
# Evaluation of the immunomodulatory effect of phagocytosis inducers on neuronal (SH-SY5Y) cell line

Master's thesis by

Kira Kuhlefelt

Pharmaceutical Sciences

Faculty of Science and Engineering

Åbo Akademi University

Åbo, Finland

2022

# Acknowledgements

This study was conducted in Professor Mikko Airavaara's laboratory at the Neuroscience Center, Helsinki Institute of Life Science (HiLIFE), University of Helsinki.

Foremost, I would like to extend my sincere gratitude to Dr. Helike Lõhelaid for being my supervisor throughout the project. Her unlimited amount of help, support, and guidance is beyond what I could have wished for. Her invaluable expertise gave me the strength and confidence to succeed in this project.

My genuine gratitude to Professor Mikko Aivaraara for giving me this opportunity to work and be a member of the Airavaara group. A group with infinite knowledge and ability to support others in their journeys. There was always someone to turn to if needed, and a solution was consistently achieved. I want to extend my special gratitude to laboratory technician Jenni Montonen, who has been a crucial help and a tremendous teacher in the laboratory. Her outstanding patience and exquisite skills have given me the best learning experience I could possibly get.

I would like to extend my sincere gratitude to Dr. Anne Filppula, my supervisor at Åbo Akademi University, for her absolute help and support. The journey felt easy with a great mentor like her by my side.

To my greatest supporters in life, my family, I would like to extend my sincerest of all gratitude.

# Abstract

Ischemic stroke is a medical emergency that cuts off oxygen supply to the brain. Within minutes, brain cells start to die. Even to date, treatment options are limited, and there is a serious need for better therapeutical alternatives.

This project is a part of a larger project, where the long-term goal is to be able to alleviate and prevent brain injury after ischemic stroke, by investigating how the overexpression of genes of interest (GOIs) affects microglia and recovery after stroke. Initial results in the mouse microglia cell line BV2 have shown, that most of the GOIs induce microglial phagocytosis or high expression of pro-inflammatory mediators. The aim of my study was to determine how the expression of GOIs in neurons affect their release of inflammatory cytokines. Neuroblastoma cells, SH-SY5Y, was subcultured and transfected. The expression of proteins was determined with Western Blot, and cytokine production with enzyme-linked immunosorbent assay (ELISA). In this cell line the GOIs did not induce any cytokine production, but most of them did express the gene after transfection. On the other hand, the protein expression in Western Blot was slightly less successful. Based on this study in SH-SY5Y cells, neurons do not seem to enhance the overall inflammatory state in the brain after stroke.

# TABLE OF CONTENTS

# List of abbreviations

**AD:** Alzheimer's disease

**C3:** Complement component 3

**C3a:** Complement component 3a

**CNS:** Central nervous system

**DAMP:** Danger-associated molecular patterns

**DMEM:** Dulbecco´s modified eagle medium

**DPBS:** Dulbecco's phosphate buffered saline

**ECL:** Enhanced chemiluminescence

**EDTA:** Ethylenediaminetetraacetic acid

**EF1a:** Human Elongation factor 1-Alpha

**ELISA:** Enzyme-linked immunosorbent assay

**Emr1:** Epidermal growth factor module-containing mucin-like receptor

**ER:** Endoplasmic reticulum

**FBS:** Fetal bovine serum

**GFP:** Green fluorescent protein

**GOI:** Genes of interest

**IL:** Interleukin

**LPS:** Lipopolysaccharide

**LV:** Lentivirus

**MANF:** Mesencephalic astrocyte-derived neurotrophic factor

**MCP-1:** Monocyte chemoattractant protein

**M-CSF:** Macrophage colony stimulating factor

**MerTK:** Mer receptor tyrosine kinase

**PD:** Parkinson's disease

**PMA:** Phorbol 12-myristate 13-acetate

**TNFα**: Tumor necrosis factor alpha

# Introduction

There is currently no effective therapy against the neurological sequelae (long-term pathological condition) of ischemic stroke. The loss of blood supply during stroke causes neuronal damage in the ischemic core and may spread to the surrounding penumbra area. However, this event could potentially be recoverable (Liu, Levine & Winn, 2010; Zhang et al., 2019).

Following an ischemic stroke, a cascade of inflammatory responses is initiated through the release of danger-associated molecular patterns (DAMPs) by the injured neurons, which is an endogenous way to limit injury. However, continuous encounter with dead neurons leads to excessive inflammation, preventing brain repair and potentiating secondary brain injury. Phagocytes are cells, which restore the function of damaged tissue and resolve local inflammation by engulfing particles via their plasma membranes in a process called phagocytosis. A rapid disposal of dead cells and waste promotes a permissive environment that facilitates tissue regeneration. However, the cellular and molecular mechanisms promoting phagocytosis in the brain after ischemic stroke, as well as its impact on brain injury and recovery, are so far poorly understood (Zhang et al., 2019).

This Master's thesis project is a part of a larger project, where the long-term goal is to investigate how the overexpression of genes of interest (GOIs) affects microglia and the recovery after stroke *in vivo*. Various proteins, including several ones used in this project, induce the recruitment of macrophages and microglia, or phagocytosis (Hedtjärn, Mallard & Hagberg, 2004).

The immunomodulatory and phagocytosis-inducing effect of GOIs has been tested on the rodent microglia cell line BV2 *in vitro*, but the effect of GOIs on neurons, oligodendrocytes and astrocytes are unknown. The hypothesis of the main project is that enhancement of the phagocytosis process could improve recovery after stroke and help to clean up the affected area. The main project involves the use of lentiviral particles with different GOIs to study their effect after stroke *in vivo*. Those lentiviral particles are generated by using the same plasmids as *in vitro*. Unfortunately, lentiviruses will infect all cells, not just

microglia, since they are suitable for infecting many different cells (Pandya, Klimatcheva & Planelles, 2005).

The hypothesis of the current subproject is that transfection and expression of GOIs affect brain cells differently, and induce specific cytokine production, generating a specific cytokine profile. This hypothesis will be tested in brain-derived cell lines representing neurons, astrocytes and oligodendrocytes. In this Master's thesis, I focus on neurons.

In the current project, we aimed to investigate how the overexpression of GOIs in neurons affects their production of cytokines. **Figure 1** presents the research activities of this project. Briefly, neuroblastoma cell line (SH-SY5Y) was cultured and transfected with lentivirus plasmids containing GOIs, and the transfection efficiency was determined using a fluorescence microscopy. The expression of GOI proteins was determined with Western Blot, and the inflammatory profile was determined with enzyme-linked immunosorbent assay (ELISA).

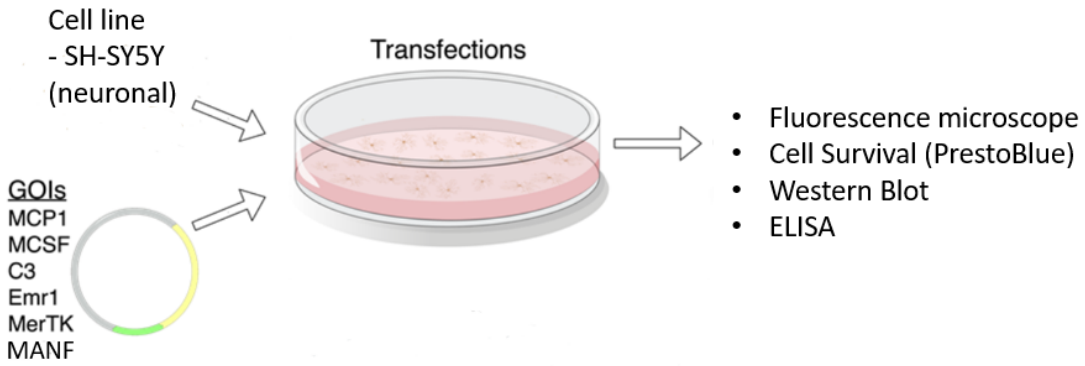


*Figure 1. Description of research activities.*

# Literature review

## Ischemic stroke

The most common type of stroke is ischemic stroke, comprising 87% of all incidents (Kuriakose & Xiao, 2020). It is a consequence of a blood vessel blockage in the brain. This blockage can be caused either by thrombosis, embolism, or stenosis. Thrombosis is a formation of a blood clot in a vessel, leading to reduced blood flow through the vessel. Brain embolism occurs when a clot moves to the brain from elsewhere in the body and stops the blood flow to the brain by being stuck in a vessel, which it is unable to pass. This leads to starvation of oxygen in the brain and, therefore, ischemic stroke. Stenosis is caused by narrowed arteries or plaque build-ups, also leading to reduced blood flow to the brain (Randolph, 2016).

Stroke, or medically termed cerebrovascular accident (Paul & Candelario-Jalil, 2021), has an annual mortality number of 5.5 million. It is the second leading cause of mortality and the leading cause of disability worldwide (Kuriakose & Xiao, 2020). Approximately 50% of patients surviving stroke are chronically disabled (Donkor, 2018). Recent research shows that the incidence of stroke is increasing, suggesting that one in four people worldwide will experience stroke in their lifetime. Factors contributing to the increasing incidence of stroke are, for example, an aging population paired with the burden of compiled risk factors. For younger adults, the increase in socioeconomic status has led to an epidemic rise (Paul & Candelario-Jalil, 2021) in the risk factors for stroke, including hypertension, hyperlipidemia, atrial fibrillation, diabetes, smoking, abdominal obesity, alcohol consumption, unhealthy diet, and lack of physical activity (O'Donnell et al., 2010). The treatment options for stroke are even today limited, but progress has been made due to improvement in recanalization therapy, where both mechanical and pharmacological thrombolysis are used. In spite of this, there is still a vast need of developing therapeutic agents in order to protect the brain from damage, both prior to and during recanalization, as well as to extend the therapeutic time window for intervention and further enhance functional outcome (Paul & Candelario-Jalil, 2021).

## Neurons and microglia

Neurons are considered the basic units of the nervous system, creating a complex network of communication, and are needed for every function and action that our body or brain performs. They respond to stimulus and send impulses to the rest of the body, as well as transmit the information in between (Newman, 2017). Neurons use electrical impulses and chemical signals for transmitting information (Faber & Pereda, 2018). Even if glial cells (microglia, astrocytes, and oligodendrocytes) outnumber neurons in some parts of the brain (Kandel, 1991), neurons are still considered the most important cells of the brain. Neurons consist of three basic parts: a cell body, an axon, and a dendrite (NINDS, 2019). Dendrites receive and transmit messages from the surroundings to the cell, and the axon transmits messages from the cell. Inside the cell body, the nucleus holds the genetic material and controls the cell's activities. Communication between neurons is conducted via neurotransmitters through a synapse (Kandel, 1991). Neurogenesis (birth of neurons) is believed to be a lifelong process, however, most of the neurons are already present in our brains when we are born. Many diseases lead to an unnatural death of neurons, e.g., Parkinson's disease (PD), Alzheimer's disease (AD), as well as stroke. Neurons are widely studied in order to gain more information about their impact on the brain to be able to develop new therapeutics to cure and alleviate various brain damages (NINDS, 2019).

Microglia represent the major types of immune cells in the central nervous system (CNS), and are important for neuronal plasticity, neurogenesis, and tissue homeostasis (Lindahl, Saarma & Lindholm, 2016). Microglia are macrophages found in the brain, and they form the first active immune barrier in the CNS. They play an important role during both child development and adulthood, as well as under disease conditions (Stratoulias et al., 2019). Microglia are activated by misfolded protein in several neurodegenerative diseases (e.g., AD, PD, ischemia), which leads to the release of pro-inflammatory and cytotoxic factors that promote neuronal damage (Lindahl, Saarma & Lindholm, 2016). The early response of microglia limits the spread of injuries (Galloway et al., 2019). Microglia cells are considered neuroprotective after an acute neuronal injury. Studies suggest that the facilitation of neuroprotection depends on neuronal-microglial crosstalk. Microglial activation is thought to be triggered by signals from injured neurons, and activated microglia are believed to produce factors that help damaged neurons recover from injury. Studies also suggest that

with aging, microglial cells become increasingly dysfunctional. The loss of microglial cells may involve the loss of neuroprotective properties and, therefore, aging-related neurodegeneration is due to the loss of microglial cells (Streit, 2005).

Depending on the needs of the tissue, microglia have different states, of which the two main ones are resting and activated. In normal, healthy conditions, microglia are resting, also called quiescent. Despite the term resting, the cells are moving and constantly surveilling the environment. If there are no pathological signals in the surrounding environment, the resting state is dominant. If the resting microglia cells detect possibly dangerous signals or molecules, or shortage of normal signaling from neurons or glial cells, the cells are activated and undergo morphological and functional changes. The activated microglia have a thick pseudopodium (extension of the cytoplasm), which allows them to move quickly to the danger zone, releasing cytotoxic substances to kill the pathogen and to phagocytose (Jurga, Paleczna & Kuter, 2020). The activated state of microglia can generally exist in two different forms, classical activation or alternative activation. Classical activation is characterized by the production of inflammatory cytokines and reactive oxygen species. In the second one, alternative activation, microglia take on an anti-inflammatory phenotype involved in wound repair and clearance of waste (Cherry, Olschowka & O´Banion, 2014). Discussion and research are ongoing regarding how to differentiate the forms of activated microglia.

## Phagocytosis

In order to maintain homeostasis in multicellular organisms, clearance of cellular waste is required. It is essential for processes like tissue growth and remodeling, as well as for regeneration and resolution of injury and inflammation. The damaged cells are mostly removed by phagocytosis, performed by macrophages and neutrophils, and by microglia in CNS. Phagocytosis is a complex process, including ingestion and degradation of the disposable particles (Westman, Grinstein & Marques, 2020). The process is generally divided into five steps, particle recognition being the first. Phagocytes recognize the particles to be ingested by using specific receptors that cooperate to achieve efficient recognition. The receptors move fast in the membrane to aggregate around the target and

then engage multiple ligands on the particle. Next step is particle ingestion, where the receptors initiate a cascade of signals, change lipids in the cell membrane, and organize the actin cytoskeleton to grow the cell membrane around the target particle. Third step is early phagosome formation, a vacuole is formed to bring the particle inside the cell, and it rapidly fuses with early endosomes to change the composition of its membrane. In the fourth step (late phagosome formation), the early phagosome fuses with vesicles from the endoplasmic reticulum (ER) and the Golgi complex. The fifth and last step is the phagolysosome formation, where an intermediary phagosome fuses with lysosomes and changes the membrane and interior components. Then, the phagosome turns into a microbicidal vacuole called the phagolysosome (Uribe-Querol & Rosales, 2021). Phagocytosis has been long known to mediate classical innate and adaptive immune responses. Today, it is also known to be critical for early neuronal development, homeostasis, as well as the start of repair mechanisms. Modulating phagocytosis is, therefore, widely explored to develop new therapeutics that promote both repair and regeneration in the CNS (Galloway et al., 2019).

