while the neuronal cells appeared in higher number. After 7 days of the co-culture the differences were only minor when visually interpreted.

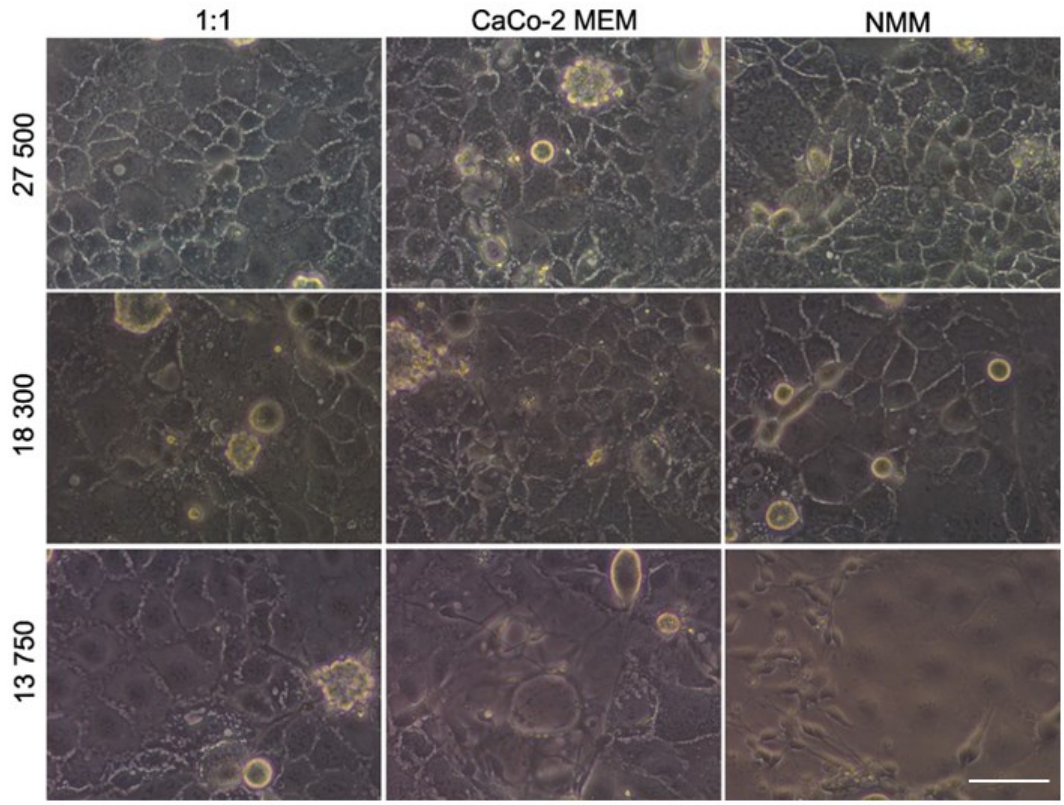

Figure 17. *Phasecontrast microscopy pictures of co-cultures in three different media options with different CaCo-2 proportions: 27500, 18300 and 13750 cells per well. Neuronal cell number 55000 in each well. Scalebar is 20µm.*

Final 2D co-cultures were conducted on PLO and LN521 coated 48-wellplates with 1:1 mixture medium and with different CaCo-2 cell proportions. Interaction between the cells was evaluated with indirect immunofluorescence staining. Indirect immunofluorescence staining with intestinal marker ZO-1 for tight junctions of CaCo-2 cells and neuronal markers NF-H and βIII-tubulin for axons and MAP2 for neuronal cells showed that after 7 days of co-culture there were differentiated CaCo-2 cells with visible tight junctions and in between low number of neuronal cells (figure 18). Axons of the neurons appear short and low in number opposite to the normal morphology. However, different CaCo-2 cell proportions showed no visible noticeable differences in the co-cultures. The appearance of neuronal cells stayed very similar regardless of the CaCo-2 cell proportions, culturing period or if the neuronal cells were plated earlier than the CaCo-2 cells. Low number of neuronal cells and their morphology with short axons indicated that experiments on 2D were discontinued and experiments on 3D3C-chips were started.

