Toll-like receptors on enteric neurons: relevance for Parkinson's disease

Carmen Desirée Rietdijk

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Toll-like receptors on enteric neurons: relevance for Parkinson's disease

Toll-like receptoren op enterische neuronen:
relevantie voor de ziekte van Parkinson
(met een samenvatting in het Nederlands)

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Copromotor:	Dr. A. D. Kraneveld

"… but his eyes gleamed like stars that shine the brighter as the night deepens."

- Lord of the Rings by J.R.R. Tolkien -

Voor Trix, Hennie en Wim. Mijn lieve grootouders.

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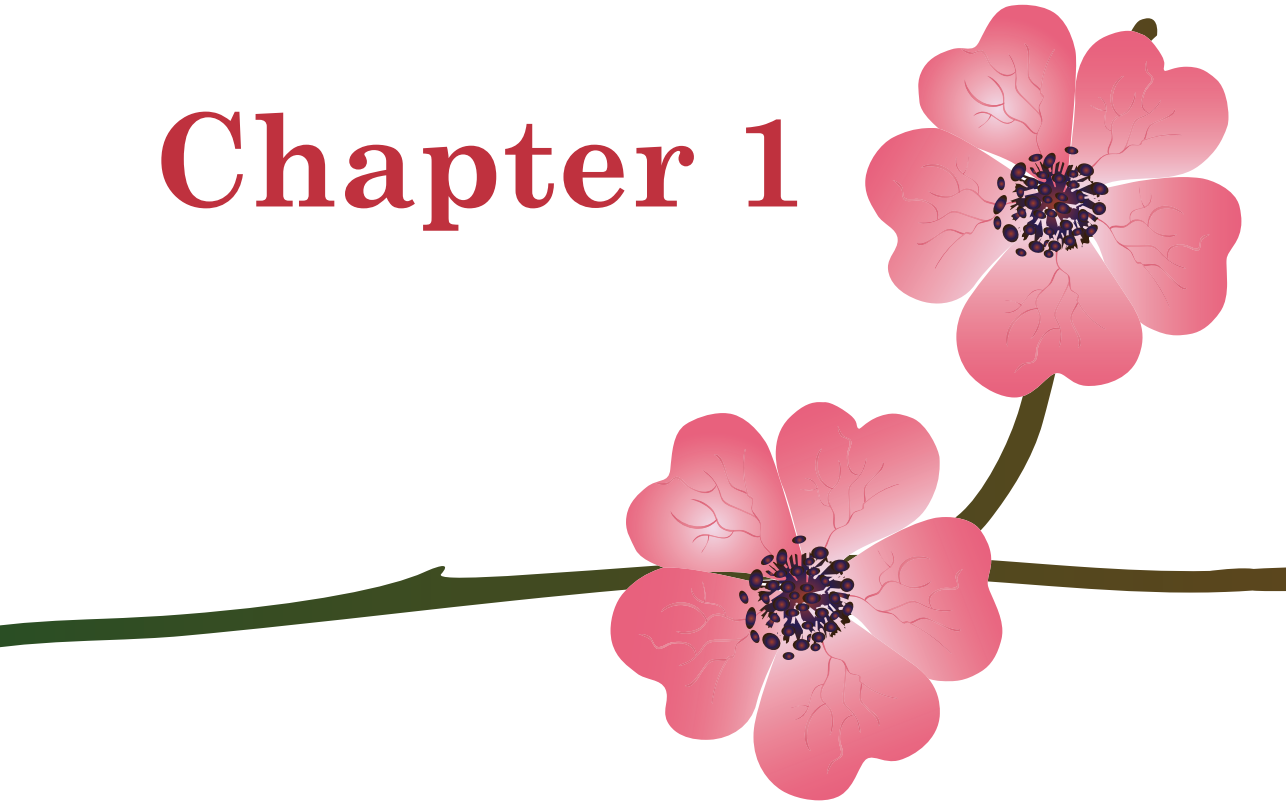
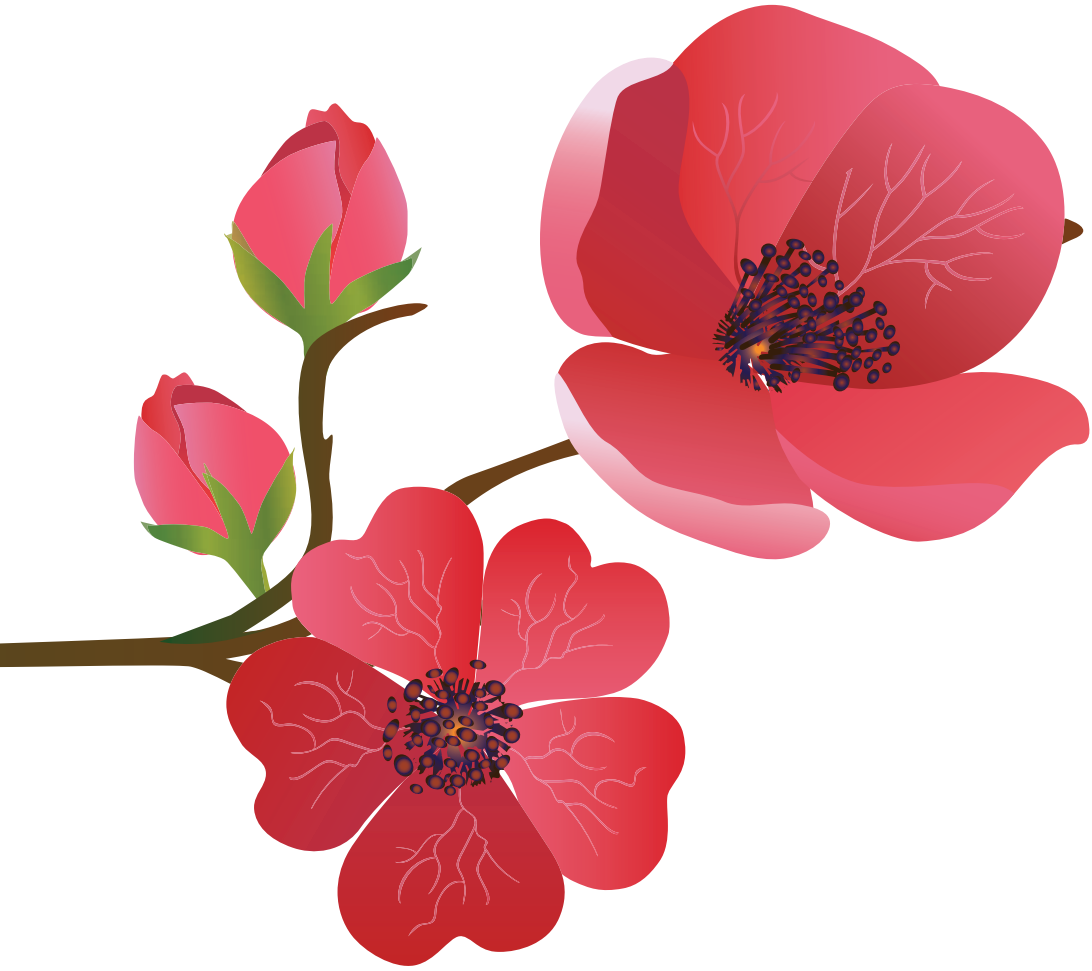
Chapter 1



None of us is who we were seven years ago.
None of us is who we will be seven years from now.
Except our brains.
Brains are forever.



Outline of the Thesis

We are all born with a large variety of cell types, and most cells will die and be replaced at some point in our lives. Some cell types have a short cycle of replacement, like the epithelial cells in our intestines, which are replaced on average every five days[1]. Other cell types have a none-existent cycle of replacement because they are never replaced, noticeably most (but not all!) of our neurons fall into this category[2]. Therefore it is very difficult to repair damage done to the nervous system, since there is no replacement system active to produce new cells.

Unfortunately, we are not able to effectively prevent damage to the nervous system, since there are many possible causes. The damage can for instance occur as physical trauma after an accident, as the result of a tumor growing in our brain, or as neuronal loss due to a neurodegenerative disease. Neurodegenerative diseases specifically damage the neurons in our nervous system, and occur more often in the elderly. Therefore, in a growing and ageing population neurodegenerative diseases receive significant attention, for instance through the EU Joint Programme – Neurodegenerative Disease Research. The prospect of increasing patient numbers offer a strong incentive to develop better treatments, or even cures.

One such neurodegenerative disease is Parkinson's disease. This disease affected about 1.5-5 per 1,000 people in the Netherlands in 2011, and rarely occurs before age 50, as reported by the Rijksinstituut voor Volksgezondheid en Milieu. The hallmark symptoms of Parkinson's disease are deficiencies in motor functioning due to dopaminergic neuronal cell loss in the substantia nigra in the brain[3]. Other symptoms are also known to ensue, some which have been found to precede the motor symptoms and diagnosis by years, like gastrointestinal problems[4,5]. This has led to the hypothesis that damage to neurons in the gastrointestinal tract occurs long before the substantia nigra are affected, and that this peripheral damage is caused by an invading pathogen[6–9]. This theory is known as the Braak's hypothesis.

The gastrointestinal and motor problems in the context of PD are potentially related to changes in the microbiome of patients, increased epithelial barrier permeability, bacterial translocation, and colonic inflammation[10–12]. These changes indicate a shift towards a pro-inflammatory milieu in the environment of the neurons of the gastrointestinal tract, so-called enteric neurons.

The enteric nervous system, often regarded as our 'second brain', consists of around 500 million neurons and is embedded in the gastrointestinal wall[13]. The majority of the neurons are found in the myenteric and submucosal plexuses, which include efferent-, afferent- and interneurons[13]. The enteric nervous system has sensory and motor functions, and is able to act autonomously to coordinate reflexes[13]. It senses chemical and mechanical changes, and regulates motility, the movement of water and electrolytes between the gut lumen and tissue fluid, and enzyme

secretion[13]. The major neurotransmitters and neuropeptides used by enteric neurons are acetylcholine, dopamine, glutamate, serotonin, vasoactive intestinal peptide, and substance P[14–17].
Additionally, the mucosal plexus extends to the lamina propria mucosae beneath/within the epithelial lining[18], and neurons in the gut express immune receptors, such as Toll-like receptors[19]. This allows the neurons to possibly sense bacteria and/or modulate immune functions, such as epithelial permeability, mucin secretion, immunoglobulin A secretion, and microbial composition[20]. Additionally, neurons are able to produce cytokines, as well as neurotransmitters and neuropeptides such as γ-aminobutyric acid, substance P, and vasoactive intestinal peptide. These substances can modify the functions of immune cells, while immune related signals can influence the functioning of the enteric nervous system[16,18,19,21]. Disruption of the balance between the immune system and the nervous system in the gut can lead to gastrointestinal deficits related to Parkinson's disease, and plays an important role in the Braak's hypothesis.
To confirm or deny the Braak's hypothesis, one aspect to study is the immune signaling of enteric neurons in the context of Parkinson's disease. In this thesis a cell line of enteric neurons has been used as a model for the neurons in the gastrointestinal tract[22]. The expression and function of a specific group of innate immune receptors, Toll-like receptors, which are important for the recognition of microbes such as bacteria[23,24], was studied on the cells of this cell line.