## Inflammation

Inflammation leads to the induction, production, and release of pro-inflammatory mediators. These mediators include cytokines, such as TNFα and IL-6 (Kany, Vollrath & Relja, 2019). According to several studies, both IL-6 and TNFα are expressed in neurons (Erta, Guintana & Hidalgo, 2012; Park & Bowers, 2010). Neurons are considered one of the essential sources of IL-6, as they have been demonstrated to produce the cytokine, as well as to secrete it upon stimuli (Erta, Guintana & Hidalgo, 2012; Park & Bowers, 2010). However, another study showed no secretion of TNFα from neurons (Viviani et al., 1998), and a third study demonstrated very low secretion of both IL-6 and TNFα in neurons (Klegeris & McGeer, 2001). Pro-inflammatory cytokines are mainly produced by activated macrophages and participate in the upregulation of inflammatory reactions. TNF-α acts through two cell receptors (TNFR1 and TNFR2) on various signalling pathways and is considered having both anti-inflammatory and pro-inflammatory effects (Masli & Turpie, 2009). It regulates apoptotic pathways, nuclear factor-kB activation of inflammation, and activates stress-activated protein kinases. TNF-α receptors are found both in glial and

neuron cells. TNF-α has shown to be significant for both inflammatory and neuropathic hyperalgesia (Zhang & An, 2009). Additionally, TNFα affects neuronal excitability. Glial cells can detect neuronal activity in a still unknown manner. Once the neuronal activity levels are low, glial cells begin to release TNFα. A blockage of TNFα signalling prevents or reverses the effect of neurons' ability to alter their level of excitatory synaptic inputs. The neuronal excitability is, thus, dependent on the glial-derived TNFα. There is a possibility that TNFα released during inflammation is simply an improvement of this process (Galic, Riazi & Pittman, 2012). **Figure 2** illustrates the main effects of TNFα.

IL-6, another inflammatory cytokine, activates and differentiates different immune cells including T and B lymphocytes (Moulton, 2016). It plays an important role in the neuronal reaction to nerve injury. An *in vivo* application of anti-IL-6R (interleukin-6 receptor) antibodies resulted in reduced regenerative effects. The cytokine is also involved in regulation of neuronal neuropeptides expression, and in microglial and astrocytic activation (Zhang & An, 2009). IL-6 is known to influence both neurons and glial cells via participation in neurogenesis. It is also known to engage in the response of mature neurons and glial cells, both under normal conditions and in a broad range of injury models. Many of the main brain diseases are affected by IL-6 expression. Because of its potential role in neuropathology, IL-6 is considered a clear target of strategic therapies (Erta, Guintana & Hidalgo, 2012). **Figure 3** illustrates the main effects of IL-6.

Together with pro-inflammatory cytokines, the anti-inflammatory cytokines regulate the human immune response, and specific cytokine inhibitors and soluble cytokine receptors are also involved. The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response. The most important anti-inflammatory cytokines include IL-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13. Of all the anti-inflammatory cytokines, IL-10 has most effective anti-inflammatory properties, and the highest ability to restrain the expression of inflammatory cytokines by activated macrophages. It can also down-regulate pro-inflammatory cytokine receptors and up-regulate endogenous anti-cytokines, hence, it can counter-regulate the production and function of pro-inflammatory cytokines at various levels. IL-10 has been shown to suppress the development of spinally mediated pain facilitation, such as peripheral neuritis and nerve injury, by acute administration in various animal models. When blocking spinal IL-10,

contrarily, it has shown to prevent and even reverse established neuropathic pain behaviors (Zhang & An, 2009). In addition to its anti-inflammatory effects, IL-10 has been shown to have direct effects on neurons. The cytokine activates signalling pathways that are included in neuronal survival and growth. Neuronal survival increases significantly when treated with IL-10 expressing vectors. Evidence based on *in vitro* data shows that IL-10 can signal through the neuronal IL-10 receptor and, thereby, provide direct neuroprotective effects (Zhou et al., 2009).

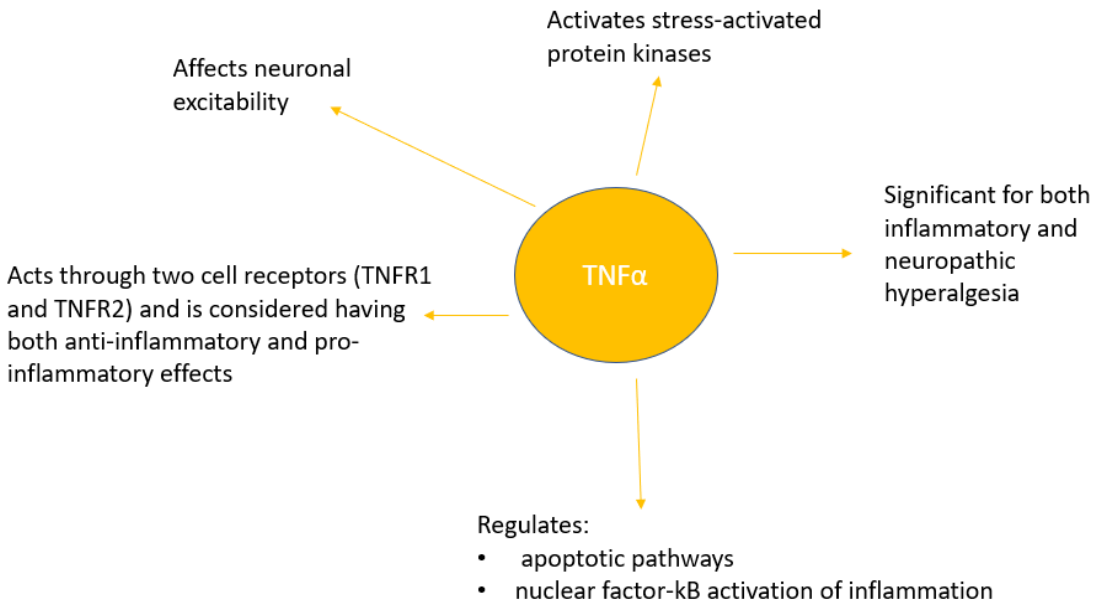


***Figure 2. Main effects of the cytokine TNFα.***

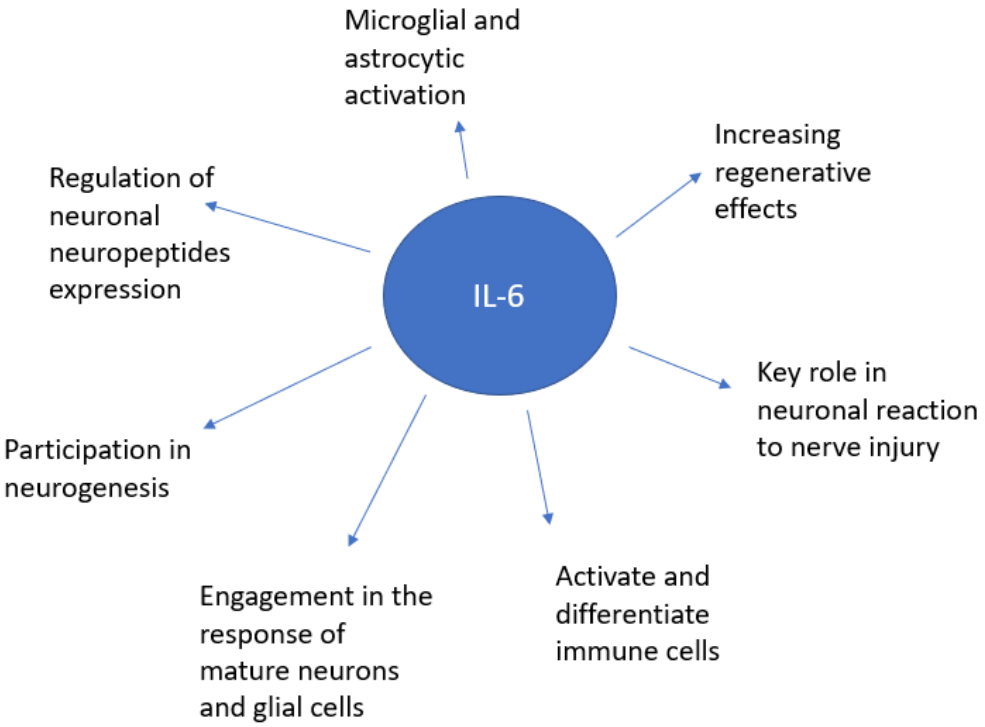


*Figure 3. Main effects of the cytokine TNFα.*

## Genes of interest

All GOIs used in this study are presented below. Their main function in phagocytosis, is presented in **Table 1**.

*Table 1. Presentation of GOIs and their main function in phagocytosis.*

| GOI | Characterization |
|---|---|
| Monocyte chemoattractant protein **(MCP-1)** | Recruitment in phagocytosis |
| Macrophage colony stimulating factor **(M-CSF)** | Inducing phagocytosis |
| Complement component 3 **(C3)** | Recruitment in phagocytosis |
| Complement component 3a **(C3a)** | Recruitment in phagocytosis |
| Epidermal growth factor module-containing mucin-like receptor 1 **(Emr1)** | Enhancing phagocytosis |
| Mer receptor tyrosine kinase **(MerTK)** | Enhancing phagocytosis |
| Mesencephalic astrocyte-derived neurotrophic factor **(MANF)** | Enhancing phagocytosis |

### Mesencephalic astrocyte-derived neurotrophic factor

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a protein localized in the ER, and functions as an ER stress response protein (Gao et al., 2020). Many pathophysiological conditions (e.g., ischemia) and neurodegenerative diseases (e.g., AD, PD) are known to be associated with chronic ER stress (Lindahl, Saarma & Lindholm, 2016). The expression of MANF is especially high in secretory tissues with extensive protein production, hence a high ER protein folding load. MANF is a relatively small protein, with a molecular weight of 18 kDa, and it is highly soluble and monomeric in neutral solution. The primary sequence of MANF contains an amino-terminal signal peptide, which directs it to

the ER (Lindahl, Saarma & Lindholm, 2016). MANF is recognized to contribute to neuroprotection, even if the mechanism is not fully understood (Gao et al., 2020). Neuroprotective effects of MANF in cerebral ischemia and spinocerebellar ataxia have been demonstrated in rodent models. Studies also suggest that MANF is important for protein homeostasis in the ER, since deletion of MANF in cultured cells, mice and fruit flies results in the activation of unfolded protein response. Unfolded protein response is a cellular defense mechanism to relieve stress by eliminating protein translation, degrading misfolded proteins through ER-associated protein degradation and activating signaling pathways. This leads to the production of molecular chaperons that are involved in protein folding (Lindahl, Saarma & Lindholm, 2016).

### Monocyte chemoattractant protein-1

Monocyte chemoattractant protein-1 (MCP-1/CCL2) regulates the migration and infiltration of monocytes/macrophages, memory T lymphocytes, and natural killer cells, being one of the key chemokines (small proteins that stimulate migration of cells) (Deshmane et al., 2009; Hughes & Nibbs, 2018). The protein has been suggested to be involved in multiple disease conditions, such as, cancers, neuroinflammatory diseases, rheumatoid arthritis and cardiovascular diseases (Singh, Anshita & Ravichandiran, 2021). Immunological surveillance of tissues, and the response to inflammation, require the migration of monocytes from the blood stream through the vascular endothelium. A various number of cells produce CCL2, including endothelial, fibroblast, smooth muscle, and microglial cells. It is produced either constitutively, or after induction by oxidative stress, cytokines, or growth factors (Deshmane et al., 2009).

### Macrophage colony-stimulating factor

Macrophage colony-stimulating factor (M-CSF), encoded by the *CSF-1* gene, supports the survival, proliferation, and functions of mature monocytes/macrophages. M-CSF also enhances myelopoiesis through the amplified production of granulocyte colony-stimulating factor and granulocyte macrophage-colony stimulating factor by monocytes, and has antitumor activity (Aronson, 2016). CSF-1 is produced by a selection of cell types, and acts in paracrine (targeting a nearby cell) and autocrine (targeting itself) manner, both

locally and humorally. Various developmental abnormalities like, skeletal, neurological, growth and fertility defects are the outcome when CSF-1 is absent. This is primarily due to severe deficiency in tissue macrophages (Jones & Ricardo, 2013).

### Complement component 3

Complement component 3 (C3) is a major protein of innate immunity and the complement system (Ricklin et al., 2017). The complement system plays an important role in the activation of microglia, and for cleavage products of C3, such as, C3a (Fumagalli et al., 2015). C3 has crucial role in apoptotic cell clearance, microbial killing, immune complex handling, and modulation of adaptive immune responses. C3 is a well-known pro-inflammatory molecule and is upregulated in most inflammatory conditions. It is induced by an upregulation of TNFα (Page et al., 2018). C3 has a central position in immune surveillance. It clears pathogens and is involved in several homeostatic processes e.g., tissue regeneration and clearing debris. However, if improperly engaged it can be a trigger point for a wide range of clinical conditions (Ricklin et al., 2017).

### Complement component 3a

Complement component 3a (C3a), is a small peptide that consists of 77 amino acids and has four anti-parallel helical structures trapped by three disulfide bridges (Gao, Cui & Zhao, 2020). It mediates phagocytosis and stimulates cytokine production by microglia (Fumagalli et al., 2015). In the immune system, C3a is a critical chemotactic mediator and has both anti-inflammatory and pro-inflammatory roles in various cells and diseases. It acts on resident immune cells to up-regulate or down-regulate different cytokines, and to activate dendritic cells. It also regulates T cell signalling between lymphocytes and antigen presenting cells. C3a has an anti-inflammatory effect on pathogens (i.e., bacteria), neutrophils in the bone marrow reservoir, and natural killer cells. C3a can directly inhibit neutrophil mobilizing factors and by that prohibiting the migration of neutrophils from the bone marrow into the circulation. Furthermore, C3a can inhibit natural killer cell cytotoxicity *in vivo*, and down-regulate the expression of interferon-γ. C3a is therefore a negative regulator in natural killer cells. It exploits the pro-inflammatory functions in mast

cells, macrophages/monocytes, T cells and antigen presenting cells. In monocytes or macrophages, C3a induces the release of pro-inflammatory mediators. In inflammation, C3a generally plays a catalytic role when the activity of monocytes is dominant over neutrophils. In T cells, C3a acts to decrease T cell apoptosis and enhance their proliferation. If C3a is absent the proliferation and differentiation of T cells is limited by insufficient antigen presentation (Gao, Cui & Zhao, 2020).