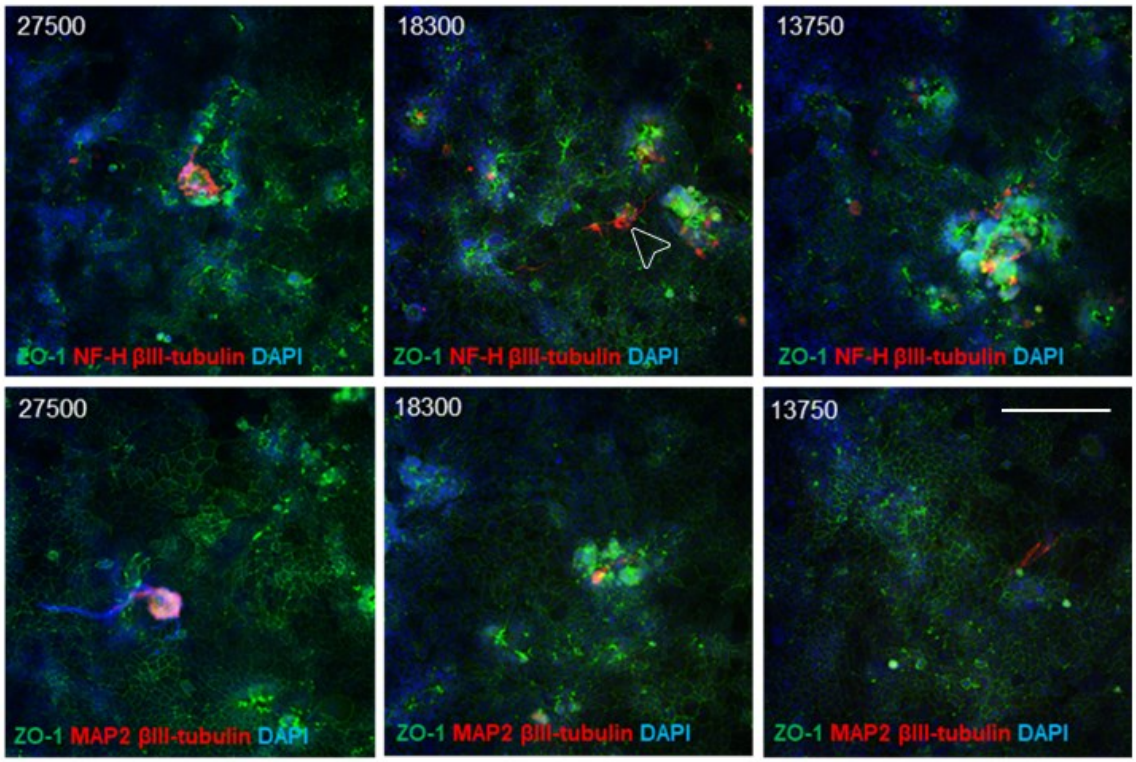

Figure 18. *Expression of intestinal marker ZO-1 and neuronal markers MAP2, NF-H and βIII-tubulin in 2D co-culture. Co-culture was continued for 7 days. Arrowhead points to the neuronal cell and its axon. Scalebar 200 µm.*

5.2 3D culture

5.2.1 Culture substrata and media

The 3D3C-chip has three compartments which are connected with microtunnels. It allows culturing even three different cell types in one chip at the same time. Here the 3D3C-chip was used in co-culture of hiPSC-neurons and CaCo-2 cells where the chip allowed to culture neuronal somas in their own compartment and axonal growth through the microtunnels to the middle compartment. In the middle compartment were seeded CaCo-2 cells and two different study designs were used to examine the interaction between the cells: neuronal cells in both side compartments or in one side compartment. Interaction between the cells happened through the microtunnels where axons stretch through. Separate medium chambers allowed culturing the cells in their optimized media which in this case were CaCo-2 MEM for CaCo-2 cells and NMM for neuronal cells.

3D3C-chips were coated with PLO and LN521 as previously described. CaCo-2 cells and neurons were cultured on control chips separately with no interaction as seen on figure 19 to determine the morphology of CaCo-2 cells on 3D3C-chip. Morphology of the

cells resembled the one on 48-wellplate with visible tight junctions. Different CaCo-2 densities were tested with no significant differences. Lower CaCo-2 density resulted in less amount of death cells packing inside the chip and experiments were continued with lowest number of cells per compartment. Phasecontrast imaging showed that when cultured without CaCo-2 cells neurons were viable and their axons began to stretch trough the microtunnels to the middle compartment immediately and continued to elongate through the culturing period.