Overview

In **chapter 2** the current scientific literature on Toll-like receptors on neurons in the context of stroke, Alzheimer's disease and Parkinson's disease is presented. The distinct importance of Toll-like receptor-2 and -4 in Parkinson's disease is apparent, as well as the need to obtain more knowledge about all Toll-like receptors in Parkinson's disease.

In **chapter 3** a review of the current scientific literature on the Braak's hypothesis is presented. The support for the hypothesis is weighed against the criticism, and it is concluded that the Braak's hypothesis is most likely relevant for a large subset of patients suffering from Parkinson's disease.

In **chapter 4** the technical aspects of culturing the enteric neuronal cell line are described. An effort is made to identify predictors for the unstable growth rate of the cells. Such predictors were not found, but the results were still useful to guide future research into these cell culturing techniques towards a better understanding of the growth rate.

In **chapter 5** the expression of Toll-like receptors on the enteric neuronal cell line, and on primary enteric neurons is described. Expression of Toll-like receptors-2, -3, -4 and -7 by the enteric neuronal cell line was observed, as well as expression of Toll-like receptors-2 and -4 on primary enteric neurons.

In **chapter 6** the function of Toll-like receptors on the enteric neuronal cell line in the context of Parkinson's disease is presented. Distinct immune-related signaling was observed in response to the Parkinson's disease-related protein α-Synuclein.

In **chapter 7** a summary and general discussion of the obtained results presented in this thesis is given.

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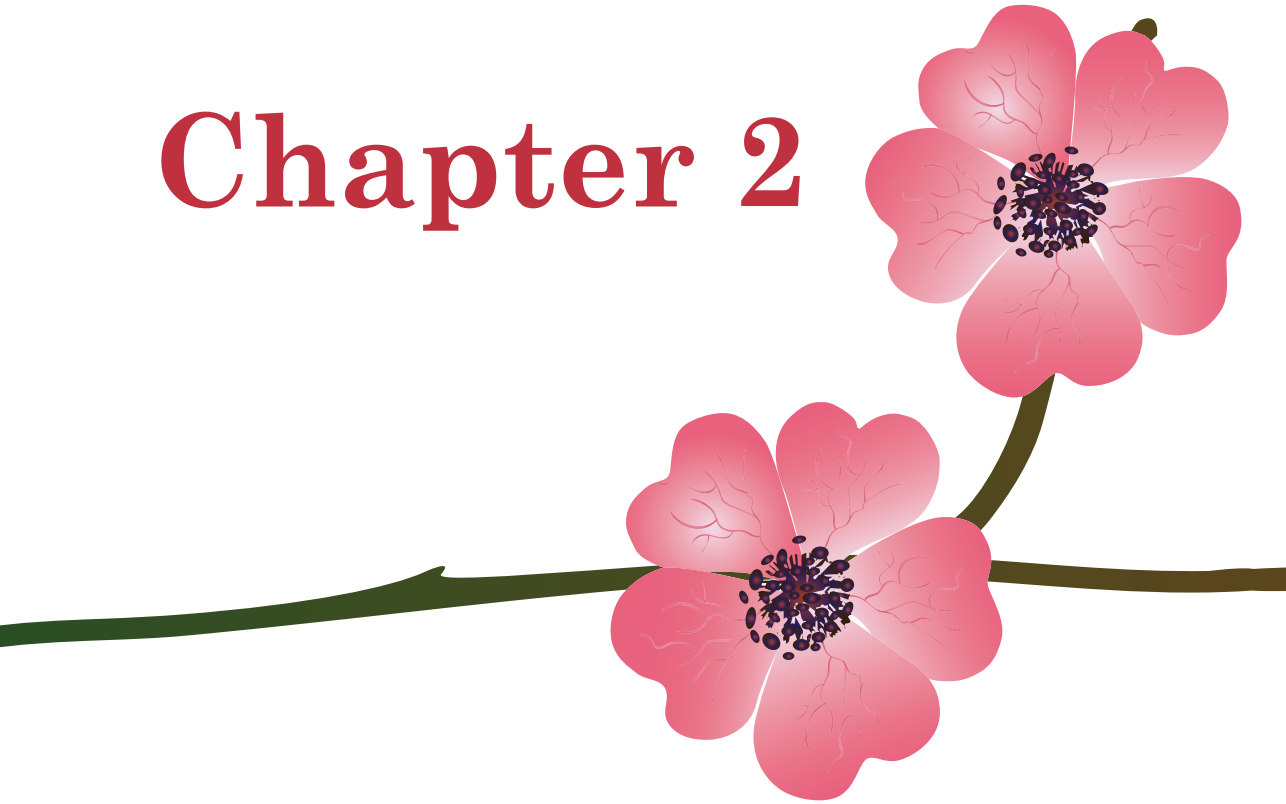
Chapter 2



Carmen D. Rietdijk[1], Richard J.A. van Wezel[1,2,3], Johan Garssen[1,4], Aletta D. Kraneveld[1]

1. Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, 3584 CG Utrecht, the Netherlands.
2. Department of Biomedical Signals and Systems, MIRA, University of Twente, 7522 NB Enschede, the Netherlands.
3. Department of Biophysics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, 6500 GL Nijmegen, the Netherlands.
4. Nutricia Research, Utrecht Science Park, 3584 CT Utrecht, the Netherlands.

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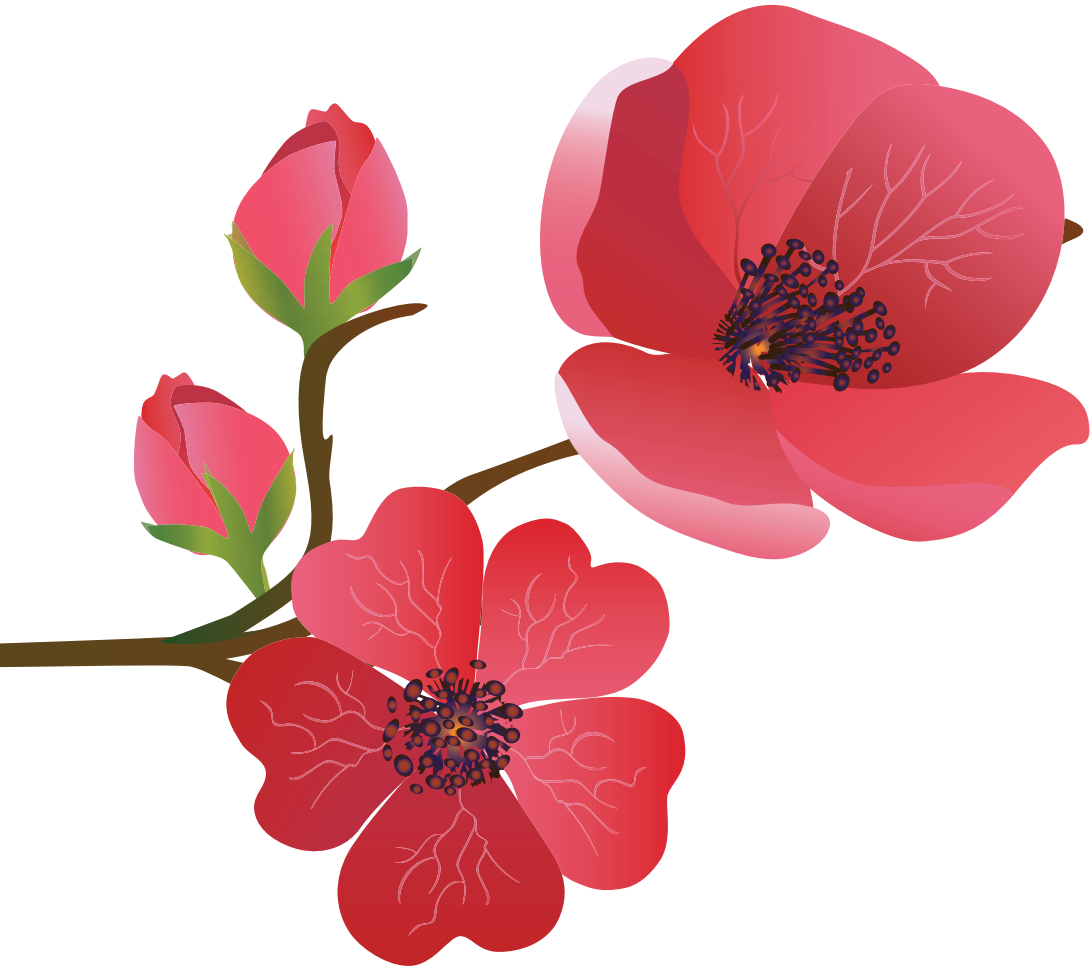

Neuronal toll-like receptors and neuro-immunity in Parkinson's disease, Alzheimer's disease and stroke

Abstract

Toll-like receptors (TLRs) are part of the innate immune system and can initiate an immune response upon exposure to harmful microorganisms. Neuronal TLRs are considered to be part of an established framework of interactions between the immune system and the nervous system, the major sensing systems in mammals. TLRs in the nervous system and neuronal TLRs are suspected to be important during inflammation and neurodegenerative diseases. The aim of this review is to offer an overview of the current knowledge about TLRs in neurodegenerative pathologies, with a focus on Parkinson's disease (PD). More research focusing on the role of TLRs in health and disease of the nervous system is needed and remains to be explored.

Abbreviations

TLR – Toll-like receptor
PD – Parkinson's disease
LPS – Lipopolysaccharide
MyD88 – Myeloid differentiation primary response gene 88
NF-κB - nuclear factor κ-light-chain-enhancer of activated B cells
IFN – Interferon
PNS – Peripheral nervous system
CNS – Central nervous system
JNK – Jun-N-terminal kinase
AD – Alzheimer's disease
MSA – Multiple systems atrophy
WT – Wild type
HNE – Hydroxynonenal
I/R – Ischemia/reperfusion
NPC – Neural progenitor cell
HIV – human immunodeficiency virus
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Literature search

Publications were first selected concerning Toll-like receptors on neurons, and on TLRs for which functional information in neurons was available. Publications were also selected for their focus on neurodegenerative diseases. The last literature search was performed on April 14th 2015.

Introduction

This review aims to offer an overview of the current knowledge about Toll-like receptors (TLRs) in the nervous system and to show the relevance of these receptors in neurodegenerative pathologies, with a focus on Parkinson's disease (PD).