### Epidermal growth factor module-containing mucin-like receptor

Epidermal growth factor module-containing mucin-like receptor F4/80 (Emr1), is an antigen, encoded by the Adgre1 locus and is widely used in mice as a monocyte-macrophage marker. The expression of F4/80 protein varies amongst mouse mononuclear populations. It is very low, or even absent in osteoclasts, macrophages of T cell areas and marginal zone, lung alveolar macrophages, and in the majority of classical dendritic cells. Emr1 is mainly expressed in eosinophilic granulocytes. When F4/80 cDNA was isolated, it revealed that it encodes a large extracellular domain, containing various epidermal growth factors-like calcium-binding domains, linked to a seven-transmembrane domain characteristic of G protein coupled-receptors. The gene in mice encoding the F4/80 antigen was given the name *Emr1*, based on homology to previously identified human cDNA (Waddell et al., 2018).

### Mer receptor tyrosine kinase

Mer receptor tyrosine kinase (MerTK)/c-mer, is a gene expressed by microglia and macrophages when they are activated (Zizzo et al., 2013; Shen et al., 2021). MerTK has been demonstrated to have an impact on the resolution phase of inflammation by influencing the phagocytosis of apoptic neurons (Scott et al., 2001). It has also shown to enhance the resolution of inflammation in macrophages (Cai et al., 2018).

## Research on Genes of interest

Based on literature, MANF alleviates secretion of inflammatory cytokines in astrocytes that are induced by oxygen–glucose deprivation (Zhao et al., 2013). MCP-1 has shown to inhibit the production of cytokines by astrocytes, after stimulation of IL-1b. According to one study, it is likely that astrocytes are responsible for the exacerbated cytokine response seen *in vivo* post-injury, in the absence of MCP-1 (Semple, Frugier & Morganti-Kossmann, 2010). Since microglia are the principal immune cells of the brain, and the first line of defence against pathophysiology induced by stroke, they are widely investigated. The role of microglia is suggested to depend on their dynamic interactions with other cells present in the brain, e.g., astrocytes and neurons. The detailed mechanisms are unclear, but several experiments have shown microglia-astrocyte crosstalk in normal conditions. For example, M-CSF has shown to induce primary microglial ramification in mice. Astrocytes have also shown to contribute to microglial morphology, and their regional density correlates with microglial regional density, suggesting that the dynamics of the microglia process could be partly directed toward astrocytes (Li et al., 2020). C3a promotes astrocyte survival after ischemia through its inhibitory effect on extracellular signal-regulated kinase, signalling-mediated apoptotic pathway and caspase-3 (crucial mediator of apoptosis) cleavage. When neurons are co-cultured with astrocytes, C3a protects neurons against excitotoxicity-induced cell death (Pekna, Stokowska & Pekny, 2021). MerTK is upregulated after transient focal ischemia. Animals that lacked MerTK showed a greatly reduced brain damage after ischemia once the phagocytic protein was absent. The total number of microglia and inflammatory mediators was the same as in animals that had MerTK, still the ones lacking MerTK had fewer microglia that contained neuronal material. Meaning that the absence of MerTK inhibits the engulfment of neurons after ischemia (Brown & Vilalta, 2015). Another study demonstrates that knockout of MerTK improves neurobehavioral recovery after ischemic stroke (Shi et al., 2021). Both studies show that MerTK can also have negative effects on the outcome of ischemic events.

## Enzyme-linked immunosorbent assay

ELISA is considered the gold standard of immunoassays. It is a very sensitive immunological test that is used to detect and quantify substances (e.g. antibodies, antigens, protein, glycoproteins, and hormones). To detect the products, antibodies and antigens are complexed to produce a measurable result. The interaction between antigens and antibodies is utilized in ELISA testing, and it allows identification of specific protein antibodies and antigens with only a small amount of test sample. Polystyrene plates, typically 96-well plates, are used in ELISAs, and for detection, a substrate that can generate a colour is added. In the protocol, there is usually a serial dilution of concentrations placed in the wells of the plate. Once the results are measured, a standard curve from the serial dilutions data is plotted with a concentration on the x-axis, using a linear scale. ELISAs are used for several different diagnostic tests including detecting and measuring the presence of antibodies e.g., in the blood, detecting and estimating the level of tumor markers or hormone levels, tracking disease outbreaks, and detecting drug abuse. The data gathered from ELISAs can be quantitative (concentration results plotted and compared to a standard curve), qualitative (confirming or denying the presence of a particular antigen/antibody in a sample) or semi-quantitative (comparing the intensity of the signals to compare relative antigen levels in a sample) (Alhajj & Farhana, 2021).

## Western Blot assay

Western Blot is a technique widely used in cell and molecular biology. The technique is used to identify specific proteins from a complex mixture of proteins that are extracted from cells. To succeed in identifying proteins three elements are used: separation by size, transfer to a solid support, and marking the target protein (Mahmood & Yang, 2012).

# Aims

The aim of the current project was to detect how transient expression of GOIs in neurons affect their release of inflammatory cytokines. More specifically, the project aims were to:

1) optimize the conditions of GOI transient transfection,
2) detect GOI expression, and
3) determine the cytokine profile in neurons.

# Material and methods

## Neuronal cell culture, subculturing

In these experiments, human neuroblastoma cells SH-SY5Y were used. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, BE12-614F), supplemented with 10% Fetal bovine serum (FBS) (Gibco, 10270106), 1% pen-strep (Lonza, DE17-602E) and 1% L-glutamine (Lonza, BE17-605E). During culturing, the cells were stored in a humified incubator at 37 °C containing 5% $CO_2$. The cells were passaged consistently in 10 cm petri dishes (Corning, 430167) with a ratio between 1:2 and 1:5, depending on the need of cells. Confluency for passaging varied between 80% and 90%. The cells were passaged by removing the medium, washed with 3 ml of Dulbecco's phosphate buffered saline (DPBS; Gibco, 14190169), detached with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco, 15400054) in DPBS, and incubated for two minutes in $CO_2$ incubator at 37 °C. Then, 4 ml of new medium was added and centrifugated for 5 minutes (1000 rpm) to collect the cells. The medium was aspirated, and the cell pellet dissolved in 1 ml of fresh medium. Depending on the ratio, the correct amount of medium and cells was placed on the petri dish with 10 ml of growth medium and stored in the incubator. All the reagents used for passaging were preheated in a water bath (37 °C) for approximately 15 to 20 minutes (excluding DPBS). Cells were counted with Corning Cytosmart cell counter using trypan blue (Bio-rad, 1450021).

## Transient transfection

The transfection was optimized on translucent 96-well plates (Corning costar, 3596) with lentiviral (LV) plasmids encoding GOIs with green fluorescent protein (GFP). Different conditions were tested:

- cell number: 10 000, 20 000 and 40 000 cells.
- Lipofectamine 2000 reagent (Invitrogen, 11668030) concentration: 0.2 µl and 0.5 µl
- DNA amount: 100 ng and 200 ng

- reverse transfection vs. forward transfection
- transfection immediately after seeding vs. after 24 hours
- medium with antibiotics (pen-strep) vs. without pen-strep

Forward transfection in this study was performed by seeding the cells in the wells and DNA-lipid complex was added on top of the cells. Reverse transfection was done in the opposite manner, DNA-lipid complex first and cells on top.

The transfection efficiency was calculated manually based on how many transfected cells there were, versus the total amount of cells. It is an approximate evaluation since it was calculated only by visual inspection. The transfected cells showing GFP were estimated by percentage of the whole image versus percentage of cells in the same image. Transfection was conducted with two repeats per GOI, and the final percentage represented is the average for both repeats. Nikon eclipse Ts2 microscope was used for imaging with a 10x magnification. Three identical transfections were conducted.

## Cell viability assay

Cell viability tests were conducted with PrestoBlue HS (Invitrogen, P50200) assay, according to the manufacturer's guidelines (Thermo Scientific). The cell viability percentage was calculated from the absorbance. First, the average for every repeat was calculated as well as for the untreated cells. The average of repeats for reference wavelength were subtracted from the average of 570 nm wavelength. All repeats were then divided with the result from untreated cells. The gained percentage represents the cell viability. Cells were seeded on a 96-well plate, 20 000 cells per well, incubated for 24 hours in $CO_2$ incubator at 37°C, after which transfection was performed with LV-GOI-GFP and LV-GOI plasmids. The day after transfection, pictures were taken with a fluorescence microscope to verify transfection, and to obtain information about the appearance of the cells. After taking pictures, cells were incubated for approximately 30 minutes for stabilization. Then, 10 µl of medium from each well was removed and replaced with PrestoBlue HS. Cells were repeatedly incubated for 10-60 minutes. Readout was made with Varioskan Flash (Thermo Scientific) by measuring the absorbance at 570 nm, with 600 nm

as reference wavelength. The readout was normalized to untreated cells. Four identical assays were conducted.

## Enzyme-linked immunosorbent assay

For this project, ELISA assay was conducted by seeding 100 000 SH-SY5Y cells per well on 24-well plates (Corning costar, 3527) and incubated in 37 °C. After 24 hours, the cells were transfected with LV-GOI or LV-GOI-GFP plasmids. LV-GFP was used as a control plasmid. Non-transfected cells functioned as negative controls, and cells treated with 10 µg/ml lipopolysaccharide (LPS) and 5 µg/ml phorbol 12-myristate 13-acetate (PMA) functioned as positive controls. 24 hours after transfection, media and cells were collected for ELISA and Western Blot assays, respectively. Three identical transfections were conducted to obtain the samples for ELISA and Western Blot. Cells were collected as described earlier in subculturing. After centrifuging and gaining the cell pellet, the pellet was washed with 1 ml DPBS and transferred to eppendorf tubes. A second centrifugation was done for 5 minutes (4 °C, 1000 rpm), after which DPBS was removed. Transfection was done with two technical repeats, which were pooled together when samples were collected. The collected samples were stored in -80 °C until assays were conducted.

ELISA assay was performed with human TNF-α and human IL-6 ELISA kits (R&D Systems, Duoset, DY210-05 & DY206-05), according to the manufacturer's protocol. With ELISA, the aim was to determine whether the cytokine profile of the brain cells change when transfected with GOIs. The concentration of pro-inflammatory cytokines TNFα and IL-6 was planned to be determined. The collected media samples were used undiluted for the assay and ran in duplicate. Measurements were done with a microplate reader (Varioskan Flash, Thermo Scientific), by measuring absorbance at 450 nm with 540 nm as wavelength correction. The measured absorbance of the reference wavelength 540 nm was subtracted from the wavelength at 450 nm. The average of the two zero wells was then subtracted from all measurements, and a standard curve was plotted. Based on the standard curve, concentrations of the studied cytokines were calculated.

## Western Blot assay

The expression of GOI proteins was determined by Western Blot assay. Before conducting assays, the cells were homogenized within 100 µl of RIPA lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.10% Sodium dodecyl sulfate, H2O) containing one EDTA-free protease inhibitor (Roche, 04693159001) and one phosphatase inhibitor (Roche, 04906837001) per 10 ml of lysis buffer. The total protein concentration of samples was determined with DC protein assay (5000111, Bio-Rad). Due to low levels of protein according to the protein assay, a double amount of sample was loaded in the gel. This manipulation was done by first loading 20 µl of sample into the gel wells. The gel run was started at 100 V for a few minutes, until the samples completely entered the stacking gel. After this, the run was stopped, and another 20 µl of sample was loaded. The run was started again for a few minutes at 100 V, until all required samples were in the gel. After this, the run was switched to 200 V for approximately 40 minutes.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was conducted to separate the proteins with ThermoFishers' NuPAGE reagents and (4% to 12% Bis-Tris) gels. Samples were prepared according to the manufacturers' guidelines, apart from heating, which was changed from 70 °C for 10 minutes to 95 °C for 5 minutes. Some samples were also preheated and sonicated due to being too viscous for loading in the gel.

After gel electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane. The membranes were washed (10 minutes x 4) with 1x Tris Buffered Saline with Tween® 20 and then blocked with 5% fat free milk in 1x Tris Buffered Saline with Tween® 20 for an hour in room temperature on an orbital shaker. After that, the membranes were incubated with the specific primary antibody (**Table 2**) overnight in 4 °C on an orbital shaker. Next day, the wash (10 minutes x 4) was repeated, and the membranes were incubated with the secondary antibody (**Table 2**) for an hour in room temperature on an orbital shaker. Proteins were visualized with Pierce enhanced chemiluminescence (ECL) plus Western Blotting substrate (Thermo Scientific, 32132), and images were taken with Syngene G:Box. After the images were taken, the membranes were washed (10 minutes x 4) and then stripped for 15 minutes (Restore plus Western Blot stripping buffer, Thermo Scientific, 46430). After stripping, the membranes were washed

again (5 minutes x 3), then blocked, and incubated in the same manner as mentioned above, but with the housekeeping protein antibody (anti actin, Sigma-Aldrich, A4700, **Table 2**). ECL plus was used for the proteins since it is more sensitive, but for the housekeeping protein it was not considered necessary, therefore, the first blotting with housekeeping protein was visualized with Pierce ECL Western Blotting substrate (Thermo Scientific, 10005943). This gave somewhat faint bands, so ECL plus was also used for the other membranes for the blotting of housekeeping protein. **Table 2** presents antibodies used, with catalogue number, host species, reactivity, producer, and dilution.

***Table 2. Primary and secondary antibodies used for Western Blot. Antibodies 1-5 represents primary antibodies, and 6-7 secondary antibodies.***

| Antibody | Catalogue number | Host species | Reactivity | Producer | Dilution |
|---|---|---|---|---|---|
| 1. Anti MCP-1 | ab9669 | Rabbit | Human | Abcam | 1:4000 |
| 2. Anti M-CSF | ab9693 | Rabbit | Human | Abcam | 1:500 |
| 3. Anti MerTK | ab52968 | Rabbit | Human | Abcam | 1:500 |
| 4. GFP Rabbit anti-tag | A11222 | Rabbit | Human | Invitrogen | 1:1000 |
| 5. Monoclonal Anti-Actin antibody | A-4700 | Mouse | Human | Sigma | 1:1000 |
| 6. HRP-linked donkey anti-rabbit | NA9340 | Donkey | Anti-rabbit | Cytiva | 1:3000 |
| 7. Mouse Immunoglobulins, Goat Anti-, Polyclonal, HRP | P044701-2 | Goat | Anti-mouse | Agilent | 1:3000 |

# Results

## Optimization of transfection conditions

The initial optimization of transient transfection was performed on 96-well plates and later also optimized on 24-well plates, due to collection of media and cells for ELISA and Western Blot assays. The cell number was clearly too high with 40 000 cells (**Figure 4**). There was not enough space in the wells for all cells. With 10 000 cells, the cell number was too low (**Figure 4**), leaving too much empty space in the wells. The more Lipofectamine reagent was used, the worse the cells appeared to be (**Figure 5**). The cells lost their shape and shrank. As there was no difference in transfection efficiency, the lower Lipofectamine concentration (0.2 µl) was used for the transfection, to be more cost-efficient. Also, the transfection itself affected the appearance of the cells. **Figure 6** represents both cells after transfection and non-transfected cells, where the transfected cells are noticeably looking worse.