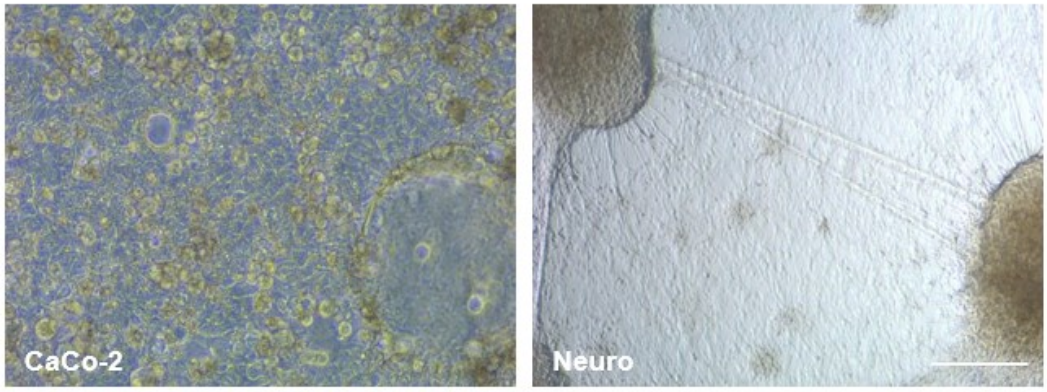

Figure 19. *Control cultures on the 3D3C-chips. On the left picture CaCo-2 cell and on the right picture neuronal cell after 14 days of the culture when cultured alone in the chip. Neurons were cultured in the side compartment and CaCo-2 cells in the middle compartment. Scalebar is 200µm.*

5.2.2 Co-culture

Co-cultures on 3D3C-chips were conducted with PLO and LN521 coated chips. On first co-culture on 3D3C-chip neurons were seeded either on one side compartment or on both sides and CaCo-2 cells one week later in the middle compartment. CaCo-2 MEM was added to the middle compartment one day before seeding CaCo-2 cells. At the time of CaCo-2 plating the axons had stretched through the microtunnels to the middle compartment and neurons had created strong neuronal network in their own compartments. Some of the neuronal cells leaked in the middle chamber of the chip during their seeding and some cells travelled through the microtunnels due to their small size.

During the 7 days of co-culture CaCo-2 cells divided creating monolayer and filled the middle compartment pushing the axons near the microtunnels. This phenomenon was examined more closely in second co-culture experiment. Indirect immunofluorescence staining with intestinal marker ZO-1 for tight junctions and neuronal marker βIII-tubulin for axons in figure 20 shows the phenomenon of CaCo-2 cells taking the space in middle compartment during five time-points. Time-points were determined based on the first co-

culture experiment on 3D3C-chip. First time-point showed strong axons in the middle compartment before the CaCo-2 plating. In the final 10 days co-culture time-point axons had developed one bulky axon strand next to the openings of the microtunnels which is pointed with white arrowhead in the picture.