TLRs are the mammalian orthologue of Drosophila Melanogaster's Toll receptor discovered in 1988.[1] TLRs are part of the innate immune system and belong to the pattern recognition receptors (fig. 1). They can recognize both small molecular motifs conserved across microbes (pathogen-associated molecular pattern) (fig. 2), and endogenous molecules generated during inflammation or tissue damage (damage associated molecular pattern).[2–5] TLRs can initiate an acute inflammatory reaction and subsequently can coordinate the activation of the adaptive immune system. To date, thirteen TLRs are known, of which ten (TLR1-10) have been described in humans.[6] The cell surface TLRs recognize pathogen associated molecular patterns that are mainly constituent of the bacterial cell wall or are expressed on the bacterial cell surface, such as lipopeptides and peptidoglycal (TLR1/TLR2, TLR2/TLR6, TLR2/TLR10), lipopolysaccharide (LPS) (TLR4) and flagellin (TLR5). In contrast, the intracellular TLRs mainly recognize microbial nucleic acid including viral double-strand RNAs (TLR3), single-strand RNAs (TLR7 and TLR8) and CpG ODN (TLR9).[7] TLRs can employ two second messenger pathways; the myeloid differentiation primary response gene 88 (MyD88) pathway, activating nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), or the TIR-domain-containing adapter-inducing interferon-β pathway, activating interferon regulatory factor 3 (fig. 2). NF-κB controls DNA transcription resulting in the production of pro-inflammatory cytokines such as tumor necrosis factor α, interleukin 1β and interleukin 6.[7,8] Interferon regulatory factor 3 is an interferon (IFN) regulatory factor leading to the production of antiviral type I IFN.[2,7]

The presence of TLRs on immune cells and epithelial cells is well known, but their expression is not restricted to these cell types. Glial cells and neurons express TLRs in both the peripheral nervous system (PNS) and the central nervous system (CNS) (fig. 3), allowing neurons to act as immune cells.[9–15] More specifically, in the CNS neurons, astrocytes and microglial cells express TLR1-9, whereas oligodendrocytes express only TLR2 and TLR3.[16–20] Peripheral neurons also express TLR1-9 and enteric glial cell express TLR1-5, TLR7 and TLR9.[13,14,21–23] Neuronal TLR signaling pathways do not necessarily employ NF-κB [24–26] and may involve the glycogen synthase kinase 3β, jun-N-terminal kinase (JNK) and phosphatidylinositol 3-kinase/protein kinase B pathways.[27–29] Interaction between neurons and the immune system has already been reported, setting the scene for neurons acting as immune cells.[30–34] It has been reported that neuronal

TLRs are involved in the development and homeostasis of the nervous system, and notably in several neurodegenerative diseases.[35,36] Both TLR2 and TLR4 are involved in neuronal apoptosis, development and survival in the context of opioids exposure, ischemia/stroke, viral infections and Alzheimer's disease (AD),[28,29,37–43] via glycogen synthase kinase 3β and JNK.[28,29] TLR3 and TLR8 negatively regulate neuronal development and axonal growth and are involved in fighting viral infections,[9,16,24,25,27,44–46] through the phosphatidylinositol 3-kinase pathway.[27] Other information also indicates a role for TLRs in the nervous system during disease. TLR1-5, TLR8 and TLR9 are overexpressed in PD and multiple systems atrophy (MSA) patients and in animal models of PD, AD, MSA and amyotrophic lateral sclerosis.[47–53] Of these receptors, TLR2 and TLR4 are of special interest in PD, as will become clear in the sections dedicated to these TLRs. The current knowledge about TLR2 and TLR4 in PD has focused heavily on microglia, and not so much on neuronal TLRs. To better understand the potential importance of neuronal TLRs in neurodegeneration and specifically in PD, AD and stroke the current knowledge about the function of neuronal TLRs in neuronal development and neurodegenerative diseases will be discussed here.

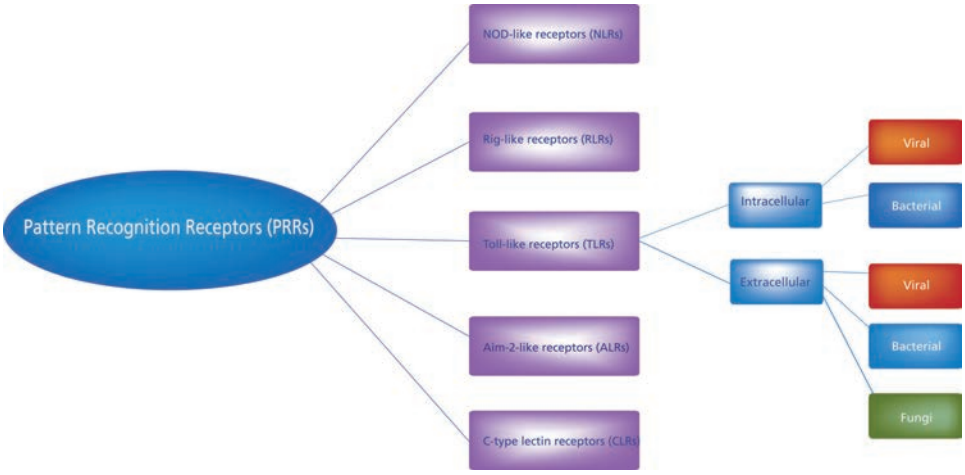

Figure 1. TLRs are part of the innate immune system and belong to the pattern recognition receptors.

Nervous system TLR expression

This section will cover the expression of TLRs on neurons and glial cells (fig. 3). To discuss the function of TLRs on neurons it is important to know whether neurons express TLRs. For the convenience of the reader, the information about expression has been organized based on species, location in the nervous system,

level of expression, and type of neurons. To discuss the function of TLRs in the nervous system it is also necessary to first address whether glial cells express TLRs, because of their biological relevance for the functioning of the CNS and PNS.

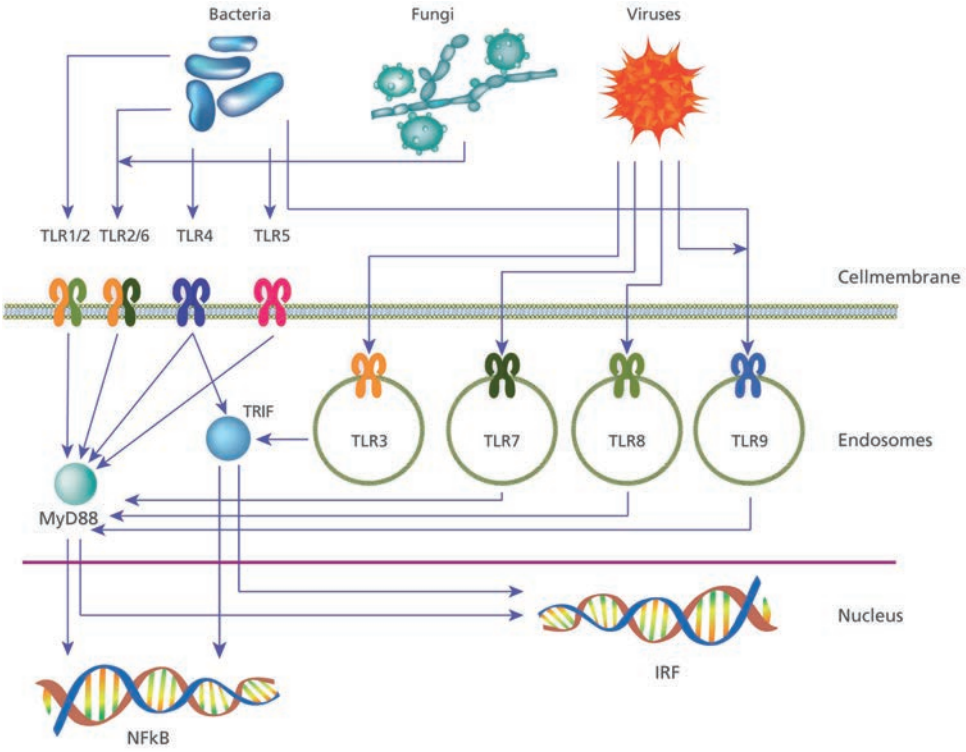

Figure 2. Different pathogens activate different Toll like receptors (TLRs). TLRs signal through two different pathways using myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon β (TRIF), leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and interferon regulating factor (IRF) respectively NF-kB leads to DNA transcription and cytokine production, while IRF leads to interferon production.

Neuronal TLR expression

TLR expression on primary neurons has been found in several species, amongst which are humans,[10,12,13,15,16,45] mice,[13,14,26,28,43,54,55] and rats.[10,27,56,57] Neuronal TLRs are present in both parts of the nervous system; the CNS[12,26,28] and the PNS.[10,13,14,43,54,57] Expression of TLRs by neurons has been confirmed at mRNA level,[14,21,24,28,43] and protein level.[10,13,24,26,28,43,54,57,58] Most results on neuronal TLRs come from experiments in embryonic neurons because these are easier to culture than neurons from adult animals. Nonetheless, neurons

express TLRs throughout life, starting at the embryonic stage, [14,24,26,28,43,58] followed by the postnatal,[54] and the young stages,[10,56] and finally in the adult stage of life.[10,12,13,21,58,55,57] Taken together, these data offer many opportunities to study neuronal TLRs for a wide range of research questions, since it is reported that TLRs are expressed by multiple species, multiple types of neurons, and throughout the lifespan of animals.

TLR expression has also been found in several neuronal cell lines. The teratocarcinoma derived human post-mitotic dopaminergic neuronal cell line NT2-N expresses TLR3.[9,59–61] TLR3 is also expressed by human neuroblastoma cell lines (including noradrenergic cell lines, CHP-212 and SK-NSH; a noradrenergic and gamma-aminobutyric cell line, SH-SY5Y; SH-SY5Y and a noradrenergic and dopaminergic cell line, BE(2)-C) and primary human neuroblastic cells.[27,44,45,59] The rat neuroblastoma cell line B103 expresses TLR2.[38] The expression of TLR1-9 has been reported in the human CHP-212 neuroblastoma cell line.[16] Although cell lines are further removed from their original biological environment than primary cells, they do offer a more readily available source of material for further research towards understanding the functions of neuronal TLRs.

Glial TLR expression

The importance of glial TLRs in neurodegeneration is indisputable.[19,32,53,62–65] Glial cells come in many shapes and sizes, and have a critical role in the CNS and PNS, such as immune surveillance, the regulation of chemical environment and the production of the myelin sheath, which makes them biologically relevant to the functioning of neurons.