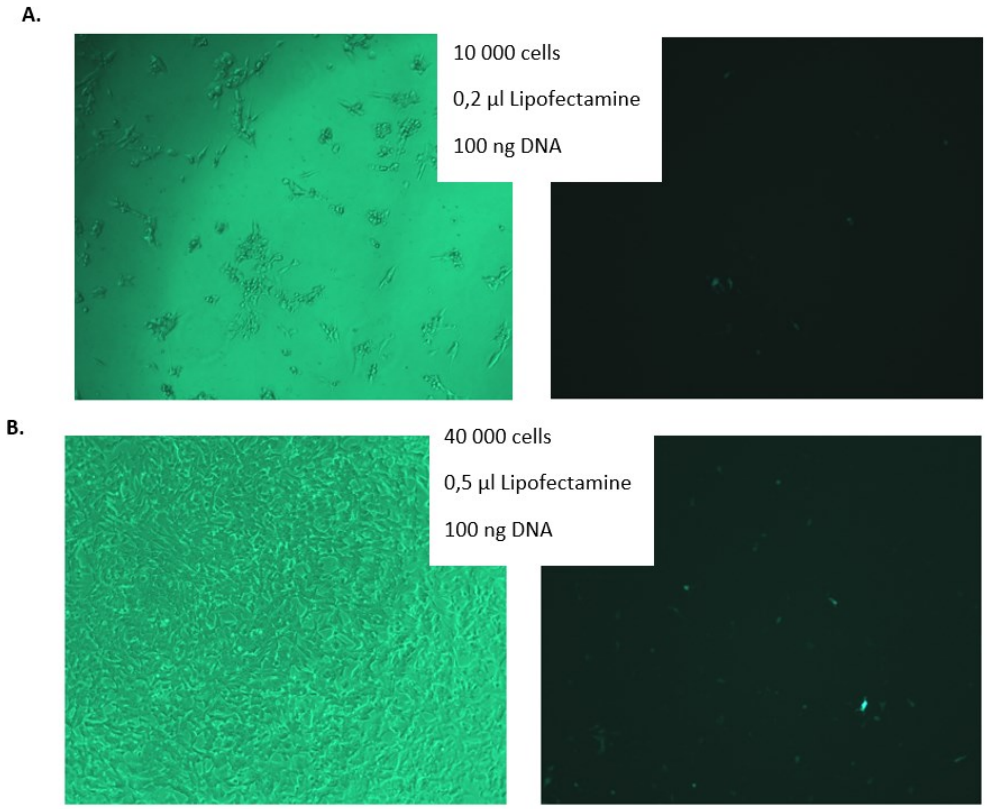


*Figure 4. Transfection of LV-GFP with A) 10 000 cells, 100 ng DNA and 0.2 µl Lipofectamine 2000 per well on a 96-well plate. B) 40 000 cells, 100 ng DNA and 0.5 µl Lipofectamine 2000 per well on a 96-well plate.*

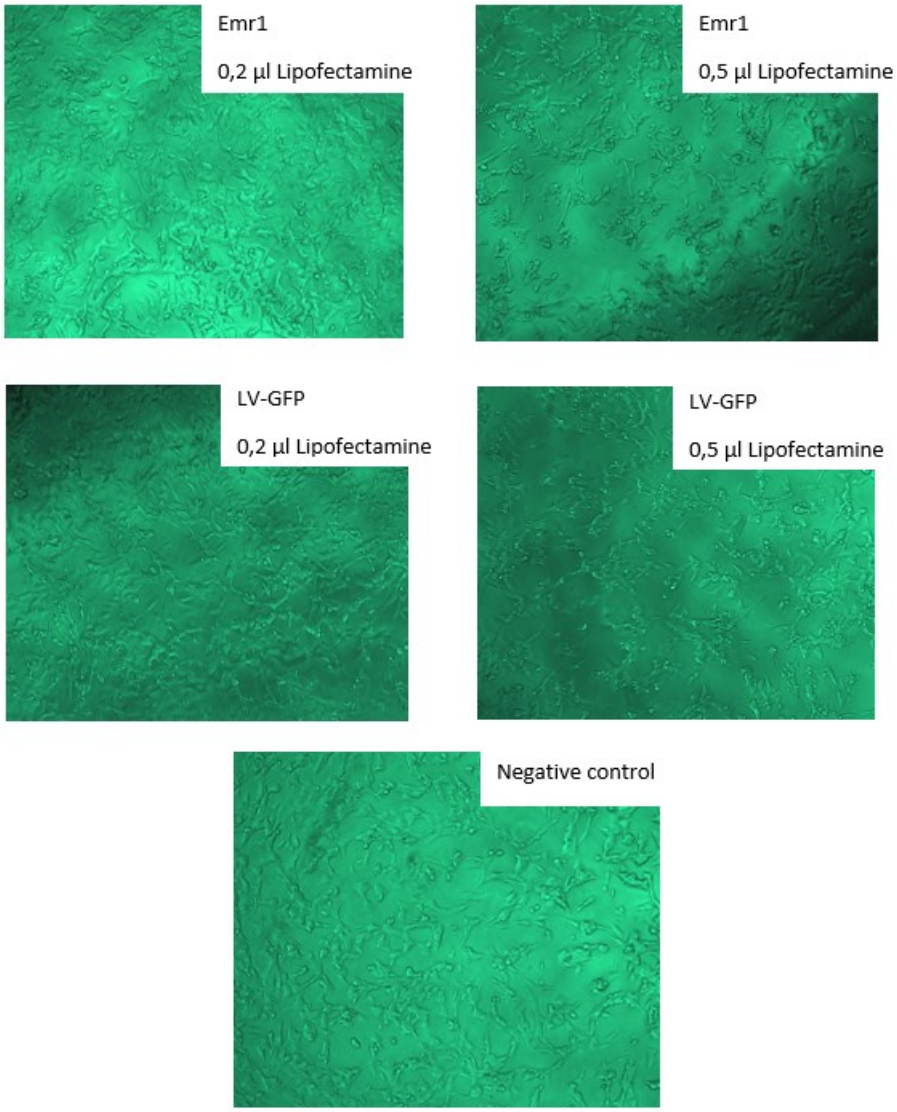


*Figure 5. Transfection with different concentrations of Lipofectamine and its effect on the cell's appearance.*

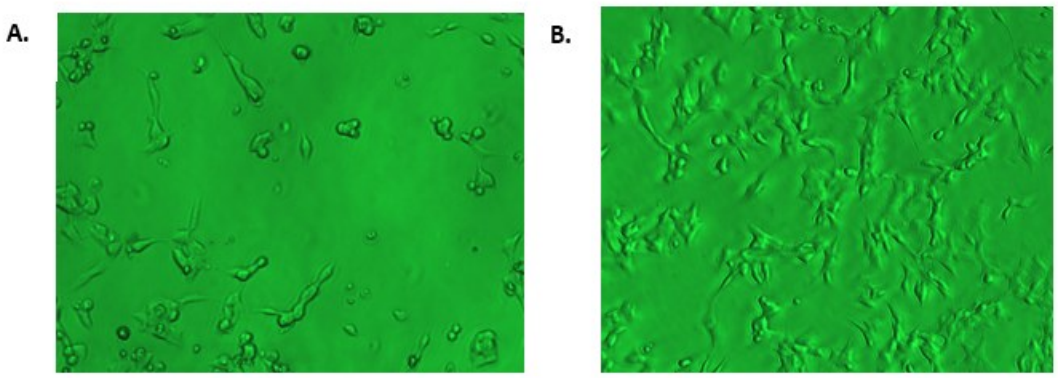


*Figure 6. Appearance of cells with and without transfection. A) Transfected cells and B) non-transfected cells.*

As the transfection efficiency was low following forward transfection, I tested the reverse transfection. In comparison, the reverse transfection with the lower amount of DNA did not show any transfection (**Figure 7**). With the lower amount of DNA (100 ng), the reverse transfection was not very successful either (**Figure 7**). Therefore, the lower amount of DNA (100 ng) with forward transfection was chosen. Forward transfection after 24 hours from seeding gave the best results compared to reverse transfection and transfection immediately after seeding (**Figure 7**). Medium without pen-strep was tested, since some studies suggest (Ryu et al., 2017) that pen-strep can interfere with the transfection. Based on optimization, the same conclusion was made, i.e., the transfection efficiency was higher without pen-strep (**Figure 7**). The same corresponding conditions as in the manufacturer's guidelines for Lipofectamine 2000 were applied for 24-well plate, i.e., 1 x $10^5$ cells per well, 500 ng of DNA and 1.0 µl Lipofectamine 2000. Both 2.0 µl and 1.0 µl of Lipofectamine 2000 was tested, as well as different DNA amounts (200 ng, 500 ng and 1000 ng) for the 24-well plate, but the corresponding results as in the manufacturer's guidelines were most efficient. The transfection was validated with a fluorescence microscope. It was notable that the adjustments and settings of the microscope can influence the results. Depending on brightness, for example, more of the cells transfected with LV-GOI-GFP can be visible. The dim dots might not be visible with low brightness. The cells did not attach evenly on the wells, leading to the chosen place of the image taken also affecting the results. In this study, pictures were taken only by me and, therefore, as far as possible done in the same manner throughout the study. The transfection was conducted with lentiviral (LV) plasmids encoding GOIs with and without GFP. **Figure 8** shows the construction of LV-GOI and LV-GOI-GFP, with MCP-1 as an example. The LVs should infect many cell types and the use of human elongation factor 1α (EF1α) promoter ensures that the GOIs are expressed in as many cells as possible.