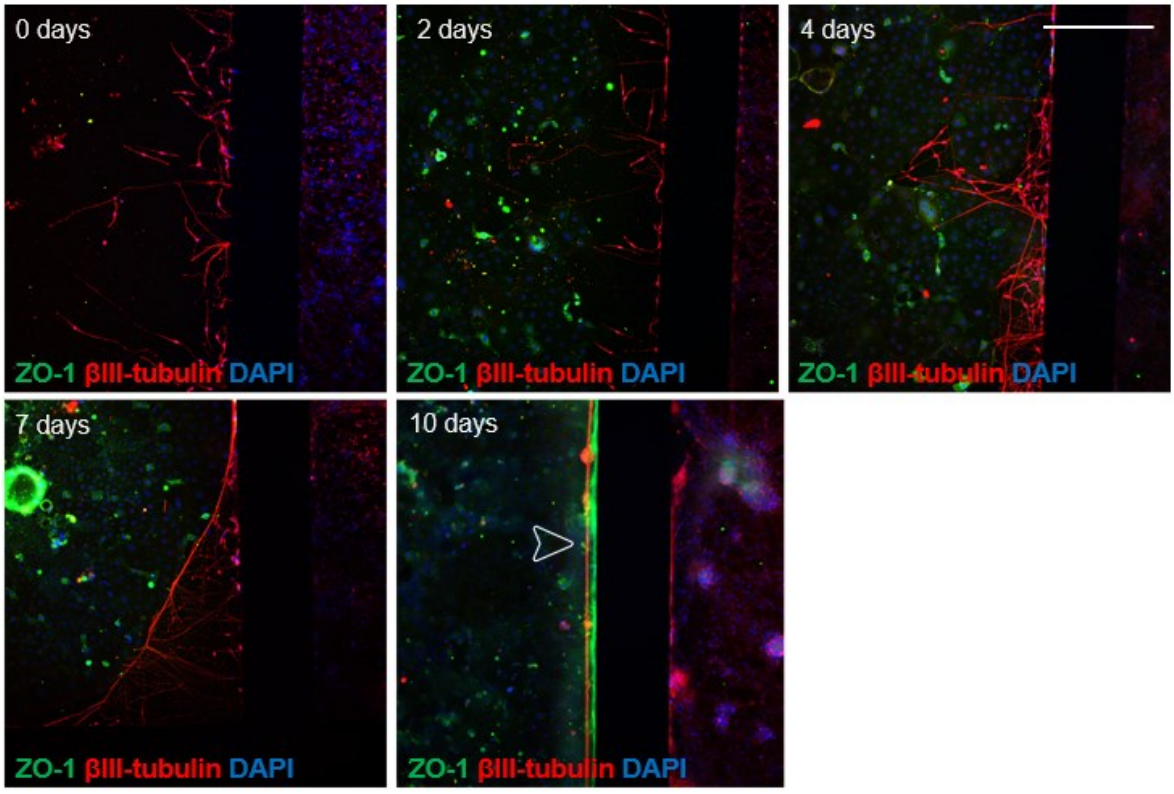

Figure 20. *Expression of intestinal marker ZO-1 and neuronal marker βIII-tubulin in 3D co-culture for 10 days co-culturing period. Arrowhead pointing at neuronal cell after 10 days of co-culture. Images show the middle compartment of the chip. Scalebar 200 µm.*

Indirect immunofluorescence staining was used to investigate the interaction between the two cell types and to study the morphology of the cells in more detail. Intestinal marker ZO-1 for tight junctions and neuronal marker βIII-tubulin for axons were used. In figure 21 co-culture on 3D3C-chip is shown in two different time-points of the experiment. After two days of the co-culture axons of the neuronal cells had stretched through the microtunnels of the chip and reached to the CaCo-2 cells. Morphology of the cells was normal, and axons were spiky, long and separated. Axons had one week to grow through the microtunnels, and CaCo-2 cells were plated on top of the axons in the middle compartment. Despite of that, neurons reach on top of the CaCo-2 cells in the first time-point. CaCo-2 cells were rapidly dividing, and they were not fully differentiated. In the second time-point the axons had started to form cluster and interaction towards the CaCo-2 cells

deteriorated. Deterioration of the axonal growth and formation of one bulky axonal strand was quick after the time-point of 4 days after starting the co-culture.