Glial cells are a heterogeneous group of cells, all of them expressing TLRs. Microglia act as immune surveillance of the CNS and express TLR1-9 in both humans and mice.[19,20] Astrocytes regulate the chemical environment of neurons, and they express TLR1-9 in mice and TLR1, TLR3-5 and TLR9 in humans, while TLR2 and TLR6-8 were not detected in human astrocytes.[17,18] Oligodendrocytes and Schwann cells respectively produce the myelin sheath around neuronal axons in the CNS and PNS. Schwann cells express TLR1-9 in mice,[14,66] while only TLR2 has been studied and detected in humans,[67] whereas TLR4 has been studied and detected in rats.[68,69] Human oligodendrocytes only express TLR2 and TLR3, while TLR1 and TLR4-9 were undetectable.[20] In glial cells of the enteric nervous system TLR2 -9 have been found, while TLR1 is absent.[13,22,70]

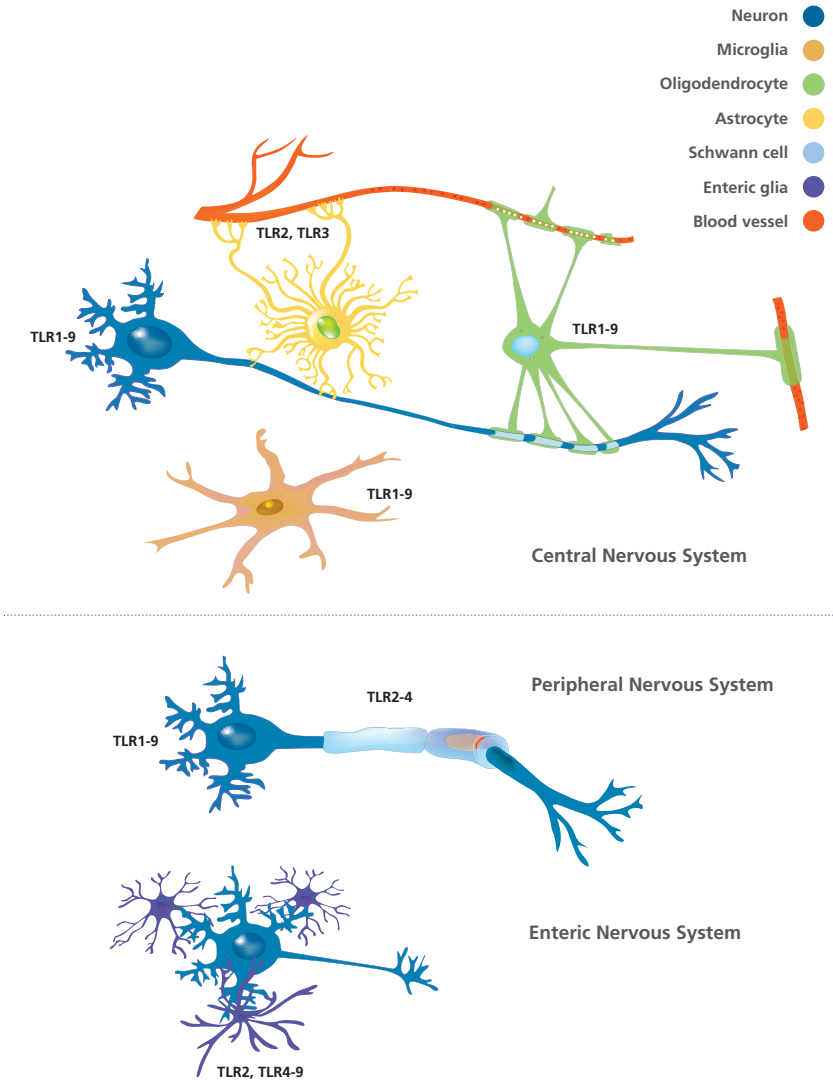

Figure 3. Toll-like receptors (TLRs) are differentially expressed by neurons and glial cells of the central, peripheral and enteric nervous system.

Neuronal TLR Functionality

Here we provide a comprehensive overview of the current knowledge about TLR functionality in the nervous system in relation to neurodegeneration. Specific information about neurons, microglia, and other cell types is included when appropriate.

TLR2

TLR2 is a TLR family member that is able to recognize bacterial lipopolypeptides and peptidoglycans. TLR2 forms heterodimers with TLR1 and TLR6 and mediates the host response to Gram-positive bacteria and yeast infections via stimulation of NF-κB signaling pathway.[71] Recently, it has been demonstrated that TLR2 can also form a heterodimer with TLR10 acting as an inhibitory receptor with immune suppressing effects.[72]

Parkinson's disease

Clinical studies have shown that TLR2 expression is increased in PD. In particular, one study revealed specific increase in microglial TLR2 in the substantia nigra and the hippocampus in the early stages of the disease, but not during the late stages,[73] while another study showed an increase in TLR2 in the striatum of advanced PD patients.[74] These results indicate that expression of TLR2 in either early or advanced PD could be region-specific, and that TLR2 is not necessarily expressed in all regions at the same time. The involvement of TLR2 in PD might be two-dimensional: microglial activation of TLR2 can induce neurotoxicity or TLR2 can be important for the clearance of α-synuclein, thus being neuroprotective.

In support of this function is the evidence that TLR2 polymorphism tends to be associated with an increased risk of PD. This polymorphism results in altered TLR2 promoter transcriptional activity leading to lower expression of TLR2.[75,76] Taken together, these findings are indirect indications of a possible role of TLR2 in the pathology of PD.

Overexpression of human α-synuclein in mice resulted in microglial activation and an increase in TLR2 expression.[74] Microglia seem to form a crucial link between TLR2 and PD pathology; an idea that is supported by results from cell culture studies: exposure to α-synuclein activates cultured microglia and increases their TLR2 expression;[49,77] it also changes the response of microglial cells to TLR1/2 stimulation by increasing their inflammatory response.[78]

It can be speculated that α-synuclein triggers neuroinflammation through microglial TLR2, initiating a positive feedback loop by increasing TLR2 expression on the microglia, resulting in neurodegeneration and disease progression. However, it is not yet clear why this process would be limited to the early disease stage in the substantia nigra and the hippocampus, while only occurring later in the striatum, or whether and how neuronal TLR2 participates in this process.

Alzheimer's disease

TLR2 expression was found on microglia surrounding amyloid β (Aβ) plaques in post-mortem brain sections and in an AD mouse model, raising the question whether and how TLR2 might be involved in AD pathology.[47,79] Injecting Aβ in the hippocampus of wild type (WT) mice increases TLR2 expression in microglia; Aβ protein was unable to induce a microglia-dependent inflammatory response in the cortex of TLR2 deficient mice.[80,81] The interaction between Aβ and TLR2 also affects behavior; TLR2 deficient mice showed more pronounced cognitive impairments, which correlated with increased levels of Aβ protein.[81] It remains to be demonstrated whether there is a direct binding between TLR2 and the Aβ protein.

AD-related damage to neurons is at least partially dependent on microglial TLR2, since the effects of Aβ protein and TLR1/2 ligand on neuronal viability are additive and dependent on microglia, and the microglial inflammatory and phagocytic response to Aβ protein is TLR2-dependent.[80,82,83] Aβ protein- and TLR1/2 ligand-induced microglial-mediated neuronal death is likely conferred through the release of inflammatory mediators.[84] There is also indication for the involvement of neuronal TLR2 in AD. Neuronal TLR2 was upregulated when neuronal cultures were exposed to hydroxynonenal (HNE), an AD-related lipid peroxidation product, but not when exposed to Aβ protein.[40] HNE exposure also resulted in an increase in both phosphorylated JNK and cleaved caspase 3; however these effects were abolished by TLR4 knock-out. Therefore, the functional consequence of TLR2 upregulation in neurons by HNE is not yet known.

In summary, microglial TLR2 is key in the neuroinflammatory response of AD pathology, but is also responsible for the clearance of Aβ protein, while neuronal TLR2 might also play a part in this neuroinflammatory environment. So TLR2 can have either a beneficial or detrimental role in AD.

Stroke

Neuronal TLR2 was studied in the cerebral ischemia/reperfusion (I/R) animal model of stroke. Cortical and hippocampal neurons of WT mice subjected to I/R injury showed transient TLR2 protein upregulation, although upregulation in the cortex may not have been exclusively neuronal.[29,37,39] TLR2-/- mice exposed to I/R showed less brain damage, smaller infarct volumes and less neurological deficits than WT mice, and TLR2-/- mice and mice treated with TLR2 antibody showed less inflammatory cell accumulation and reduced neuronal loss.[29,39,42] From a treatment perspective it is interesting that the anti-inflammatory agent baicalin, used for the treatment of stroke, reduced TLR2 expression in hippocampal neurons after I/R injury.[37] However, before suggesting a potential role of TLR2

in the treatment of stroke, clinical studies are needed to determine whether TLR2 is viable as a marker or target for treatment in stroke.
Animal models show that TLR2 is relevant in relation to stroke, however, they might obscure the specific importance of neuronal TLR2 in the context of glial cells. In order to isolate and study neuronal TLR2 in a stroke model, cultured neurons were exposed to glucose deprivation, a model of stroke.[29] Increased cell death was found in WT neurons, while TLR2-/- neurons were resistant to glucose deprivation induced cell death. In a neuronal cell line oxygen-glucose deprivation resulted in TLR2 upregulation and in an increase of non-apoptotic cell death.[85] These in vitro data confirm that stroke can result in neurodegeneration through the activation of neuronal TLR2, making neuronal TLR2 a potential player in brain damage after I/R injury in mice, independent from the influence of glial TLR2.
Information on TLR2 in the brain of patients with stroke is sorely missing and should be sought in future research, starting with investigating expression patterns in different brain regions. Also, a major focus of research should aim at distinguishing neuronal TLR2 from glial TLR2.

TLR3

TLR3 recognizes double stranded RNA associated with viral infection and host RNA. Ligand binding induces the production of anti-viral mediators like the type I IFNs, such as IFNα and –β production by leukocytes. These IFNs stimulate macrophages and natural killer cells to elicit an anti-viral response.[86]
TLR3 has not been studied in direct relationship to neurodegenerative diseases, but work has been performed on the effect of TLR3 in the development of the nervous system. TLR3 expression decreases in the embryonic CNS during neurogenesis.[58] Intrathecal injection of TLR3 agonist polyinosine: polycytidylic acid in postnatal day 4 mice resulted in sensory-motor deficits, neuroanatomical defects and fewer axons in the spinal cord, which was associated with neurodegeneration.[24] The role for TLR3 in this study was demonstrated by the fact that no anatomical or behavioral problems were found in TLR3-/- mice treated with polyinosine: polycytidylic acid.[24] It seems that TLR3 is involved in the proper development of the CNS in early fetal life, because the receptor is differentially expressed at different embryonic stages. After birth, stimulation of TLR3 results in neurodegeneration.
The decrease in expression of TLR3 during neurogenesis, as found in the embryonic brain, is also found in cultured neural progenitor cells (NPCs), making NPCs more sensitive to TLR3-mediated inhibition of proliferation than mature neurons.[58] Despite this reported decrease in TLR3 expression during neurogenesis, neurons do express functional TLR3.[24] In primary neurons, TLR3 stimulation inhibits neurite outgrowth and causes irreversible growth cone collapse, without affecting

cell survival.[24] Different results were found in the high TLR3-expressing neuroblastoma cell line SK-N-AS, where exposure to a TLR3 ligand resulted in growth inhibition and apoptosis.[44] The difference in results on cell viability could be due to the use of different cell types (dorsal root ganglia,[24] NPCs,[58] and cell lines[44]), thus revealing the limits of cell culture as a model of biological processes. Although all in vivo data are obtained from early life studies and interpretation of these data in the context of neurodegeneration must be done carefully, extrapolating these results leads to the hypothesis that stimulation of neuronal TLR3 could be detrimental in neurodegenerative diseases, especially in the context of viral infections. TLR3 is a viral sensing innate immune receptor. It is known that viral infections like influenza can cause neurodegeneration[87] and that viruses are linked to neurodegenerative diseases:[88] specifically hepatitis C virus, Eppstein-Barr virus and human immunodeficiency virus (HIV) have been associated with PD.[89–91] The involvement of neuronal TLRs during viral infections is discussed in more detail in a later part of this review.