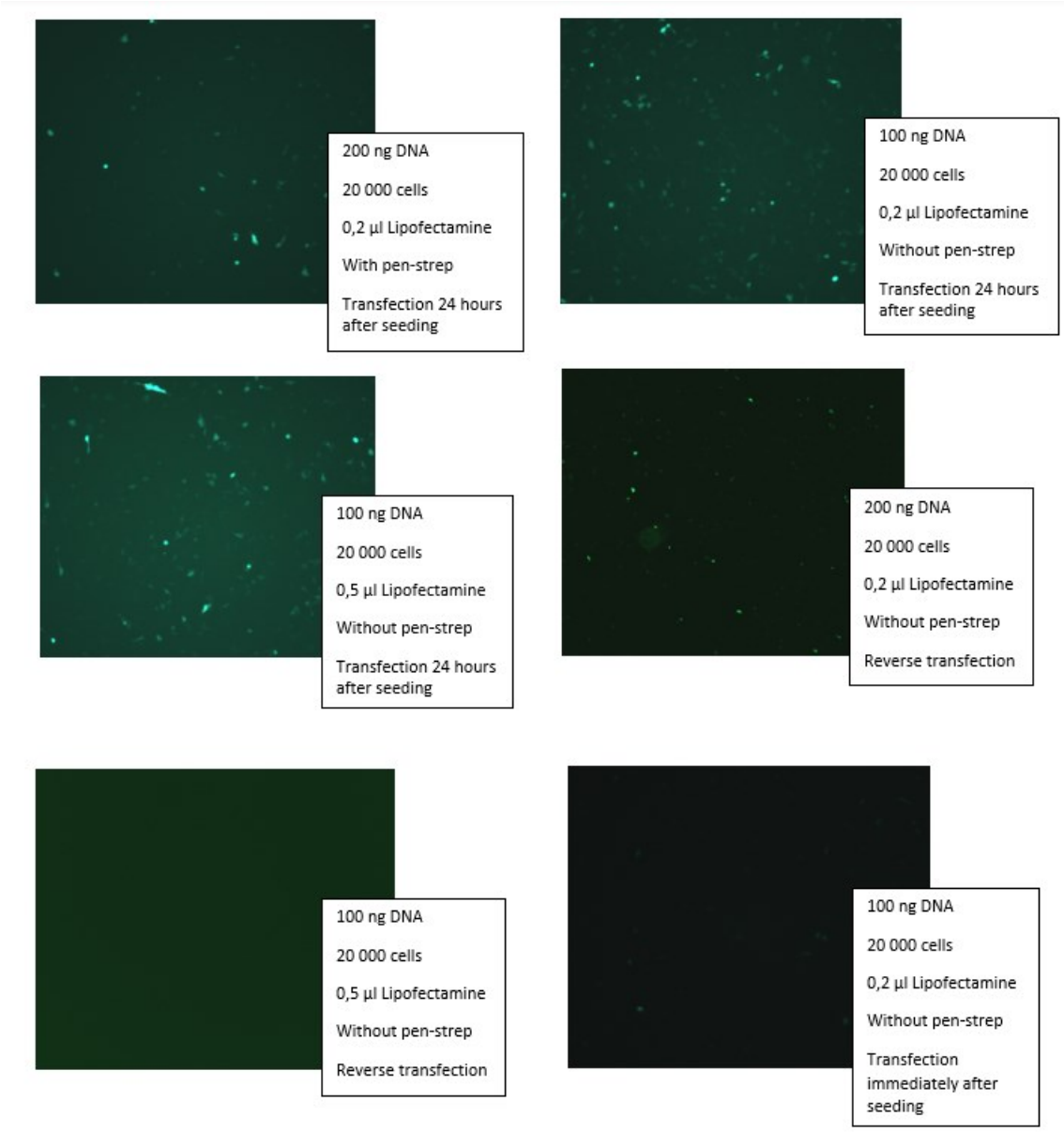


*Figure 7. Different transfection conditions for LV-GFP on a 96-well plate.*

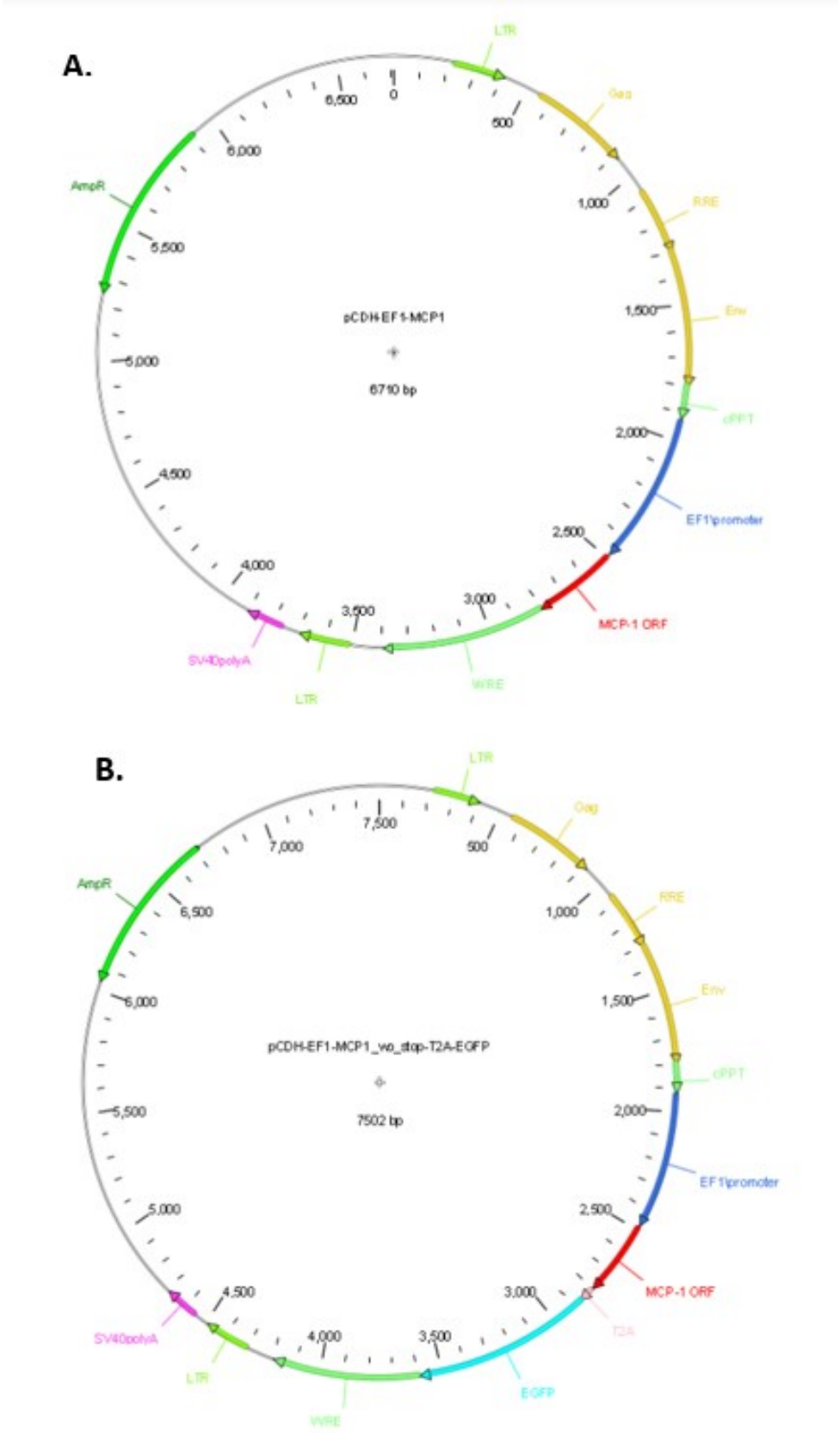


*Figure 8. Example plasmid maps of LV-GOIs and LV-GOI-GFP used in this study. In LV-GOI-GFPs the open reading frame of GOIs (red) were cloned between EF1a promoter (blue) and EGFP (cyan) or in case of LV-GOIs instead of EGFP. A) The plasmid map of LV-MCP-1 and B) LV-MCP-1-GFP.*

## Transfection efficiency

The final transfection efficiency was based on results from the last transfection giving the highest number of transfected cells. **Figure 9** shows the estimated transfection efficiencies, while **Figure 10** represents the microscope images of LV-GOI-GFP transfection, with the optimised transfection conditions on 24 well-plates. The images in **Figure 10** are corresponding to the transfection efficiencies in **Figure 9**.

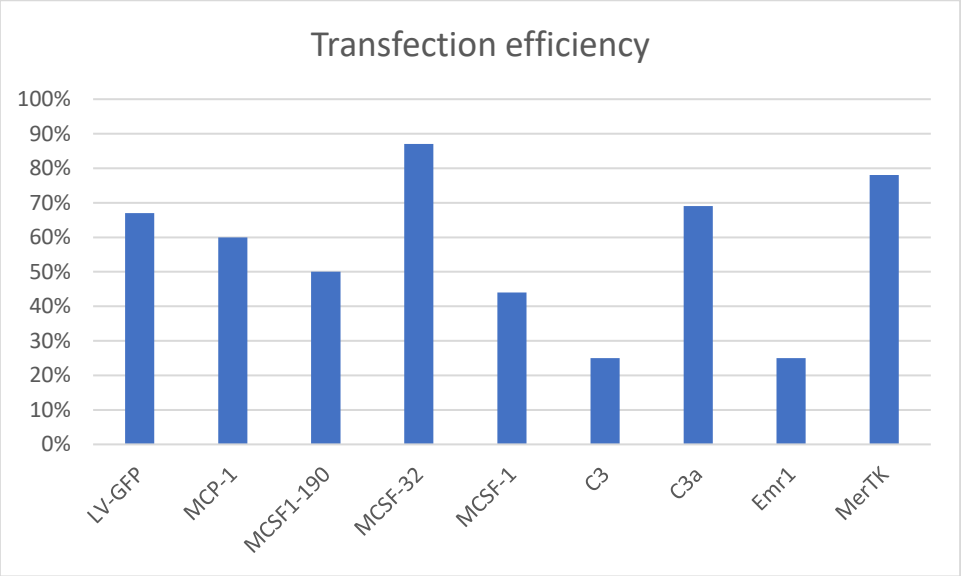


*Figure 9. Transfection efficiencies for LV-GOI-GFP on 24-well plate with 1 x $10^5$ cells, 500 ng DNA and 1.0 µl of Lipofectamine 2000 reagent per well. Results are based on the mean of two repeats for each GOI.*

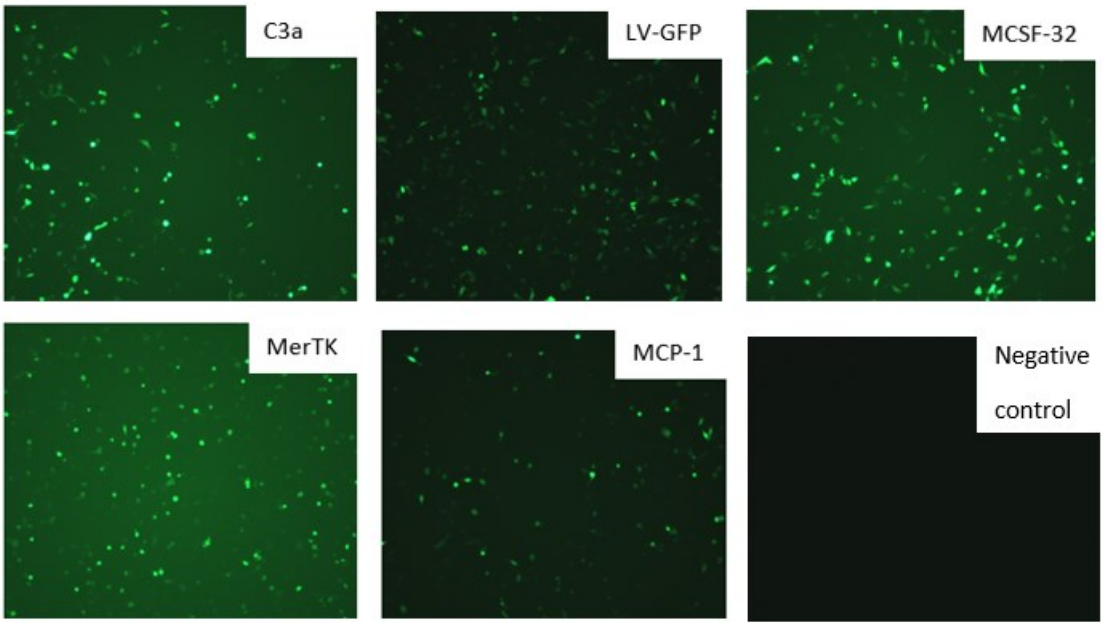


*Figure 10. Fluorescence images of LV-GOI-GFPs with best transfection efficiencies on a 24-well plate. Conditions: 1 x $10^5$ cells,500 ng DNA and 1.0 µl Lipofectamine 2000 reagent per well. Magnification of microscope is 10x.*

## Cell viability

For the cell viability, four identical transfection experiments with LV-GOI and LV-GOI-GFP were conducted, and results are presented in **Figure 11 and 12**. The results are based on the mean of two technical repeats for each GOI in every experiment. Cell viability is represented as percentage, where 100% equals that all cells survived the transfection. The GOIs show a cell viability between 1% and 270%.

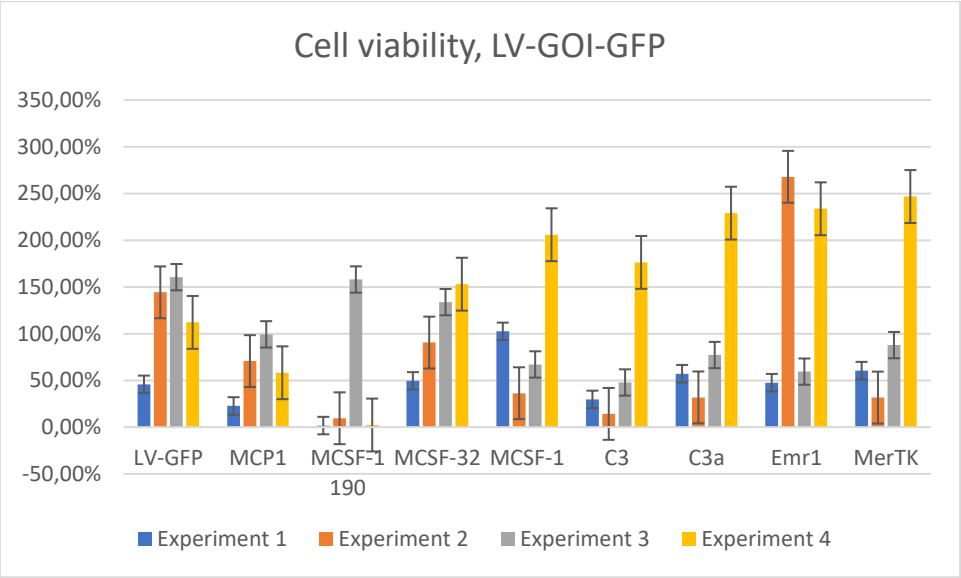


***Figure 11. Viability of cells transfected with LV-GOI-GFP. Plated on transparent 96-well plate with 20 000 cells per well. DNA concentration 100 ng per well and Lipofectamine 2000 concentration 0.2 µl per well. Results are based on the mean of two technical repeats for each GOI in every experiment.***

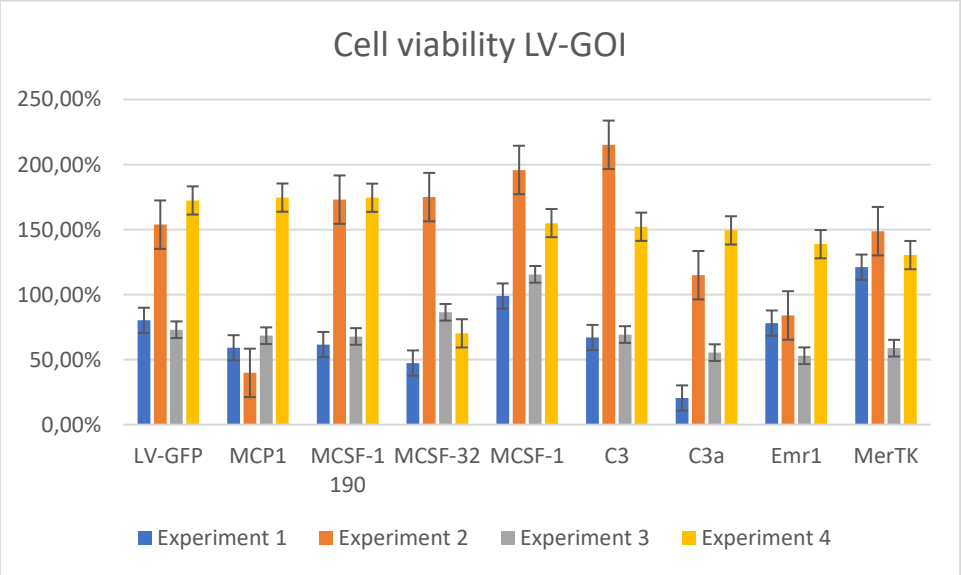


*Figure 12. Viability of cells transfected with LV-GOI. Plated on transparent 96-well plate with 20 000 cells per well. DNA concentration 100 ng per well and Lipofectamine 2000 concentration 0.2 µl per well. Results are based on the mean of two technical repeats for each GOI in every experiment.*

## Expression of GOI proteins

The expression of GOIs in neurons was determined with Western Blot. For this assay, only the GOIs with highest transfection efficiency were chosen, both with and without GFP, being M-CSF-32, MerTK and LV-GFP and just cells were used as negative control. C3a also had high transfection efficiency, but since the antibody that was available for C3 is unable to detect C3a, we determined the expression of MCP-1. Recombinant protein for MCP-1 (Abcam, 73866) and MerTK (Abcam, 167957) was used as positive control. The samples with highest protein concentration according to DC protein assay were used for Western Blot.

First, blotting was conducted for MerTK and MCP-1 samples. Specific bands for MerTK (**Figure 13 A**) were detected, as well as for β-actin (**Figure 13 B**). MCP-1 did not show any expression, besides for the recombinant protein (data not shown). For the next membrane, the ECL plus was also used for β-actin, due to faint bands from the first membrane (**Figure 13 B**). Secondly, M-CSF-32 and LV-GFP samples were blotted, also one sample of MerTK-GFP (**Figure 14 A**). M-CSF-32 gave no expression (data not shown), but LV-GFP showed bands for all different samples (**Figure 14 A**.). MerTK-GFP gave a faint band with the GFP antibody, since it also expresses GFP (**Figure 14 A**). **Figure 14 B** represents actin bands from blotting of M-CSF-32 and LV-GFP samples.