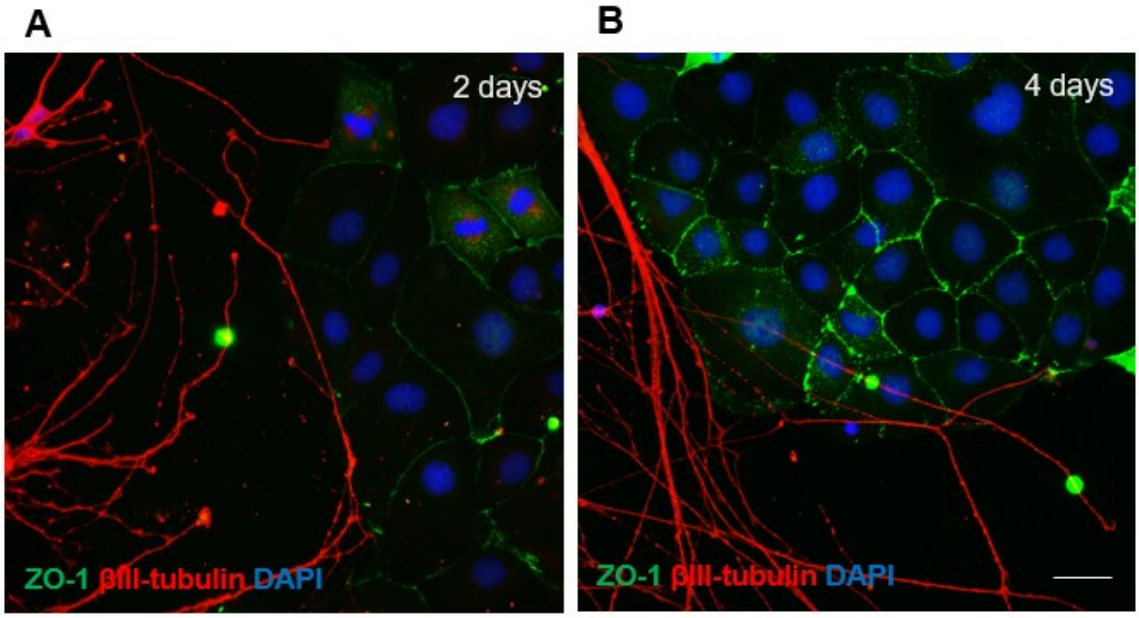

Figure 21. *Expression of intestinal marker ZO-1 and neuronal marker βIII-tubulin in 3D co-culture after A. 2 days and B. 4 days of co-culture. Images are presenting the middle compartment of the chip. Scalebar is 20 µm.*

6. DISCUSSION

This thesis aimed to optimize the co-culture conditions for the CaCo-2 cells when cultured with hiPSC-neurons to model the gut-brain axis connection from gut to the brain. These conditions included the co-culture media, cell proportions and cell culture time. The human epithelial cell line CaCo-2 is widely used intestinal epithelial model since it spontaneously differentiates into a monolayer with genetical properties typical of enterocytes in small intestine. The use of iPSCs in the other hand allows co-culturing human neurons with CaCo-2 cells since they have been challenging to isolate and culture, yet they represent the *in vivo* situation in humans the most accurately. 3D3C-chip enables mimicking enteric neurons stretching towards intestinal epithelial cells and analyses can be performed separately for both cell types.

It was shown in this thesis that CaCo-2 cells can be cultured on PLO and LN521 coated surfaces including 3D3C-chip. However, the results of this thesis indicate that the optimization of the co-culture was unsuccessful, and no clear evidence was found for physical interaction between the cell types. However, the interaction was measured only visually. After 14 days of the co-culture the morphology of the neuronal cells was abnormal as the axons were short compared to control cultures and the number of the cells in the culture was low in both 2D and 3D co-cultures.

6.1 2D culture

6.1.1 Culture substrata and media

Depending on the properties of the particular CaCo-2 cell line available, CaCo-2 cells are cultured on collagen coated plastic well plates or filter inserts (Lea, 2015). CaCo-2 cells used in this thesis have been cultured on plastic well plates with collagen based Geltrex coating where they attach to the culturing surface developing monolayer with tight junctions. Hyvärinen ym. (2019) have optimized culturing hiPSC-neurons on well plates and 3D3C-chip using PLO and LN521 coating combination, indicating that it supports functional development of neuronal networks. Geltrex have not been tested for hiPSC-neurons and previously optimized coating option was chosen. To find suitable coating for co-culture, PLO and LN521 coating combination was tested on CaCo-2 cells

and no noticeable visible difference between the Geltrex and PLO and LN521 combination coatings was evident. Experiments were continued using the PLO and LN521 combination coating for both cell types.

CaCo-2 cells are maintained in simple medium consisting of MEM supplemented with penicillin/streptomycin, NEAA, Na Pyruvate, GlutaMAX and FBS (Satsu ym., 2002). Neuronal medium consists of DMEM/F12 with Glutamax and Neurobasal, N2, B27 with Retinoic Acid, GlutaMAX, NEEA, 2-mercaptoethanol, insulin and penicillin/streptomycin. Neuronal maturation is promoted with neurotrophic factors BDNF, GDNF, cAMP and ascorbic acid added to the base medium. (Hyvärinen ym., 2019) Due to significant differences in the medium contents, three different medium options were tested on both cell types to find suitable option for co-culture: CaCo-2 MEM optimized for culturing CaCo-2 cells, NMM optimized for culturing iPSC-neurons and 1:1 mixture of both media. Since CaCo-2 MEM has no neurotrophic factors to support neuronal maturation, the morphology of the neuronal cells was compromised, and axonal growth was limited. In contrary NMM contains supplements inducing the CaCo-2 dividing resulting in quickly renewing monolayer, yet the morphology or the viability of the CaCo-2 cells was not compromised. In previous studies (Satsu ym., 1999) CaCo-2 cells have been cultured in neuronal medium with no changes in cell viability and even though the co-culture setup has been different, this thesis supports these results. Nevertheless, it seems that finding optimal medium option for co-culture is challenging since lack of optimal amount of neurotrophic factors is evident in 1:1 medium mixture as well.