TLR4

TLR4 detects LPS derived from Gram-negative bacteria and host-derived signaling molecules such as heat shock proteins, and extracellular matrix proteins, after which the innate immune system is activated, leading to an inflammatory response.[92–94]

Parkinson's disease

The expression of TLR4 is increased in PD and MSA post-mortem brain tissue, suggesting clinical relevance to TLR4 in PD and neurodegeneration in general.[50,74] Animal experiments have been used to further elucidate the role of TLR4 in PD. TLR4-/- mice were more vulnerable to dopaminergic neuronal loss and motor problems induced by α-synuclein overexpression, but less vulnerable to the induction of PD symptoms by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment.[95,96] Furthermore, TLR4 and α-synuclein are both necessary for LPS-induced neurodegeneration in mice.[97,98] Therefore, mouse models support the importance of TLR4 in PD, but make no suggestion as to the impact of TLR4 on disease development, since TLR4 was both protective and harmful in these models. Since TLR4 is suggested to be protective in the context of α-synuclein overexpression, TLR4 seems likely to be protective in the context of PD, since α-synuclein misfolding and aggregation is a hallmark of the disease. The harmful contribution of TLR4 to MPTP-induced PD seems to suggest that TLR4 might have a negative impact on the health of a subgroup of PD patients who suffer from toxin-induced PD (for instance after exposure to MPTP or rotenone). Therefore, TLR4 appears mostly protective in the context of PD, but it might be harmful in the context of toxin-induced PD.

Similar to TLR2, microglia form a link between TLR4 and PD. Microglia are necessary for LPS-induced degeneration of rat cortical neurons in cell culture since these neurons themselves do not express TLR4, and nitric oxide and superoxide seem to be at least partially responsible for microglial-induced neurodegeneration.[97,98] On the other hand, microglial TLR4 is necessary for the neuroprotective endocytosis of α-synuclein.[96] Microglia activated with α-synuclein downregulate TLR4, disabling any neuroinflammatory positive feedback loop, but also reducing the ability of the microglia to take up α-synuclein from their environment.[77] These results are contradictive and whether TLR4 is protective or injurious in PD is still a matter of debate. The balance between the contribution of microglial TLR4 to neuroinflammation and the endocytosis of α-synuclein might eventually determine whether this receptor is protective or harmful to the surrounding neurons.
TLR4 is a promising target for future PD research. The role of TLR4 during PD can be both beneficial and harmful, and the factors determining the outcome need to be investigated in more detail. It seems that neuronal TLR4 could be protective, but the role of microglial TLR4 is still not fully understood. Resolving this debate could potentially lead to approaches aimed at turning harmful TLR4 responses into protective ones.

Alzheimer's disease
One of the first lines of evidence suggesting that TLR4 might be involved in AD pathology comes from two studies where a TLR4 polymorphism was found to be protective against late onset Alzheimer's disease in the Italian population and glial cells surrounding Aβ plaques showed increased TLR4 expression in post mortem brain tissue.[99,100] In a genetic AD mouse model TLR4 knock-out reduced the expression of tumor necrosis factor α and chemokine (C-C motif) ligand 4 (also known as macrophage inflammatory protein-1β) in cortex homogenate, while increasing the amount of activated microglia, activated astrocytes, and Aβ protein in the brain.[101,102] Effects of TLR4 knock-out on behavior or disease progression have not been reported. The increase of Aβ protein in the brain of TLR4-/- animals could be due to a lack of TLR4-mediated Aβ clearance, potentially by microglia.[102,103] Cell culture data support a pro-inflammatory role for microglia, and implicate microglia-induced inflammation in neuronal degeneration. Mouse microglia initiate an inflammatory and phagocytic response to aggregated Aβ through TLR4, resulting in microglia-mediated neuronal death.[82,99] Microglia need TLR4 to initiate LPS-stimulated Aβ uptake, in fact they trigger a stronger inflammatory response to Aβ in combination with LPS.[84,102]
Neurons themselves respond to Aβ and AD-related peroxidation product HNE through TLR4, resulting in apoptosis.[40] Since little is known about the role of

neuronal TLR4 in AD, it is important to start exploring the function of this receptor in animal models and in patient tissue, in a way that differentiates glial-mediated TLR4 responses from neuronal responses, for instance by selective knock-down of TLR4 in neurons in AD mouse models.
Collectively, it seems that TLR4 induces an immune response in AD through pro-inflammatory cytokines, aimed at the removal of Aβ by microglial uptake, but also phagocytosis of neurons by microglia. Insufficient removal of Aβ results in an increase of Aβ in the extracellular space, the subsequent activation of microglia and astrocytes, and neuronal apoptosis. In light of this hypothesis, it is also interesting to note the similarity in the role of microglial TLR4 in AD and PD, where this receptor is responsible for the uptake of disease-specific aggregated protein and initiation of neuroinflammation, possibly causing neuronal death.

Stroke
TLR4 has both beneficial and detrimental effects in stroke models. Neurons of I/R treated mice show TLR4 upregulation, a first clue that TLR4 is involved in stroke-induced brain damage.[29,37] Paradoxically, mice treated with low dose systemic LPS two days before I/R injury had smaller infarct sizes and less neuroinflammation in the brain, while TLR4-/- mice had less stroke-induced brain damage and less neurological deficits after I/R treatment.[29,104] Although these results do not suggest a beneficial or harmful role of TLR4 in stroke, the data are not mutually exclusive. TLR4 stimulation before stroke seems to be protective, while TLR4 stimulation during stroke seems detrimental. This hypothesis is supported by in vitro experiments. Increased TLR4 activity does not increase neuronal death, and TLR4 stimulation can be beneficial to neuronal survival at low concentrations.[43,105,54,106] On the other hand, glucose deprivation increased TLR4 expression and cell death in neuronal cultures while TLR4-/- neurons were less susceptible to glucose deprivation induced cell death.[29,37] In agreement with the in vivo results, it seems that TLR4 stimulation per se is not harmful to neurons and it might even be beneficial. During stroke TLR4 has a negative impact on neuronal survival, possibly in part through glucose deprivation, but most likely also as the result of a more profound inflammatory process.[107] In relation to the opposing effects of TLR4 in PD, it is curious that TLR4 can have either protective or detrimental effects in the context of stroke.

TLR8

In innate immune cells TLR8 functions as an endosomal receptor that recognizes viral single-stranded RNA. Stimulation of TLR8 induces the activation of the My88 signaling pathway leading to an anti-viral response.[108]

The role of TLR8 in PD and AD has not yet been studied in detail, and is therefore unknown. In stroke patients a higher level of TLR8 mRNA expression in whole blood sample was positively correlated with poor patient outcome after three months, larger infarct volume and greater inflammatory response.[109] Similar results were found in a mouse stroke model: diseased animals had increased TLR8 mRNA expression in their brain and systemic administration of TLR8 before ischemic insult increased infarct size and neurological problems.[110] The neuronal damage in stroke patients and mice could be mediated by neuronal TLR8, since TLR8 stimulation of neurons results in fewer and shorter neurites and apoptosis and slightly but significantly increases oxygen-glucose deprivation induced cell death, while TLR8 silencing reduced oxygen-glucose deprivation induced cell death.[26,110] Interestingly, neurodevelopmental research has shown that TLR8 is differentially expressed in the embryonic and postnatal brain in mice.[25,26] In the mouse brain TLR8 expression increases between embryonic day 12 and postnatal day 1, and decreases between postnatal day 7 and adulthood.[26] During early embryonic development TLR8 expression is high in postmitotic migrating cells, but not in the periventricular proliferative area.[26] During late embryonic development, TLR8 was restricted to axonal tracts (including the olfactory nerve fiber layer, cortical intermediate zone, internal capsule, anterior commissure, fimbria of hippocampus and optic chiasm).[26] Postnatal expression is diffuse throughout the brain and located in soma.[26] This indicates a potential role of TLR8 in brain development. Considering how little is known about its function in the nervous system, specifically on neurons, both the fields of neurodevelopment and neurodegeneration have much to explore with regards to TLR8. This TLR could be considered critical to study the role of TLRs on neurons in neurodegeneration, and PD in particular.

Neuronal TLRs and viral infections

In the previous sections we have discussed that TLRs play an important role in microglial-mediated neuroinflammation of neurodegenerative diseases. In this context it is extremely relevant to investigate further the TLR-induced immune-like functions of neurons and to understand the role of neurons in neuroinflammation. Viral infections offer useful conditions to study TLR-mediated neuronal immune functions, because neurons respond to viral infections by upregulating TLR and secreting IFN.

Neurons upregulate TLR3 and TLR4 mRNA in response to HIV and adenovirus infection, they also upregulate IFN-β mRNA in response to Sendai virus, and increase IFN production after TLR3 and TLR8 stimulation.[16,27,45,111] TLR3 and TLR8 are virus-sensing receptors and virus-infected cells use IFNs to signals

to the neighboring cells that an infection is ongoing and to induce an immune response from nearby immune cells (or glial cells). Altogether, such findings suggest that neurons can act as immune cells. These neuronal TLR-mediated immune responses seem to be protective to the neurons themselves. Stimulation of TLR3 on neuronal cell lines inhibits HIV replication through IFN-λ, and TLR3 and TLR8 stimulation of a neuronal cell line results in lower susceptibility to herpes simplex virus-1 potentially through IFN-α. Moreover, TLR3-/- primary neurons showed increased infection when exposed to West Nile virus compared to WT neurons which was not due to changes in IFN-α of IFN-β production.[16,45,46] The protective effect of TLR3 during viral infection of the CNS has been confirmed in mice: infection of TLR3-/- mice with West Nile virus resulted in a higher viral burden in neurons and increased mortality compared to WT mice.[46] The viral-sensing receptors TLR3 and TLR8 are able to initiate a protective immune-like response in neurons upon viral challenge. Unfortunately, it is not clear whether TLR2 or TLR4 have a similar potential to protect neurons against pathogenic (bacterial) attack, and what the resulting immune response would be. Therefore, it is interesting to investigate a wide range of possible immune-like responses in neuronal cultures exposed to endogenous and exogenous TLR2 and TLR4 stimuli.

Conclusion

This review summarizes the relevance of TLRs in the nervous system, and especially in neurodegenerative pathologies. Current literature shows that several neuronal TLRs are involved in the development of the nervous system and in neurodegenerative diseases. Neuronal TLRs are important for NPC proliferation, axonal growth, cell survival and in defense against viral infections. The capacity of TLR-stimulated neurons to respond as immune-like cells (production of cytokines and induction of apoptosis) is of special interest for neurodegenerative diseases, since microglial-mediated neuroinflammation is a unique feature of neurodegenerative diseases. These results raise the question whether neurons are active contributing to neuroinflammatory degenerative process such as PD. TLR2, TLR3, TLR4 and TLR8 are all important for neuronal function and are implicated in PD, AD and stroke. This suggests that these TLRs should be investigated further in PD and other diseases as the first innate immune receptors on neurons.

Neuronal TLR2 has divergent functions in the nervous system. It responds to tissue damage during stroke, and allows neurons to respond to the neuroinflammatory environment of AD pathology. It is interesting to analyze the involvement of neuronal TLR2 in neurodegenerative diseases other than stroke and AD. Elucidating the role

of neuronal TLR2 in PD is very attractive, since microglial TLR2 has already been described in PD pathology.[49,73,77,78] We hypothesize that α-synuclein triggers neuroinflammation through microglial TLR2, initiating a positive feedback loop by increasing TLR2 expression on the microglia, resulting in neurodegeneration and disease progression. The contribution of neuronal TLR2 in this process is yet unknown, and could be evaluated by studying PD mouse models with a specific knock-out of TLR2 in neurons, and by studying the immune response of neurons activated with TLR2 stimuli in culture.