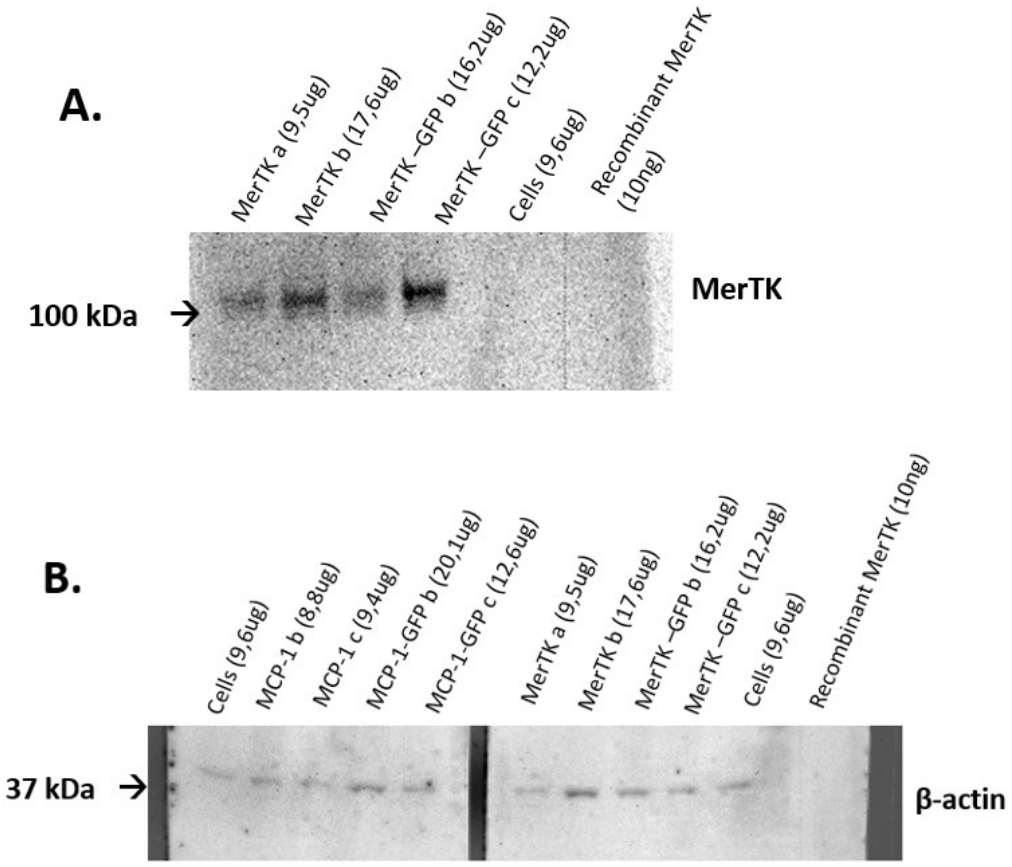


*Figure 13. Detecting the protein expression of MerTK, MCP-1 and actin in SH-SY5Y cells by Western Blot. Total amount of protein loaded shown in parentheses. Incubation with A) MerTK antibody B) Actin antibody. Letters in the sample names represent the date of transfection.*

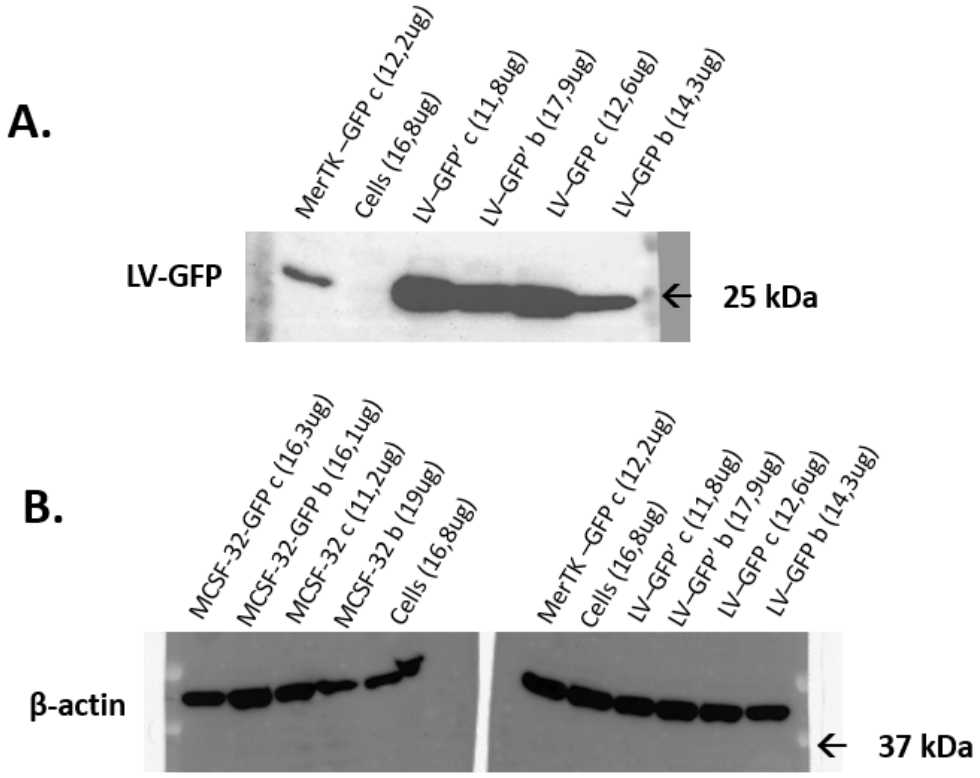


*Figure 14. Detecting the protein expression of GFP, M-CSF and actin in SH-SY5Y cells by Western Blot. Incubation with A) GFP antibody and B) actin antibody. Letters in the sample names represent the date of transfection.*

## GOI-induced cytokine production

Specific ELISA assays were conducted to determine whether the cytokine release from the neurons is affected by transfection with GOIs. The plan was to determine the concentrations of pro-inflammatory cytokines TNFα and IL-6. The concentrations obtained from TNFα ELISA assay were negative (data not shown), meaning that there was no cytokine release from neurons after transient transfection of LV-GOIs or LV-GOI-GFPs under the conditions tested. Thus, based on the results from TNFα ELISA assay, proceeding with IL-6 ELISA assay was not justified.

# Discussion

In this study, the immunomodulatory effect of GOIs was investigated in neurons. The hypothesis of the project was that transfection and expression of GOIs affect brain cells differently, as well as induce specific cytokine production. Thus, the initial plan was to study the effects also on oligodendrocytes and astrocytes but, unfortunately, due to the low transfection efficiency of neurons, the allocated time was not enough to perform all planned experiments. However, I was able to fulfil the main aim, which was to optimize transient transfection conditions, detect GOI expression and determine the cytokine profile in neurons.

The optimization process of transfection conditions was time-consuming, as the suggested transfection conditions were unable to provide sufficient transfection efficiency, and much work was done to obtain a better transfection efficiency. However, SH-SY5Y cells had not been transfected with the GOIs previously, and the outcome was lower than expected. In the end, MerTK, LV-GFP, M-CSF-32 and C3a had the best transfection efficiencies and were chosen for Western Blot assay. C3a was excluded, since the available C3 antibody is unable to recognize C3a. During the optimization of transfection conditions, it was obvious that the cells were affected by the transfection. The appearance of the cells was monitored along with the transfection efficiency, and most of the cells lost their shape and size. It was also noticeable that the cells gathered to the walls of the wells in clusters, showing no clear dispersal of the cells. However, this was not linked with the transfection since wells with only cells showed the same pattern. It was clear, though, that the transfection did not follow the same manner, as the transfected cells were more outspread in the wells, and mostly not in the clusters of cells by the walls. No clear line can be drawn between transfection and appearance of the cells but, generally, the cells expressing the GOIs also looked worse. Future studies may use the optimized transient transfection conditions as a basis, but should keep in mind that other reagents could be considered, for example Lipofectamine 3000. Also, notable is how much settings and adjustments of the fluorescence microscope affect the results, as well as which part of the well is in the picture.

Based on literature, the expectation of cytokine release from neurons was low, or even non existing (Viviani et al., 1998; Klegeris & McGeer, 2001), which justified not continuing with ELISA assays on IL-6 after gaining the results from TNFα assay. Even the positive control, cells treated with LPS and PMA, did not release any TNFα, although several studies have shown that both increase the cytokine production in several cell lines (Garrelds et al., 1999) & (von Asmuth et al., 1994). However, based on standard curves the assays do work (data not shown). In regard of the cytokine release from this cell line, based on this study, SH-SY5Y cells do not release TNFα and most likely not IL-6 either, not by its own, not after transfection with any of the GOIs, nor with stimulation of LPS and PMA. Samples from three different transfections were used, which increases the credibility of the results. However, future studies may alter the concentrations of stimulants or use other suitable stimulants.

In Western Blot, MerTK showed nice specific bands with the correct molecular weight, 110 kDa (**Figure 13 A**). Why the recombinant MerTK protein was not detected is still unknown. In the future, the purity and integrity of the recombinant protein could be evaluated with Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. When comparing appearance of the cells transfected with MerTK without GFP, there are almost no differences, but clearly the sample with more protein is showing higher expression, which is logical. However, MerTK-GFP samples show higher transfection efficiency from the sample with less protein but higher expression according to Western Blot results (**Figure 13 A**). Since the protein amount presented is the amount altogether of all proteins, not specifically MerTK, the results make sense. MCP-1 had the lowest transfection efficiency of all the GOIs used for Western Blot, yet some expression was anticipated since the cells were clearly transfected (**Figure 10**). Unfortunately, MCP-1 samples showed no expression with Western Blot but proved that the antibody works, since the recombinant protein used showed a strong band (data not shown). In parallel, β-actin was used as housekeeping protein. In this case, the protein amount is known to be different in all samples, since the amounts were so low, plus the issues with loading of the samples. However, β-actin proves that samples are loaded in all wells, even if they are not correlated with the total protein amount. β-actin bands are faint (**Figure 13 B**) in the samples with MerTK and MCP-1, but still specific and shown for all samples above 37 kDa, besides for the recombinant proteins, as it should be. The bands for β-actin in GFP and M-CSF are more expressed (**Figure 14 B**),

but still quite specific. This is most likely due to using the ECL plus for detection of the proteins. LV GFP c' and LV-GFP c samples have the highest transfection efficiencies as well as show the highest expression of the protein. MerTK-GFP showed a band for GFP (**Figure 14 A**), since it is expresses both MerTK and GFP, as it should. Based on Western Blot results, only two GOIs showed protein expression of the transfected GOI, being MerTK and LV-GFP. However, images with fluorescence microscope after transfection proved that most of the GOIs were expressed in the cells (**Figure 10**). It is still unknown why not all the GOIs that clearly were successfully transfected showed expression of the protein with Western Blot, since the plasmids were constructed in a manner that if GFP is expressed, they also express the GOI, meaning that all the GOIs should express the protein in Western Blot assay. The low amount of protein in all samples, as well as loading issues of the samples in the gel, might have an impact, but should not be a reason alone for the proteins not to be detected.

Results from cell viability experiments were unfortunately not reliable, since they did not give plausible numbers. Also, the different experiments gave very varying results. Neither was there any correlation between cell viability and the appearance of the cells, e.g., the cells that looked well, had a low cell viability and vice versa. In future studies, a longer incubation time after adding the PrestoBlue HS reagent could possibly give more reasonable results, since it increases the sensitivity according to the manufacturer. Another point to consider is that in this study the absorbance was measured. According to the manufacturer, by measuring fluorescence, the sensitivity is yet again increased and is the preferred detection method for this assay. In this study, absorbance was measured due to it being more cost-effective and the cell viability was not the assay of highest interest.

In the main project, microglia and phagocytosis are of interest. However, in the *in vivo* work there is no target cell selection when infecting the brains after stroke with LVs. Based on the results from this study, if the inflammatory state in the brain is alleviated after infecting the brain *in vivo*, it is presumably not due to the neurons. Therefore, if aiming to target a specific cell type, neurons should be excluded, since they do not induce a specific cytokine production when infected with LV-GOIs. Further studies could consider measuring the effect of co-culturing neurons with microglia, or using conditioned media from microglia on neurons, which might induce their cytokine production.

# Conclusions

The main aim of the study was to optimize most suitable transient transfection conditions for a neuronal cell line. This aim was accomplished by establishing these conditions for the SH-SY5Y cell line, using Lipofectamine 2000 reagent. The optimization on 96-well plates led to the following conditions: 20 000 cells, 0.2 µl Lipofectamine 2000 reagent, 100 ng DNA, forward transfection 24 hours after seeding and medium without pen-strep.

Based on this study and considering the results from the cell viability assay, Prestoblue HS is not suitable for this cell line.

The transient transfection of GOIs in SH-SY5Y cells does not change their inflammatory profile. Even if neurons are infected with LV-GOIs and LV-GOI-GFPs and they do express the GOIs, they most likely do not contribute to enhance the overall inflammatory state in the brain after ischemic stroke, when used *in vivo*.

# Summary in Swedish – Svensk sammanfattning

**Utvärdering av den immunmodulerande effekten hos fagocytosinducerare på neuronal cellinje SH-SY5Y**

## Introduktion

I dagens läge finns det ingen effektiv terapi för behandling av det långvariga patologiska tillståndet efter ischemisk stroke. Förlusten av blodtillförsel till hjärnan under stroke orsakar neurologiska skador i den ischemiska kärnan, som även kan sprida sig till det omgivande penumbraområdet. Penumbran kunde dock potentiellt återställas (Liu, Levine & Winn, 2010; Zhang et al., 2019). Skadade neuroner i den ischemiska kärnan frisläpper molekyler som inleder olika inflammatoriska respons. Detta fungerar som kroppens endogena försvar mot skador. Dock leder kontinuerligt bemötande av döda neuroner till överdriven inflammation som hindrar reparationen av hjärnan, samt möjliggör sekundär hjärnskada. Fagocyter återställer funktionen hos den skadade vävnaden och lindrar inflammation genom fagocytos. En snabb utplåning av döda celler och avfall främjar omgivningen för nervnybildning. I dagens läge är de cellulära och molekylära mekanismerna bakom fagocytos i hjärnan efter ischemisk stroke samt dess betydelse vid hjärnskada svagt begripna (Zhang et al., 2019).

Denna studie är en del av ett större projekt där långtidssyftet är att undersöka hur överuttryck av gener av intresse (GOI; gene of interest) påverkar mikroglia och återhämtning efter stroke *in vivo.* Flera proteiner som används i detta projekt inducerar rekryteringen av makrofager och mikroglia eller fagocytos (Hedtjärn, Mallard & Hagberg, 2004). Den immunomodulerande och fagocytosframkallande effekten av generna har testats på gnagarmikroglia-cellinje, BV2, *in vitro*, men hur generna påverkar neuroner, oligodendroglia samt astrocyter är okänt. Hypotesen för huvudprojektet är att en ökning av fagocytos kan förbättra återhämtningen efter stroke och hjälpa till att städa upp området.

Hypotesen för denna substudie var att transfektion och uttryck av de beskrivna generna påverkar hjärnceller på olika sätt och framkallar en specifik cytokinproduktion. Denna hypotes undersöktes i neuroner.