6.1.2 Culture period

Culturing period refers to the plating order of the cells in co-culture and duration of the cell cultures. IPSC-neurons are differentiated for 32 days and then used for the experiments, yet they keep maturing after this (Hyvärinen ym., 2019). CaCo-2 cells spontaneously differentiate within 14-21 days with some areas of monolayer differentiating more quickly than others resulting in fully differentiated monolayer after 21 days (Lea, 2015). The first steps in the optimization of the culture period were plating both cell types at the same time on plastic 48 well plate and culturing them for 7 days or until either cell type showed no viability to allow neuronal cells to maturate and interaction between the cells to develop. When plated at the same time, the phase-contrast imaging showed that the number of neuronal cells decreased quickly in the culture while the CaCo-2 cells remained viable. Previous studies (Häkli ym., 2022; Satsu ym., 1999) have demonstrated

that plating neuronal cells before other cell types increases the survival rate of the neuronal cells since they have time to attach to the cell surface and develop neuronal networks before interacting with the other cell type. Next experiments included tests with neuronal cell plating 3 days and 7 days before plating CaCo-2 cells. There were slightly higher number of neuronal cells in the culture compared to plating the cells at the same time.

Since there were still neuronal cells detected after 7 days of co-culture, even though with abnormal morphology, the culture was continued for total of 14 days. Between 7 days and 14 days of culturing period there were no significant differences. This indicates that the culture stabilizes after certain time.

6.1.3 Co-culture

CaCo-2 cells are typically cultured on filter inserts and previous studies conducted with CaCo-2 cells and neuronal cells have utilized this culturing method. Utilizing filter inserts limits the interaction to compounds secreted by the cells excluding the physical connection. (Lea, 2015; Satsu ym., 1999, 2002) In this thesis 2D co-culture was conducted on 48 well plate where cells have physical connection to each other. Final 2D co-culture was performed on PLO and LN521 coated plastic 48 well plates in 1:1 medium mixture with neuronal cells plated 7 days before CaCo-2 cells. The co-culture was continued for total of 7 days.

Cell proportions were optimized for CaCo-2 cells during the co-culture experiments. Neuronal cells were cultured at a density of 50,000 cells/cm^2 based on protocol of Hyvärinen ym. (2019). CaCo-2 cells were cultured on 1:2 (25,000 cells/cm^2), 1:3 (16,700 cells/cm^2) and 1:4 (12,500 cells/cm^2) densities according to neuronal cell density. CaCo-2 cells are typically cultured at a density of 400,000 cells/cm^2 (Lea, 2015). Phase-contrast imaging showed more neuronal cells at the beginning of the culture when cultured with lower densities of CaCo-2 cells. When culture was continued the differences in number of neuronal cells was stabilized between cultures with different CaCo-2 cell densities.

The indirect immunofluorescence images showed that both cell types remained viable in the co-culture for 7 days, although the morphology of the neuronal cells was abnormal, and the number of the neuronal cells was low. With limited axonal growth, it is unlikely that these neuronal cells interacted with CaCo-2 cells in the culture although this was not demonstrated with further testing. Taken together, these 2D cultures suggest that co-culture on well plate is not suitable for culturing iPSC-neurons and CaCo-2 cells mainly due to different medium needs of the cells.

6.2 3D culture

6.2.1 Culture substrata and media

Gut-brain axis has been studied utilizing different chip technologies, mostly focusing on complex organ-on-chip structures (Moysidou & Owens, 2021). 3D3C-chip containing three compartments which are connected with microtunnels was used in this thesis. Interaction between the cells in 3D3C-chip was restricted to happen between axons and CaCo-2 cells. 3D3C-chip resembles the *in vivo* situation better than 2D co-culture since the enteric neurons mostly reside in the deeper layers of the intestinal wall and their axons connect with the epithelial cells (Duan ym., 2021).