Neuronal TLR3 regulates cortical development and neurogenesis and is able to initiate immune-like responses in response to viral infections. This provides an interesting perspective to explore the function of neuronal TLR3 in neurodegenerative diseases, since we hypothesize that neuronal TLR3 will be detrimental in this context, and because viral infections could cause PD through the development of encephalopathy.[112] Expression patterns of TLR3 in brain tissue of early and late PD would shed light on whether TLR3 is indeed an interesting candidate for future PD research, and what role neuronal TLR3 might play in disease development.

TLR4 is a very promising target for future PD research. The effect of TLR4 during PD can be both beneficial and harmful, but the factors determining the outcome are yet unknown. It has is conceivable that neuronal TLR4 could be protective, but the role of microglial TLR4 is still in uncertain. One explanation for the confounding function of TLR4 in PD needs to be sought in the interaction between TLR4 stimulation and the stimulation of other receptors. Such interactions are known to occur for TLR4, in fact neuronal TLR4 interacts with the transient receptor potential cation channel V1 receptor to transduce itch itch signals and possibly to transduce pain caused by bacterial infections.[55,113] These data open up a new scenario of research in PD, particularly using specific neuronal TLR4 deficient animals, especially if this knock-out can be initiated before, during and after PD initiation, since the beneficial or detrimental effects of TLR4 in stroke seem to be dependent on the timing of TLR4 stimulation in relation to stroke. Similarly, the study of the effects of TLR4 stimulation in neuronal cultures in combination with (microglial) immune signals known to be important in PD pathology represents also another research direction.

TLR8 influences neuronal growth and survival and is also important for the initiation of the immune response during neuronal viral infections. The implications for neurodegenerative diseases are manifold, since viral infections have been linked to PD and a better understanding of the mechanisms underlying neuronal survival could help to reduce neuronal death.[112] Since so little is known about TLR8, further investigation about the role of this receptor represents an opportunity for future PD research.

Neuronal TLRs are an emerging research area, which will have implications for neurodevelopmental, neurodegenerative and neuroinflammatory research. To date neuronal TLR2-4 and neuronal TLR8 are known to be promising candidates for future studies. Elucidation of the function of other neuronal TLRs requires further research that would lead to a better understanding of the interaction between the nervous system and the immune system.

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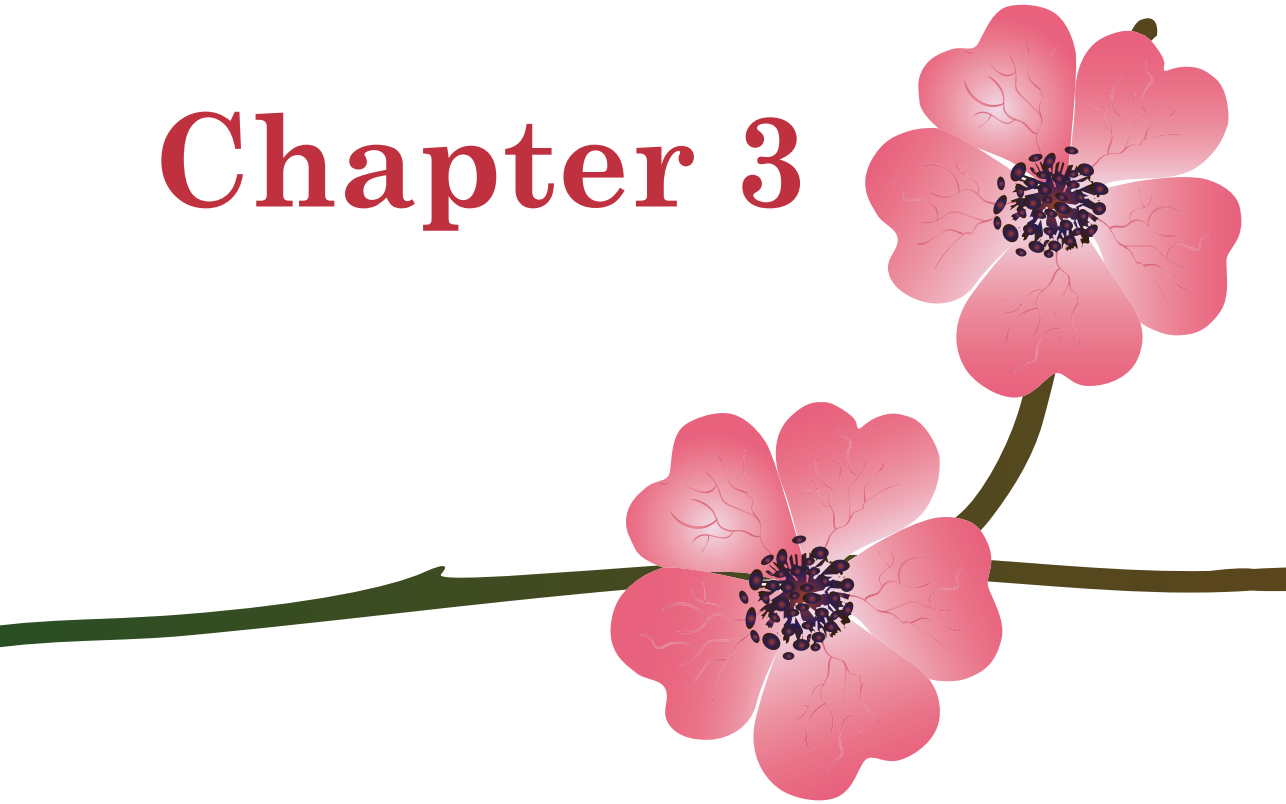
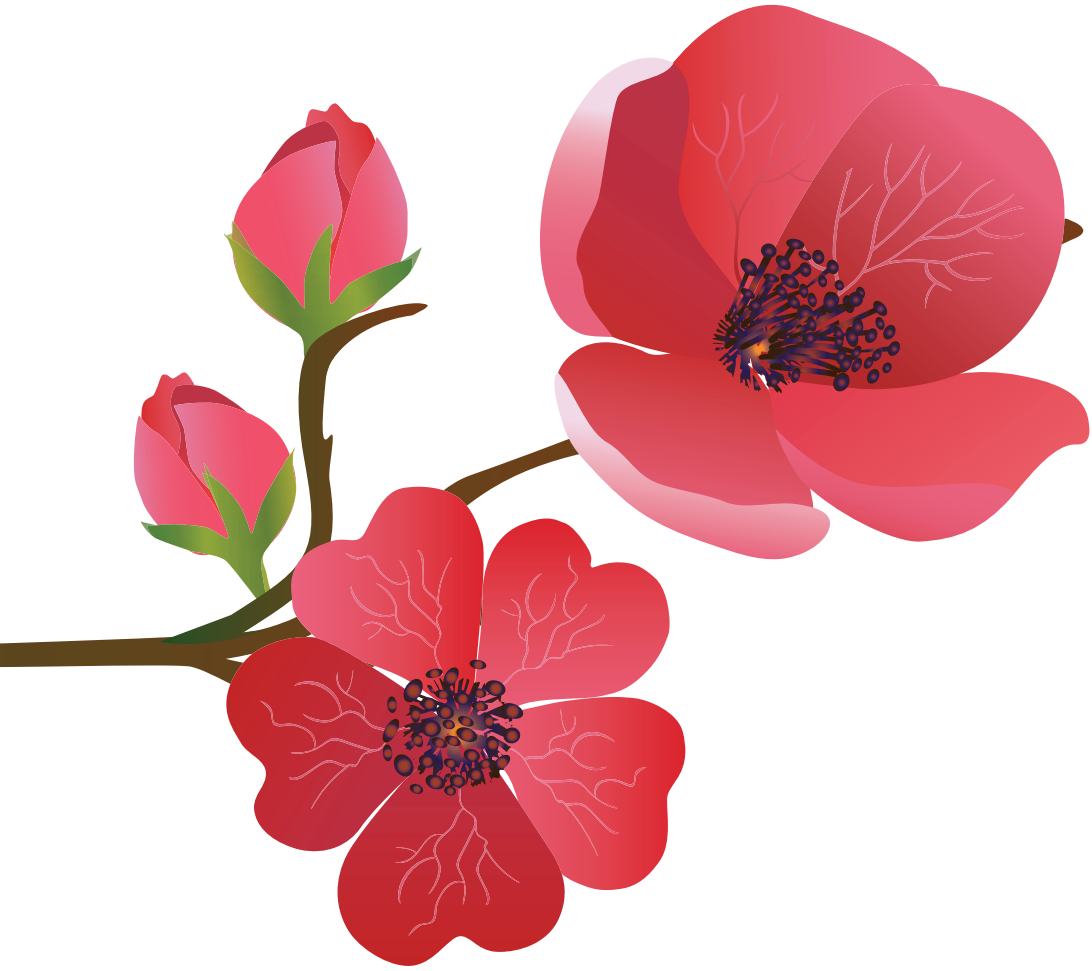
Chapter 3



C.D. Rietdijk[1], P. Perez-Pardo[1], J. Garssen[1,2], R.J.A. van Wezel[3,4], A.D. Kraneveld[1]

1. Div. of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, the Netherlands
2. Nutricia Research, Utrecht Science Park, Uppsalalaan 12, 3584 CT Utrecht
3. Dept. of Biomedical Signals and Systems, MIRA, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, the Netherlands
4. Dept. of Biophysics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, PO box 9010, 6500 GL, Nijmegen, the Netherlands

To be submitted



Exploring the Braak's hypothesis of Parkinson's disease

Abstract

Parkinson's disease (PD) is a neurodegenerative disorder for which there is no cure. Most patients suffer from sporadic PD, which is likely caused by a combination of genetic and environmental factors. The Braak's hypothesis states that sporadic PD is caused by a pathogen that enters the body via the nasal cavity, and subsequently is swallowed and reaches the gut, initiating Lewy pathology (LP) in the nose and the digestive tract. A staging system describing the spread of LP from the peripheral to the central nervous system was also postulated by the same research group. There has been criticism to the Braak's hypothesis, in part because not all patients follow the proposed staging system. Here, we review literature that either supports or criticizes the Braak's hypothesis, focused on the enteric route, digestive problems in patients, the spread of LP on a tissue and a cellular level, and the toxicity of the protein αSynuclein (αSyn), which is the major constituent of LP. We conclude that the Braak's hypothesis is supported by *in vitro, in vivo* and clinical evidence. However, we also conclude that the staging system of Braak only describes a specific subset of patients with young onset and long duration of the disease.