Neuroblastomcellinjen SH-SY5Y odlades och transfekterades med lentivirus (LV)-plasmider innehållande GOI. Transfektionens effektivitet bestämdes med fluorescerande mikroskop. Uttryck av genernas proteiner bestämdes med Western Blot genom att använda genspecifika antikroppar och den inflammatoriska profilen bestämdes med tumörnekrosfaktor (TNFα) och interleukin-6 (IL-6) enzymkopplad immunadsorberande analys (ELISA) 24 timmar efter transfektion. **Figur 1** (**Figure 1**) beskriver forskningsaktiviteterna för projektet.

# Bakgrund

## Ischemisk stroke

Ischemisk stroke är den vanligaste typen av stroke. Den leder till syrebrist orsakad av blodpropp i hjärnan (Randolph, 2016). Den årliga mortaliteten för stroke är 5,5 miljoner som är den näst vanligaste dödsorsaken och främsta orsaken till funktionshinder i världen (Kuriakose & Xiao, 2020). Ungefär 50 % av patienterna som överlever stroke blir kroniskt funktionshindrade (Donkor,2018). Ny forskning visar att förekomsten av stroke ökar och att en av fyra människor globalt kommer att uppleva stroke. Orsakerna bakom detta är den åldrande befolkningen ihop parat med den summerade bördan av olika riskfaktorer. Än i dagens läge är vårdalternativen begränsade, fastän framsteg också har gjorts. Oavsett finns det ett stort behov att utveckla terapeutiska medel för att skydda hjärnan från skada efter ischemisk stroke (Paul & Candelario-Jalil, 2021).

## Neuroner och mikroglia

Nervceller, neuroner, anses vara basenheterna för nervsystemet. Nervcellerna skapar ett komplext kommunikationsnätverk, och behövs för varje funktion och handling som vår kropp eller hjärna utför. De svarar på stimulans och skickar impulser till resten av kroppen, samt överför informationen däremellan (Newman, 2017). Flera sjukdomar som till exempel Parkinsons sjukdom, Alzheimers sjukdom och stroke leder till onaturlig död av neuroner. Neuroner studeras flitigt för att erhålla mer information om deras inverkan på hjärnan för att kunna utveckla nya terapeutiska medel och därmed bota samt lindra olika hjärnskador (NINDS, 2019).

Mikrogliaceller representerar huvudtypen av immunceller i centrala nervsystemet (CNS; central nervous system) och är viktiga för neuroplasticitet, neurogenes och vävnadshomeostas (Lindahl, Saarma & Lindholm, 2016). Mikrogliaceller är makrofager som finns i hjärnan och bildar den första aktiva immunbarriären i CNS. Cellerna spelar en stor roll under både barndomen och vuxen åldern, lika så vid sjukdomstillstånd (Stratoulias et al., 2019). Felvikta proteiner aktiverar mikroglia vid flera olika neurodegenerativa sjukdomar, vilket leder till frisättning av proinflammatoriska och cytotoxiska faktorer som främjar neuronskada (Lindahl, Saarma & Lindholm, 2016). En tidig respons av mikrogliaceller begränsar spridningen av skador (Galloway et al., 2019). Mikroglia anses skydda neuroner efter en akut skada och de aktiveras genom signaler från skadade neuroner (Streit, 2005).

## Fagocytos och inflammation

För att upprätthålla homeostas i flercelliga organismer krävs utrensning av cellavfall. Det är viktigt för processer som vävnadstillväxt och ombyggnad, såväl som för nybildning och upplösning av skada och inflammation. De skadade cellerna avlägsnas för det mesta genom fagocytos, utfört av makrofager och neutrofiler, till exempel mikrogliaceller. Fagocytos är ett komplext fenomen som inkluderar intag och nedbrytning av partiklar (Westman, Grinstein & Marques, 2020). Det har länge varit känt att fagocytos förmedlar det medfödda och specifika immunförsvaret. Idag vet vi även att den är central för tidig utveckling av neuroner, homeostas, samt grunden för olika reparationsmekanismer. Därför är modulering av fagocytos vitt utforskat för att utveckla nya terapeutiska medel som både främjar reparation och nybildning i CNS (Galloway et al., 2019).

Induktion, produktion och frisläppning av proinflammatoriska mediatorer är en följd av inflammation. Cytokiner som TNFα och IL-6 är exempel på dessa mediatorer (Kany, Vollrath & Relja, 2019). Flera studier har påvisat att IL-6 och TNFα uttrycks i neuroner (Erta, Guintana & Hidalgo, 2012; Park & Bowers, 2010). Neuroner anses vara en av de väsentliga källorna till IL-6 och har påvisats producera samt utsöndra cytokinen i fråga vid stimuli (Erta, Guintana & Hidalgo, 2012; Park & Bowers, 2010). Däremot redovisar en annan studie ingen utsöndring av TNFα från neuroner (Viviani et al., 1998). Cytokinen IL-6 spelar en stor roll i neurala reaktioner mot nervskada (Zhang & An, 2009). Flera av de huvudsakliga hjärnsjukdomarna påverkas av IL-6 och cytokinen anses därför vara ett tydligt mål för strategiska terapier (Erta, Guintana & Hidalgo, 2012). En annan cytokin, TNFα, har bevisats vara betydelsefull för både inflammation och neuropatisk hyperalgesi (Zhang & An, 2009). Gliaceller kan upptäcka neuronal aktivitet på ett ännu okänt sätt. När den neuronala aktiviteten är låg börjar gliaceller frisätta TNFα (Galic, Riazi & Pittman, 2011).

## Generna

Tabellen nedan **(Tabell 3)** presenterar GOI:er som användes i projektet, samt beskriver kortfattat deras inverkan på fagocytos.

*Tabell 3. Presentation av GOIs som användes i projektet, samt deras inverkan på fagocytos.*

| GOI | Karakterisering |
|---|---|
| *Monocyt-kemoattraherande* protein **(MCP-1)** / CCL2 | Rekrytering i fagocytos |
| Makrofagkolonistimulerande faktor **(M-CSF)** / CSF-1 | Framkallar fagocytos |
| Komplementfragment 3 **(C3)** | Rekrytering i fagocytos |
| Komplementfragment 3a **(C3a)** | Rekrytering i fagocytos |
| EGF Modulinnehållande mucinliknande receptor **(Emr1)** | Ökar fagocytos |
| Mer receptortyrosinkinas **(MerTK)** / c-mer | Ökar fagocytos |
| Mesencefalisk astrocyt-härledd neurotrofisk faktor **(MANF)** | Ökar fagocytos |

## Syften

Syftet med detta projekt var att utforska hur övergående transfektion av GOI-uttryck i neuroner påverkar deras frisättning av inflammatoriska cytokiner. Mer specifikt var syftena att:

1) optimera omständigheterna för GOI-transfektion,
2) utforska uttryck av GOI, och
3) bestämma cytokinprofilen i neuroner.

## Material och metoder

I projektet användes neuroblastomcellinjen SH-SY5Y. Cellerna odlades i odlingssubstrat och lagrades i en inkubator. Transfektionen optimerades på 96-hålsplatta med LV-plasmider som kodar för GFP. Olika experimentella omständigheter utvärderades: cellmängden, koncentrationen av Lipofectaminereagens, mängden DNA, omvänd vs. vanlig transfektion, transfektion direkt vs. efter 24 timmar, och odlingssubstrat med eller utan antibiotika.

Transfektionens effektivitet beräknades på basen av bilder tagna med ett fluorescerande mikroskop. Uppskattningen är ungefärlig, eftersom den är beräknad med blotta ögat genom att jämföra den totala mängden celler med mängden celler som uttrycker GFP. Cellöverlevnad kontrollerades med PrestoBlue HS enligt tillverkarens instruktioner. Resultaten baserar sig på absorbans. Innan ELISA och Western Blot-testerna utfördes, samlades celler och odlingssubstrat tre gånger från 24-hålsplattor. ELISA-uppsättningar för TNF-α och IL-6 användes enligt tillverkarens instruktioner. Den totala proteinkoncentrationen i proven mättes innan Western Blot med DC Protein-assay. På grund av låga koncentrationer protein matades en dubbel mängd av proven i Western Blot. Proven homogeniserades i lyseringsbuffer och kördes med gelektrofores, varefter proteinerna överflyttades till ett polyvinylidendifluoridmembran. Proteinerna visualiserades med Pierce enhanced chemiluminescence (ECL) plus, bortsett från första visualiseringen av β-aktin. Analysen gjordes enligt tillverkarens instruktioner, förutom uppvärmningen som ändrades från 10 minuter i 70 °C till 5 minuter i 95 °C. Uppvärmningen ändrades på basen av tidigare erfarenhet hos forskarna i Airavaaras forskningsgrupp. Vissa prov sonikerades och uppvärmdes två gånger, eftersom de var för viskösa och svåra att lasta in i gelen. β-aktin fungerade som hushållsprotein.

Optimering av transfektionen ledde till följande resultat på 96-hålsplatta: 20 000 celler, 100 ng DNA, 0,2 µl Lipofectamine 2000, vanlig transfektion 24 timmar efter odling med odlingssubstrat utan antiobiotika. **Figur 7 (Figure 7)** presenterar olika experimentella omständigheter som prövades. För 24-hålsplatta användes motsvarande mängder reagens samt DNA enligt tillverkarens instruktioner, även om högre samt lägre koncentrationer också prövades. **Figur 9 (Figure 9)** visar transfektionseffektiviteten för den sista transfektionen som gav de bästa resultaten. Det var tydligt att cellerna påverkades av transfektionen, **Figur 6 (Figure 6)** visar A) transfekterade celler och B) celler utan transfektion. De transfekterade cellerna tappade formen och krympte. Det var även noterbart att cellerna samlades till plattans väggar i klungor, istället än att sprida ut sig jämnt över plattan. Detta var dock inte knutet till transfektionen, eftersom cellerna utan transfektion även visade samma mönster. De transfekterade cellerna däremot visade sig vara mer utspridda i plattan. Det finns ingen tydlig linje att dra emellan transfektion och cellernas utseende, men i allmänhet visade sig de transfekterade cellerna må sämre.

Resultaten från cellöverlevnadstesterna är presenterade i **Figur 8 och 9 (Figure 8 & 9)**, både för GOI:er med GFP samt utan GFP. Dessvärre var resultaten inte pålitliga, eftersom procenttalen var orimliga. Olika experiment gav även mycket varierande resultat. Det fanns inte heller någon korrelation mellan cellöverlevnad och cellernas utseende, vilket betyder att celler som såg välmående ut hade låg cellöverlevnad och vice versa. Med tanke på resultaten verkar PrestoBlue HS inte vara ett fungerande alternativ för denna cellinje.

Uttrycket av GOI:er i neuroner bestämdes med Western Blot. Endast de GOI:er som visade den högsta transfektionseffektiviteten valdes för denna analys, både med och utan GFP. GOI:er som valdes var följande: MSCF-32, MerTK och LV-GFP, och celler utan transfektion användes som negativ kontroll. C3a hade också hög effektivitet, men antikroppen som fanns tillgänglig känner inte igen C3a och därför användes MCP-1 i stället. Rekombinanta proteiner för MCP-1 och MerTK användes som positiva kontroller. Proven med den högsta proteinkoncentrationen användes för analysen. Första blotting gjordes med MerTK och MCP-1-proven. Specifika band för MerTK upptäcktes **(Figur 13 A; Figure 13 A)**, likaså för β-

aktin **(Figur 13 B; Figure 13 B)**, båda med rätt molekylvikt. MCP-1 uttrycktes inte, förutom dess rekombinanta protein (inga data). Varför inte det rekombinanta proteinet för MerTK uttrycktes är ett frågetecken. I framtiden kunde det rekombinanta proteinets renhet och integritet undersökas med Sodium dodecyl sulfate–polyacrylamide gelelektrofores. I jämförelse med MerTK-proven utan GFP är det inga tydliga skillnader, men proven med högre proteinkoncentration är mera uttryckta. MerTK proven med GFP däremot visar högre transfektionseffektivitet för proven med lägre proteinkoncentration men högre uttryck av proteinet enligt Western Blot-resultaten **(Figur 13 A; Figure 13 A)**. Proteinkoncentrationen beskriver den totala koncentrationen protein, inte specifikt MerTK, vilket gör att resultaten är logiska. Av GOI:er som användes för Western Blot hade MCP-1 lägsta transfektionseffektiviteten, oavsett förväntades uttryck av proteinet eftersom det tydligt var transfekterat **(Figur 10; Figure 10)**. M-CSF-32 och LV-GFP-proven var blottade som följande, även ett prov av MerTK-GFP **(Figur 14)**. M-CSF-32 uttrycktes inte (inga data), men LV-GFP visade band för alla olika proven. MerTK-GFP visade också ett svagt band med GFP-antikroppen, eftersom den också uttrycker GFP **(Figur 14 A; Figure 14 A)**. LV-GFP c' och LV-GFP c-proven (bokstäverna indikerar provdatum) hade högsta transfektionseffektiviteten samt högsta uttryck av proteinet. I denna studie är det känt att proteinmängden i de olika proven inte är samma, på grund av låga proteinkoncentrationer samt problemen med viskösa prov. Oavsett, bevisar β-aktin att alla hål i gelen är laddade med prov, även om det inte korrelerar med den totala proteinmängden. I första blotting var β-aktin-banden svaga **(Figur 13 B; Figure 13 B)** med MerTK och MCP-1-proven, men ändå specifika och vid rätt molekylvikt, bortsett för rekombinanta proteinen, som det ska vara. I proven med LV-GFP och M-CSF är β-aktin-banden mer uttryckta **(Figur 14 B; Figure 14 B)**, men ändå ganska specifika. Detta beror högst troligen på att ECL plus användes för proven med LV-GFP och M-CSF. Överlag bevisar Western Blot resultaten att proteinet av transfekterade GOI är uttryckt, i detta projekt gäller det för MerTK och LV-GFP. Bilderna med det fluorescerande mikroskopet efter transfektion bevisar att de flesta generna är uttryckta i cellerna **(Figur 10; Figure 10)**. Det är ännu oklart varför inte alla GOI:er som tydligt var transfekterade uttrycks i Western Blot. Plasmiderna är uppbyggda på ett sätt att då GFP uttrycks, uttrycks även GOI, betydande att alla GOI:er borde ha uttryckt proteinet i Western Blot-analysen. **Figur 8 (Figure 8)** visar uppbyggnaden av plasmiderna med MCP-1 som exempel.