3D3C-chips were coated with PLO and LN521 combination. The separate medium chambers of the chip allowed co-culturing the neuronal cells and CaCo-2 cells in their optimized medium option. This eliminated the need to adjust the medium needs of each cell type and especially provided neurotrophic factors for the neuronal cells to support their maturation. Häkli ym. (2022) utilized 3D3C-chip in their study with hiPSC-neurons and reported no significant effect on axonal growth even though the axons were exposed to wrong medium in the middle compartment while the somas of the cells were in neuronal medium. This indicates that exposure to the CaCo-2 MEM in the middle compartment was not crucial to the axons.

6.2.2 Co-culture

3D co-culture of the hiPSC-neurons and CaCo-2 cells was performed on PLO and LN521 coated 3D3C-chip. Neuronal cells were seeded on either one side compartment or in both side compartments one week before CaCo-2 cells and CaCo-2 cells were seeded in the middle compartment. First experiment was continued for total of 14 days and results were analysed at the end of the experiment. Experiment was repeated with analysis of five different time-points during the experiment.

CaCo-2 densities were tested on the first 3D co-culture experiment. Intestinal cells renew quickly, and their lifespan is less than a week (de Santa Barbara ym., 2003). Structure of the 3D3C-chip limits the removal of the dead cells from the cell culturing compartment which is why the lowest CaCo-2 cell density was chosen for the next experiment to avoid crowding the compartment with dead cells.

Indirect immunofluorescence imaging showed that both cell types were viable at the end of the culture. At the beginning of the experiment, axons were expanding, and neurons were creating denser and longer network, yet time-point analysis demonstrated phenomenon of axons in the middle compartment deteriorating during the co-culture resulting in one bulky axon strand next to the opening of microtunnels. Neuronal somas in the side compartments remained strong and viable. This indicates that axons were not able to interact with the CaCo-2 cells.

6.3 Future perspectives

CaCo-2 cells have been widely used as an intestinal model since they spontaneously differentiate and have functional characteristics of small intestinal enterocytes (Lea, 2015). There are several limitations concerning the use of CaCo-2 cells as intestinal model. Normal intestinal epithelium contains more than one cell type and several non-cellular parameters which affect the function of the small intestine (Lea, 2015). Addition to that, CaCo-2 cells have narrower tight junctions compared to the human intestine (Sun ym., 2008). This indicates that despite of being used as small intestine epithelium model, CaCo-2 cells, being derived from the colon, have closer resemblance to the colon (Press & Grandi, 2008). Taken together, the co-culture could be conducted using different intestinal epithelial model, such as iPSC-derived intestinal cells to avoid these shortfalls with CaCo-2 cells. Currently, there are no co-cultures tested utilizing iPSC-derived intestinal cells.

Neuronal cells used in this thesis were differentiated based on Hyvärinen ym. (2019) protocol. Neuronal cells were differentiated to cortical neurons due to which they do not fully represent the enteric neurons. Utilizing peripheral neurons in further experiments could better represent the in *vivo* situation and support the interaction between the cells in co-culture. IPSC-derived peripheral neurons have not been used in similar co-culture setups yet.

Major limitation of this thesis was the challenge to measure the interaction between the cells in the co-culture since it was only measured by immunofluorescence staining analysis. This gives limited overview of the connection. Further research on gut-brain axis co-culture should focus on determining the factors that indicate interaction between neuronal cells and intestinal epithelium. These could be biochemical or genetical factors. Research on this is still limited.

7. CONCLUSIONS

The aim of this thesis was to optimize the culturing conditions for iPSC-neurons and CaCo-2 cells. These conditions included co-culture media, cell co-culture proportions and cell culture time. Several experiments were conducted including co-cultures on 2D and 3D platforms. These experiments confirmed that CaCo-2 cells can be cultured on PLO and LN521 coated 3D3C-chips, although the co-culture was unable to prove visible interaction between neuronal cells and CaCo-2 cells when co-cultures were visually interpreted.

Despite its exploratory nature, this thesis gives preliminary insight into the optimization process of the co-culture for intestinal epithelial cells and neuronal cells. However, since CaCo-2 cells poorly represent the intestinal epithelium, iPSC-derived intestinal epithelial cells could be used for further co-culture testing. Different biochemical and genetical factors which indicate interaction between the cell types should be determined since currently the measuring of interaction relies on visual methods.

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