Abbreviations

PD – Parkinson's Disease
LP – Lewy Pathology
SN – Substantia Nigra
αSyn – αSynuclein
CNS – Central nervous system
ENS – Enteric nervous system
DMV – Dorsal motor nucleus of the vagus
LB – Lewy Body

An introduction to Parkinson's disease

Parkinson's disease (PD) is an incurable neurodegenerative disease hallmarked by damage to the dopaminergic neurons of the substantia nigra (SN), and αSynuclein (αSyn) containing inclusion bodies (Lewy pathology; LP[1]) in the surviving neurons, resulting in characteristic motor impairment[2]. In the Netherlands the incidence of PD is 0.2 per 1000 patients per year[3]. Over the age of 74 this number rises to 2.7 for men and 1.8 for women[3]. The concomitant prevalence is 1.8 per 1000 for men and 1.6 per 1000 for women[4].The prevalence of PD in Europe ranges between 65.6 to 12,500 per 100,000, and the annual incidence rate ranges between 5 to 346 per 100,000[5]. The variation in these prevalence and incidence rates could be due to genetic or environmental factors, differences in case ascertainment or diagnostic criteria, or different age distributions in the populations (countries) studied[5]. In the US population of 65 years and older, PD is more common in Caucasians and Hispanics, than Afro-Americans and Asians[6,7], indicating a genetic factor may be (partially) responsible for the differences found in the European study. Current treatments for PD include medicinal treatment using high-dose levodopa[8], and surgical treatment using deep brain stimulation[9]. Although these treatments offer relief of symptoms, they do not cure the disease. All in all it is clear that PD is an important neurodegenerative disorder to study, even with the more conservative estimations of prevalence and incidence, since currently no cure or preventative treatment exists.

There are two forms of PD; familial and sporadic. The familial form is caused by genetic aberrations, amongst others in the gene for αSyn (point mutations A30P[10], A53T[11], E46K[12], H50Q[13,14], and G51D[15], or locus duplication[16,17] or triplication[18,19]). The cause for sporadic PD is not known, but some progress has been made in the search for potential causes, implicating both genetic and environmental factors. The pesticides rotenone[20] and paraquat[20], and the toxin MPTP[21] (1-methyl-4-fenyl-1,2,3,6-tetrahydropyridine; a toxic byproduct of the opioid analgesic desmethylprodine, MPPP, a synthetic heroin), are known to cause PD in humans, explaining some cases of sporadic PD. Additionally, two twin studies have found that sporadic PD has a significant genetic component[22,23]. As mentioned above, in the US a difference was found in the incidence and prevalence of PD between the Caucasian and Hispanic vs Afro-American and Asian population, also showing a genetic influence[6]. On the other hand, a recent review by Pan-Montojo and Heinz Reichmann suggests an important role of toxic environmental substances in the etiology of sporadic PD[24]. Although the exact influence of genetic and environmental factors in sporadic PD may not be known, some elements of disease development have been identified, most importantly neuroinflammation, oxidative stress,

and αSyn misfolding and aggregation[25-32]. Misfolding and aggregation of αSyn is suspected to lead to LP in surviving neurons, and thus combatting αSyn aggregation has been suggested to be of potential therapeutic value[33]. It seems likely that both environmental and genetic factors interact to cause sporadic PD. As a result, the search for potential environmental factors has been ongoing in PD research.

The Braak's hypothesis

Introduction

In 2003 Braak et al. postulated the hypothesis that an unknown pathogen (virus or bacterium) in the gut could be responsible for the initiation of sporadic PD[34], and they presented an associated staging system for PD based on a specific pattern of αSyn spreading[35]. These publications were followed by the more encompassing dual-hit hypothesis, stating that sporadic PD starts in two places; the neurons of the nasal cavity and the neurons in the gut[36,37]. This is now known as the Braak's hypothesis. From these places the pathology is hypothesized to spread according to a specific pattern, via the olfactory tract and the vagal nerve respectively, towards and within the central nervous system (CNS). This process has been visualized in figure 1. Interestingly, the hypothesized spread of disease to the spinal cord only takes place after the CNS has already become involved, and so the spinal cord is not considered to be a potential route for the spread of the disease from the periphery to the brain[36,38].

Pre-clinical and clinical evidence

There is experimental and clinical evidence supporting Braak's hypothesis. Gastrointestinal problems like dysphagia, nausea, constipation and defecatory difficulty[39,40], and the olfactory problem of the loss of smell[41] have been reported in PD. Additionally, the presence of LP in the neurons of the olfactory tract[42,43] and the enteric nervous system (ENS) [44-46] has been confirmed. Severe LP in the ENS is positively correlated with constipation and motor problems in PD patients[47]. There is also clinical evidence that LP in the nasal and gastrointestinal regions potentially precedes the diagnosis of the disease[35,46,48], leading to complaints of the digestive tract[49,50] and problems with olfaction[51,52] during the earlier stages of PD, before the onset of motor symptoms (this stage is also known as incidental Lewy Body Disease[53]).

In animal models similar results have been found. Gastrointestinal problems have been described in models of advanced PD suffering from motor impairment [54-61], and in both genetic and toxin-induced models for earlier stages of PD without motor problems[62-64]. Additionally, αSyn aggregations were found in the gastrointestinal tract of animal models of early[62,63,65] and advanced[54,58] PD.

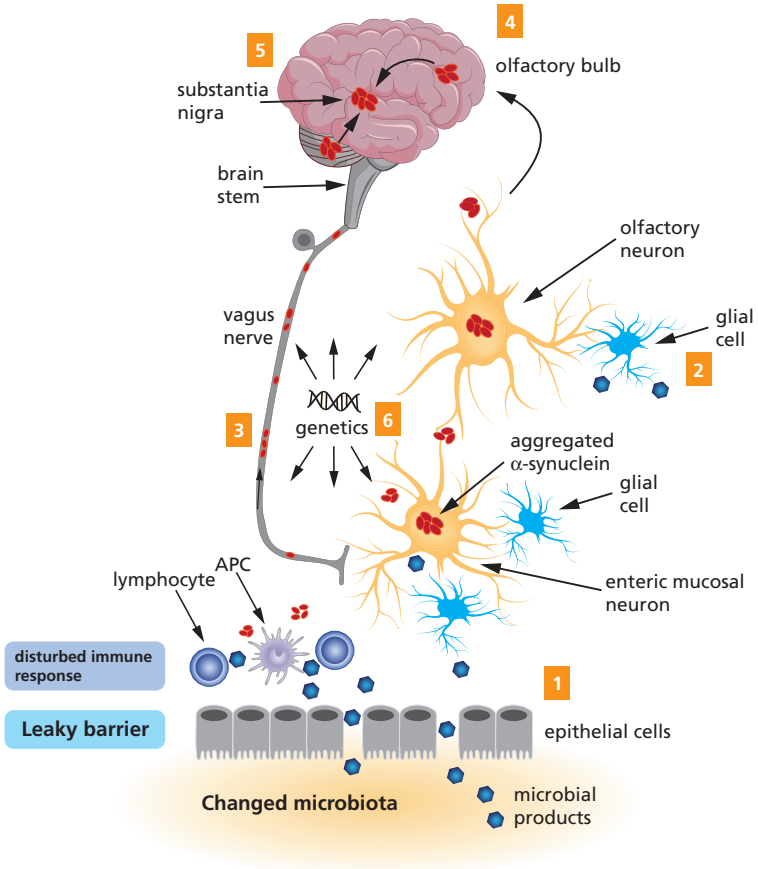


Figure 1. A schematic representation of the Braak's hypothesis of Parkinson's disease. Microbial products come into contact with olfactory and/or enteric neurons, which triggers the aggregation of α-Synuclein (1&2). The aggregated α-Synuclein spreads towards the central nervous system via the olfactory bulb and the vagus nerve (3&4). Eventually the aggregated α-Synuclein arrives at the substantia nigra (5). Geneticfactors are likely to contribute to Parkinson's disease, but the exact mechanism remains to be elucidated (6).

Enteric route: clinical evidence

From here on this review will focus on the enteric route of the Braak's hypothesis. The importance of the ENS for PD is emphasized by circumstantial clinical evidence. The microbiome of control subjects contains a higher relative abundance of Prevotellaceae bacteria compared to PD patients, and within PD patients a higher relative abundance of Enterobacteriaceae is associated with more postural and gait symptoms and less tremors[66]. PD patients also suffer from increased inflammation in the colon, although colonic inflammation does not seem to be related to severity of gastrointestinal or motor problems[67]. However, in PD patients another sign of intestinal inflammation, an increased permeability of the intestinal

barrier, seems to be related to increased staining in the intestinal mucosa for bacteria, oxidative stress and αSyn[68]. If changes in the microbiome predispose the (future) PD patient to a more pro-inflammatory environment in the intestines and increased barrier permeability, this could potentially lead to oxidative stress in the ENS. This oxidative stress could then trigger αSyn misfolding and aggregation, which could potentially spread from the ENS to the CNS, and eventually cause the hallmark motor problems. Therefore, changes in the microbiome and increased inflammation could directly negatively affect neurons of the ENS, and be related to PD development, which is in accordance with Braak's hypothesis.

Enteric route: αSyn spreading via vagal nerve

Another vital part of the Braak's hypothesis is the spread of αSyn pathology from the ENS to the CNS via the vagal nerve and the dorsal motor nucleus of the vagus (DMV) in the medulla oblongata, and the spread of pathology within the CNS from lower brainstem regions, towards the SN, and eventually the neocortex. Although these specific areas of the nervous system are affected by PD, certain neighboring areas seem to be spared, such as the nucleus tractus solitarius that is located next to and connected to the DMV. This indicates a non-uniform and specific pattern of the spreading of disease, which cannot be explained by the nearest neighbor rule[69]. This specific pattern of spreading is supported by experimental and clinical evidence, although discussion about the validity of the Braak's hypothesis is still ongoing. In PD patients LP has been found in the vagal nerve[70,71] and the DMV [70,72-75], and cell loss in the DMV of PD patients has also been reported[76]. Lewy pathology has been shown to occur in vagal nerves and DMV before it spreads to other parts of the CNS[35,48,73,77], like the locus coeruleus and the SN, the mesocortex, the neocortex and the prefrontal cortex[35]. Additionally, truncal vagotomy might be associated with a decreased long-term risk of developing PD, which could be related to a hindrance of the spreading of disease via the vagal nerve, although this cannot yet be concluded from this single study[78]. The spread of αSyn from the ENS to the CNS has also been studied in animal models. When the protein αSyn was injected in the wall of the stomach and duodenum of rats it was able to spread through the vagal nerve to the DMV[79]. Additionally, intragastric rotenone treatment of mice resulted in αSyn inclusions in the ENS, DMV and SN, and cell loss in the SN[80]. This rotenone-induced αSyn spreading could be stopped by vagotomy[81]. These results show the vagus nerve is involved in and essential for the spread of αSyn pathology from the ENS to the CNS in both rats and mice.

Enteric route: spread of αSyn within CNS

Clinical evidence for the cellular transport of LP within the CNS comes from studies of PD patients whose grafts of fetal dopaminergic neurons showed LP and degeneration, indicating potential spread of pathology from host cells to graft cells[82-87]. Host-to-graft transmission of αSyn has also been shown for mouse cortical neuronal stem cells[88] and mouse embryonic dopaminergic neurons[89] implanted in transgenic mice overexpressing human αSyn, and for rat embryonic dopaminergic neurons implanted in human αSyn overexpressing rats with[90] or without[91] striatal dopamine depletion. These results show that healthy neurons in the CNS are vulnerable to spread of disease by taking up LP from surrounding LP-affected neurons, although it does not indicate any specific pattern for this spreading.