ELISA-analys gjordes för att bestämma ifall frisläppandet av cytokiner i neuronerna ändras efter transfektion med GOIs. Planen var att bestämma koncentrationen TNFα och IL-6. Koncentrationen TNFα var negativ (ingen data), vilket betyder att neuronerna inte frisläpper någon TNFα efter transfektion av GOIs. På basen av litteraturen var förväntningarna för frisläppandet av cytokinerna lågt, nästan osannolik (Viviani et al., 1998) & (Klegeris & McGeer, 2001), vilket gav motiveringen att inte fortskrida med IL-6 ELISA-analys efter erhållna resultat från TNFα-analysen. Även celler behandlade med LPS och PMA frisläppte ingen TNFα, fastän flera undersökningar har bevisat att båda ökar cytokinproduktionen i flera olika cellinjer (Garrelds et al., 1999) & (Asmuth et al., 1994). Oavsett, bevisar standardkurvan (inga data) att analysen fungerar. På basen av detta kan endast konstateras att SH-SY5Y celler inte frisläpper TNFα och högst antagligen inte IL-6 heller. Inte av sig själv, inte efter transfektion med någon av GOI:erna och inte heller efter stimulans med LPS och PMA.

Som slutsats kan dras att transfektion av GOI:er i SH-SY5Y celler ändrar inte deras inflammatoriska profil. Även om neuronerna infekteras av LV-GOI:er och LV-GOI-GFP:er och de uttrycker GOI:er, bidrar de högst antagligen inte till att förbättra inflammatoriska tillståndet i hjärnan efter ischemisk stroke vid *in vivo*-användning.

# References


Alhajj, M. & Farhana, A. (2021). StatPearls. *Enzyme Linked Immunosorbent Assay.* Treasure Island (FL): StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK555922/

Aronson, J.K. (2016). *Macrophage colony-stimulating factor (M-CSF)*. Meyler's Side Effects of Drugs (Sixteenth Edition), Elsevier, Pages 726-727. https://doi.org/10.1016/B978-0-444-53717-1.01010-6.

Brown, G. C. & Viltala, A. (2015). How microglia kill neurons. *Brain research,* 1628 (B). https://doi.org/10.1016/j.brainres.2015.08.031

Cai, B., Kasikara, C., Doran, A.C., Ramakrishnan, R., Birge, R.B. & Tabas, I. (2018). MerTK signaling in macrophages promotes the synthesis of inflammation resolution mediators by suppressing CaMKII activity. *Science Signaling,* 11 (549). doi: 10.1126/scisignal.aar3721

Cherry, J.D., Olschowka, J.A. & O'Banion. (2014). M.K. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J Neuroinflammation* 11, 98. https://doi.org/10.1186/1742-2094-11-98

Deshmane, S. L., Kremlev, S., Amini, S., & Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research*, *29* (6). https://doi.org/10.1089/jir.2008.0027

Donkor E. S. (2018). Stroke in the 21st Century: A Snapshot of the Burden, Epidemiology, and Quality of Life. *Stroke research and treatment*, 3238165. https://doi.org/10.1155/2018/3238165

Erta, M., Quintana, A., & Hidalgo, J. (2012). Interleukin-6, a major cytokine in the central nervous system. *International journal of biological sciences*, *8* (9). https://doi.org/10.7150/ijbs.4679

Faber, D. S., & Pereda, A. E. (2018). Two Forms of Electrical Transmission Between Neurons. *Frontiers in molecular neuroscience*, *11*, (427). https://doi.org/10.3389/fnmol.2018.00427

Fumagalli, S., Perego, C., Pischiutta, F., Zanier, E.R. & De Simoni, M.G. (2015). The ischemic environment drives microglia and macrophage function. *Frontiers in Neurology*. doi: 10.3389/fneur.2015.00081

Galic, M. A., Riazi, K., & Pittman, Q. J. (2012). Cytokines and brain excitability. *Frontiers in neuroendocrinology*, *33* (1). https://doi.org/10.1016/j.yfrne.2011.12.002

Galloway, D. A., Phillips, A., Owen, D., & Moore, C. S. (2019). Phagocytosis in the Brain: Homeostasis and Disease. *Frontiers in immunology*, *10* (790). https://doi.org/10.3389/fimmu.2019.00790

Gao, B., Jing, D., Xiaogang, Z., Houchao, S., Gongwei, J., Jiani, L., Keming, Z., Chengqun W., Linxi, W. & Liang-Jun, Y.. (2020). Effects of mesencephalic astrocyte-derived neurotrophic factor on cerebral angiogenesis in a rat model of cerebral ischemia. *Neuroscience letters,* 715. https://doi.org/10.1016/j.neulet.2019.134657

Gao, S., Cui, Z. & Zhao, M. (2020). The Complement C3a and C3a Receptor Pathway in Kidney Diseases. *Frontiers in immunology,* 11. https://doi.org/10.3389/fimmu.2020.01875

Garrelds, I. M., Van Hal, P., Haakmat, R. C., Hoogsteden, H.C., Saxena, P. R. & Zijlstra, F. J. (1999). Time dependent production of cytokines and eicosanoids by human monocytic leukaemia U937 cells; effects of glucocorticosteroids. *Mediators of Inflammation*, 8. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1781809/pdf/10704077.pdf

Hedtjärn, M., Mallard, C. and Hagberg, H. (2004). Inflammatory gene profiling in the developing mouse brain after hypoxia-ischemia. *Journal of Cerebral Blood Flow & Metabolism*, 24 (12). doi: 10.1097/01.wcb.0000141559.17620.36.

Hughes, C. E., & Nibbs, R. (2018). A guide to chemokines and their receptors. *The FEBS journal*, *285* (16). https://doi.org/10.1111/febs.14466

Jones, C. V., & Ricardo, S. D. (2013). Macrophages and CSF-1: implications for development and beyond. *Organogenesis*, 9 (4). https://doi.org/10.4161/org.25676

Jurga, A.M., Paleczna, M. & Kuter, K. (2020). Overview of General and Discriminating Markers of Differential Microglia Phenotypes. *Frontiers in cellular neuroscience,* 14. https://doi.org/10.3389/fncel.2020.00198

Kandel, E. R. (1991). Nerve cells and behavior. *Principles of neural science*, *3*.

Kany, S., Vollrath, J. T., & Relja, B. (2019). Cytokines in Inflammatory Disease. *International journal of molecular sciences*, *20* (23). https://doi.org/10.3390/ijms20236008

Klegeris, A. & McGeer, P.L. (2001). Inflammatory cytokine levels are influenced by interactions between THP-1 monocytic, U-373 MG astrocytic, and SH-SY5Y neuronal cell lines of human origin. *Neuroscience Letters,* 313 (1-2). https://doi.org/10.1016/S0304-3940(01)02251-0

Kuriakose, D. & Xiao, Z. (2020). Pathophysiology and Treatment of Stroke: Present Status and Future Perspectives. *International Journal of Molecular Sciences*, 21 (20). https://doi.org/10.3390/ijms21207609

Li, L. Z., Huang, Y. Y., Yang, Z. H., Zhang, S. J., Han, Z. P., & Luo, Y. M. (2020). Potential microglia-based interventions for stroke. *CNS neuroscience & therapeutics*, *26* (3). https://doi.org/10.1111/cns.13291

Lindahl, M., Saarma, M. & Lindholm, P. (2016). Unconventional neurotrophic factors CDNF and MANF: Structure, physiological functions and therapeutic potential. *Neurobiology of disease.* doi: 97. 10.1016/j.nbd.2016.07.009

Liu, S., Levine, S. R., & Winn, H. R. (2010). Targeting ischemic penumbra: part I - from pathophysiology to therapeutic strategy. *Journal of experimental stroke & translational medicine*, *3* (1). https://doi.org/10.6030/1939-067x-3.1.47

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences*, *4* (9). https://doi.org/10.4103/1947-2714.100998

Masli, S., & Turpie, B. (2009). Anti-inflammatory effects of tumour necrosis factor (TNF)-alpha are mediated via TNF-R2 (p75) in tolerogenic transforming growth factor-beta-treated antigen-presenting cells. *Immunology*, *127* (1). https://doi.org/10.1111/j.1365-2567.2008.02933.x

Moulton, R. (2016). Chapter 17 - Cytokines, *Systemic Lupus Erythematosus*, Academic Press, 137-141, ISBN 9780128019177. https://doi.org/10.1016/B978-0-12-801917-7.00017-6.

National Institute of Neurological Disorders and Stroke (NINDS). (2019). Brain Basics: The Life and Death of a Neuron. Available at: https://www.ninds.nih.gov/disorders/patient-caregiver-education/life-and-death-neuron

Newman, T. (2017): All you need to know about neurons. *Medical news today.* Available at: https://www.medicalnewstoday.com/articles/320289

O'Donnell, M. J., Xavier, D., Liu, L., Zhang, H., Chin, S.L., Rao-Melacini, P., Rangarajan, S., Islam, S., Pais, P., McQueen, M.J., Mondo, C., Damasceno, A., Lopez-Jaramillo, P., Hankey, G.J., Dans, A.L., Yusoff, K., Truelsen, T., Diener, H.C., Sacco, R.L., Ryglewicz, D., Czlonkowska, A., Weimar, C, Wang X, Yusuf S (2010). Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *The Lancet*, 376 (9735). doi: 10.1016/S0140-6736(10)60834-3.

Pandya, S., Klimatcheva, E. & Planelles, V. (2001) Lentivirus and foamy virus vectors: novel gene therapy tools. *Expert Opinion on Biological Therapy*, 1. doi: 10.1517/14712598.1.1.17

Park, K. M., & Bowers, W. J. (2010). Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. *Cellular signalling*, *22* (7). https://doi.org/10.1016/j.cellsig.2010.01.010

Paul, S. & Candelario-Jalil, E. (2021). Emerging neuroprotective strategies for the treatment of ischemic stroke: An overview of clinical and preclinical studies. *Experimental neurology,* 335. https://doi.org/10.1016/j.expneurol.2020.113518

Pekna, M., Stokowska, A. & Pekny, M. (2021). Targeting Complement C3a Receptor to Improve Outcome After Ischemic Brain Injury. *Neurochemical Research* 46. https://doi.org/10.1007/s11064-021-03419-6

Randolph, S.A. (2016). Ischemic Stroke. *Sage journals,* 64 (9). https://doi.org/10.1177/2165079916665400

Ricklin, D., Reis, E. S., Mastellos, D. C., Gros, P., & Lambris, J. D. (2016). Complement component C3 - The "Swiss Army Knife" of innate immunity and host defense. *Immunological reviews*, *274* (1). https://doi.org/10.1111/imr.12500

Ryu, A. H., Eckalbar, W. L., Kreimer, A., Yosef, N., & Ahituv, N. (2017). Use antibiotics in cell culture with caution: genome-wide identification of antibiotic-induced changes in gene expression and regulation. *Scientific reports*, *7* (1). https://doi.org/10.1038/s41598-017-07757-w

Semple, B., Frugier, T., Morganti-Kossmann, C. M. (2010). CCL2 modulates cytokine production in cultured mouse astrocytes. *Journal of neuroinflammation,* 7 (67). https://doi.org/10.1186/1742-2094-7-67

Shen, K., Reichelt, M., Kyauk, R. V., Friedman, B. A., Sheng, M. & Yuen, T.J. (2021). Multiple sclerosis risk gene Mertk is required for microglial activation and subsequent remyelination. *Cell reports,* 34 (10). https://doi.org/10.1016/j.celrep.2021.108835

Shi, X., Luo, L., Zhang, Z., Tang, Y. & Yang, G-Y. (2021). Abstract 64: Scar-Forming Glial Cells Engulf Synapse and Worsen Stroke Outcome Through Megf10 and Mertk. *Stroke,* 52. https://doi.org/10.1161/str.52.suppl_1.64

Singh, S., Anshita, D., & Ravichandiran, V. (2021). MCP-1: Function, regulation, and involvement in disease. *International immunopharmacology*, *101*. https://doi.org/10.1016/j.intimp.2021.107598

Stratoulias, V., Venero, J.L, Tremblay, M-E. & Bertrand, J. (2019) Microglial subtypes: diversity within the microglial community. *The embo journal,* 38. doi: 10.15252/embj.2019101997

Streit, W.J. (2005). Microglia and neuroprotection: implications for Alzheimer's disease. Brain Research Reviews, 48 (2). doi: 10.1016/j.brainresrev.2004.12.013

Uribe-Querol, E. & Rosales, C. (2021). Phagocytosis*. Encyclopedia of Infection and Immunity,* 1. https://doi.org/10.1016/B978-0-12-818731-9.00049-5

Viviani, B., Corsini, E., Galli, C.L. & Marinovich, M. (1998). Glia Increase Degeneration of Hippocampal Neurons through Release of Tumor Necrosis Factor-α. *Toxicology and Applied Pharmacology,* 150 (2). https://doi.org/10.1006/taap.1998.8406

von Asmuth, E.J., Dentener, M.A., Ceska, M. & Buurman, W.A. (1994). IL-6, IL-8 and TNF production by cytokine and lipopolysaccharide-stimulated human renal cortical epithelial cells in vitro. *Eur Cytokine Netw,* 5 (3). https://pubmed.ncbi.nlm.nih.gov/7524716/

Waddell, L. A., Lefevre, L., Bush, S. J., Raper, A., Young, R., Lisowski, Z. M., McCulloch, M., Muriuki, C., Sauter, K. A., Clark, E. L., Irvine, K. M., Pridans, C., Hope, J. C., & Hume, D. A. (2018). ADGRE1 (EMR1, F4/80) Is a Rapidly-Evolving Gene Expressed in Mammalian Monocyte-Macrophages. *Frontiers in immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02246

Westman, J., Grinstein, S. & Marques, P. E. (2020): Phagocytosis of Necrotic Debris at Sites of Injury and Inflammation. *Frontiers in immunology,* 10. https://doi.org/10.3389/fimmu.2019.03030

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International anesthesiology clinics*, *45* (2). https://doi.org/10.1097/AIA.0b013e318034194e

Zhang, W., Zhao, J., Wang, R., Jiang, M., Ye, Q., Smith, A. D., Chen, J., & Shi, Y. (2019). Macrophages reprogram after ischemic stroke and promote efferocytosis and inflammation resolution in the mouse brain. *CNS neuroscience & therapeutics*, *25* (12). https://doi.org/10.1111/cns.13256

Zhao, H., Liu, Y., Cheng, L., Zhang, W., Guo, Y-J. & Nie, L. (2013). Mesencephalic Astrocyte-Derived Neurotrophic Factor Inhibits Oxygen–Glucose Deprivation-Induced Cell Damage and Inflammation by Suppressing Endoplasmic Reticulum Stress in Rat Primary

Astrocytes. *Journal of Molecular Neuroscience,* 51. https://doi.org/10.1007/s12031-013-0042-4

Zhou, Z., Peng, X., Insolera, R., Fink, D.J. & Mata, M. (2009). IL-10 promotes neuronal survival following spinal cord injury. *Experimental Neurology*, 220 (1). doi: 10.1016/j.expneurol.2009.08.018.

Zizzo, G., Hilliard, B. A., Monestier, M., & Cohen, P. L. (2012). Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. *Journal of immunology*, *189* (7). https://doi.org/10.4049/jimmunol.1200662