Transport of αSyn between neurons

The ability of LP to spread through the nervous system raises the question what is the exact mechanism of transport of LP between neurons, and why the spread of LP follows a specific pattern, as suggested by the Braak's hypothesis. Both neuronal cell lines and primary neurons are able to excrete αSyn monomers, oligomers and fibrils through unconventional calcium dependent exocytosis from large dense core vesicles or via exosomes[81,92-94]. Once the αSyn is present in their environment, both neuronal cell lines and primary neurons seem able to take up free or exosome bound fibrils and oligomers by endocytosis after which they are degraded in lysosomes (SH-SY5Y cells), while monomers seem to diffuse across the cell membrane and are not degraded[88,94,95]. In a different study the uptake was only found in proliferating SH-SY5Y neurons, but not in differentiated SH-SY5Y neurons, which could be due to the type of αSyn which was different from the other studies (radioactively labelled cell produced αSyn, versus different forms of recombinant human or non-human αSyn)[93]. The transfer of specific αSyn molecules between by cells of neuronal cell lines was proven in a coculture study of SH-SY5Y neurons expressing the same human αSyn labelled either green or red[89]. Coculture resulted in double labelled neurons, showing the process of subsequent excretion and uptake of αSyn by neighboring cells. After uptake αSyn can be transported anterograde or retrograde through axons and passed on to other neurons[79,81,96-98], providing a potential highway for the spread of LP between connected nervous system regions in PD patients.

Furthermore, it is known that the neurons in the area's affected by LP in PD have specific characteristics that cause a high metabolic burden, which seems to make these neurons especially sensitive to oxidative stress and αSyn misfolding. These neurons have high levels of endogenous αSyn, they use monoamine

neurotransmitters, have long and highly branched axons with no or poor myelination, and characteristic continuous activity patterns[69,99,100]. Together this could explain why PD pathology develops in the specific pattern proposed by Braak, specifically affecting interconnected regions with vulnerable neurons like the DMV, while sparing neighboring areas like the nucleus tractus solitarius[69].

Neurotoxicity of αSyn

It has been suggested that αSyn acts prion-like in PD. In this theory pathologic, misfolded αSyn is an infectious protein spreading toxicity by forming a toxic template that seeds misfolding for nearby αSyn protein, turning the previously healthy protein into a toxic protein, causing LP. Excellent reviews on the prion-like theory of αSyn have been previously published[101,102]. The prion-like theory fits into the Braak's hypothesis, since the staging system of Braak is based on the regional presence (or absence) of LP and the spreading of LP, linking LP to severity of disease[35]. The toxicity of αSyn in its different forms is still undecided and remains the topic of many experiments, with one study reporting a cytoprotective function of αSyn aggregation[103], while others suggest the oligomeric form of αSyn is the most toxic form of the protein[104-106]. Foreign αSyn induces LP-resembling inclusion bodies in recipient neurons[88], caused by fibrils acting as exogenous seeds and recruiting endogenous αSyn into the inclusion body[89,107], even in cells not overexpressing αSyn[98]. Neuronal death resulting from αSyn exposure has also been shown[88], with a higher toxicity for oligomeric compared to monomeric species[93], and a higher toxicity of exosome bound oligomers compared to free oligomers[94]. Inclusion bodies are linked to cell death, involving the loss of synaptic proteins and reduction in network connectivity[98].

In animal studies injection of aggregated αSyn (derived from symptomatic transgenic mice) or synthetic αSyn fibrils into the brain of young, asymptomatic transgenic mice accelerated the formation and spread of αSyn inclusions throughout the brain, resulted in early onset motor symptoms, and reduced the lifespan of these mice[108,109]. Synthetic αSyn fibrils injected in the striatum also induced widespread LP, cell death of dopamine neurons in the SN and motor deficits in wild type mice[110]. It has even been shown that fibril-seeded αSyn inclusions specifically increase neuronal death in αSyn transgenic mice in an experiment where neurons with or without inclusions were followed *in vivo*, providing direct evidence that αSyn inclusions were responsible for neuronal death[111]. Injection of wild type mice with patient-derived Lewy Body (LB) αSyn just above the SN resulted in degeneration of the dopamine fibers and cell bodies in the SN, and concomitant development of inclusion bodies exclusively consisting of endogenous αSyn, and reduced motor coordination and balance[112]. Mice treated with non-LB αSyn (monomers) did not

develop these lesions. Similar results were found in rhesus monkeys; injection of patient-derived LB αSyn in the striatum or SN resulted in reduced nigrostriatal dopaminergic innervation, increased αSyn immunoreactivity in connected brain regions after striatal injection (but not after SN injection), without LP or motor symptoms[112]. Taken together, these results do not definitively confirm or reject the prion-like theory in the context of the Braak's hypothesis. However a picture emerges where αSyn oligomers are likely toxic to neurons, and inclusion bodies are linked to neuronal death, which might or might not lead to motor symptoms. Although the studies included here were performed in the CNS, the emerging picture of oligomer toxicity and inclusion body-induced neuronal death could also be applicable to the ENS and other parts of the peripheral nervous system.

Criticism to the Braak's hypothesis

Criticism to the specific pattern of spreading

Despite the *in vitro*, *in vivo* and clinical support for the Braak's hypothesis, there is also doubt whether it accurately describes the development of PD in all patients[113,114]. A large subset of 51%-83% of PD patients follow Braak's staging, while a smaller subset of 7%-11% do not have LP in the DMV while higher brain regions are affected[115-120]. Additionally, there is no correlation between severity of LP in the DMV, and in the limbic system or neocortex[121]. Also, LP in the ENS is not correlated to olfactory problems, and 27%-33% of PD patients did not show any LP in the ENS, which does not support the dual-hit hypothesis[67,122], although it is known LP can be restricted to the olfactory system in the early stage of the disease[120]. Additionally, people with incidental Lewy Body Disease seem to have a similar distribution but milder expression of LP compared to PD patients[53,123], and can show LP in the SN and other areas of the brain without LP or neuronal loss in the DMV[74,118,124,125] or LP in the vagus nerve[48], favoring multiple origination sites for LP instead of a spread from ENS to CNS via the vagus nerve. Additionally, Braak's hypothesis does not explain how or why cardiac sympathetic nerves are affected in early PD[125]. Therefore it seems safe to conclude that not all PD patients adhere to the specific pattern of LP spread proposed by Braak.

Criticism to the link between LP, neuronal loss, and PD symptoms

Other studies have shown that the link between LP and clinical PD symptoms should be questioned. Only 45% of people with widespread LP in the brain are diagnosed with dementia or motor symptoms[117] and only about 10% of people with LP in the SN, DMV and/or basal forebrain are diagnosed with PD[126]. Additionally, neurodegeneration in the SN might precede LP[127]. Therefore the

spreading of LP, whether according to Braak's staging system or not, might not be as tightly bound to clinical symptoms as has been suggested by Braak. The basic science underlying the Braak's hypothesis has also been questioned[114,128], because in the initial studies all cases were preselected for LP in the DMV[35,73], systematically excluding any cases where LP in higher brain regions was found in the absence of LP in the DMV, which seems to have led to a selection bias and the inclusion of non-representative samples in the pre-clinical PD group in the original research [128]. The limited clinical information on the pre-clinical PD group and the absence of information on neuronal cell loss in the original Braak papers has also been criticised[113,114,128]. It has been suggested that neuronal loss and activation of glial cells should be part of future pathological analysis of PD to better describe disease progression, since the clinical significance of LP is not yet clear and might be less important than previously thought[117,126,127].

Studying neuronal loss and glial activation in future PD research

Studying neuronal loss together with LP during PD development is important because neuronal loss in the SN shows a linear relationship with motor symptoms[129], while LP in the overall brain only shows a trend for positive correlation with motor symptoms[120]. Additionally, LP is not related to dopaminergic cell loss in the striatum[120], and may[120] or may not[130] be related to dopaminergic cell loss in the SN of PD patients. Therefore it can be concluded that neuronal loss and LP are not interchangeable hallmarks for PD progression or severity of disease, but should rather be seen as complimentary to each other.

Studying the activation of glial cells is important because neuroinflammation is an important factor in PD development, and glial cells are major contributors to neuroinflammation, partially through Toll-like receptors (TLRs)[25-30]. Especially TLR2 and -4 are important in PD, since their expression is increased in the brain of PD patients, and a polymorphism resulting in lower expression of TLR2 tends to be linked to an increased risk of PD[131-134]. Pre-clinical research has confirmed the importance of TLR2 and -4 for PD, and has specifically shown their importance in the context of glial-induced inflammation and αSyn uptake by glial cells[134-145].

Concluding on the Braak's hypothesis

Reviewing the current literature it can be concluded that there is much evidence to support the Braak's hypothesis. Enteric and olfactory pathology and dysfunction are well known characteristics of early and late PD. The vagus nerve and DMV form a likely route for αSyn pathology to spread from the ENS to the CNS, and αSyn is able to spread cellularly within the CNS. Neurons are able to transmit

different forms of αSyn protein to each other, and to transport αSyn via their axons, which enables the spread of the potentially toxic oligomeric variety of the protein, which could be the basic mechanism underlying the specific pattern of LP spread in PD as proposed by Braak. It seems then possible that a pathogen or environmental toxin might provoke local inflammation and oxidative stress in the gut, thereby initiating αSyn deposition that is subsequently disseminated to the CNS. Hypothetically, the toxic αSyn can lead to neuronal death. (Micro)glial cells and surviving neurons can then be activated through the release of danger associated molecular patterns and subsequent activation of Toll-like receptors. This would trigger a vicious circle of neuroinflammation.

However, it can also be concluded that a significant portion of PD patients do not follow Braak's staging system. It has been discovered that a subgroup of levodopa-responsive PD patients who develop PD at a young age and have a long duration clinical course with predominantly motor symptoms, and dementia only at the later stages, seem to follow Braak's staging, while other levodopa-responsive PD patients did not[77]. In addition to this, a Lewy Body staging system has been proposed which encompasses all patient groups, a system wherein LP staging correlates well with motor symptoms and cognitive decline[120], and allowing for patients who show a spread of LP not accounted for in the Braak's hypothesis. Unfortunately the staging system is only describing the different observed patterns of LP spread, while not answering the question as to the cause of the non-Braak patterns. What is the reason or explanation for these other types of patterns to occur? This question remains to be answered.

We conclude that the Braak's hypothesis and the Braak staging system are valuable and useful for the future study of PD, and these theories are likely to accurately describe disease initiation and progression in a subgroup of PD patients with young onset and long duration of disease. However, a similar theory describing the initiation and disease progression in other PD patients is still sorely lacking and deserves to be elucidated. To better understand the progression of LP and PD in different patient groups it is necessary to study people longitudinally during disease development, and especially in the earliest stages of PD. This should lead to a larger theory describing different disease processes, all leading to PD, including the Braak's hypothesis. This theory could offer useful insight into specific targets for disease prevention or disease treatment, dependent on the type of LP disease the patient is likely suffering from. Either more optimal treatment with currently available drugs and technology, or the development of new treatments could be the result.

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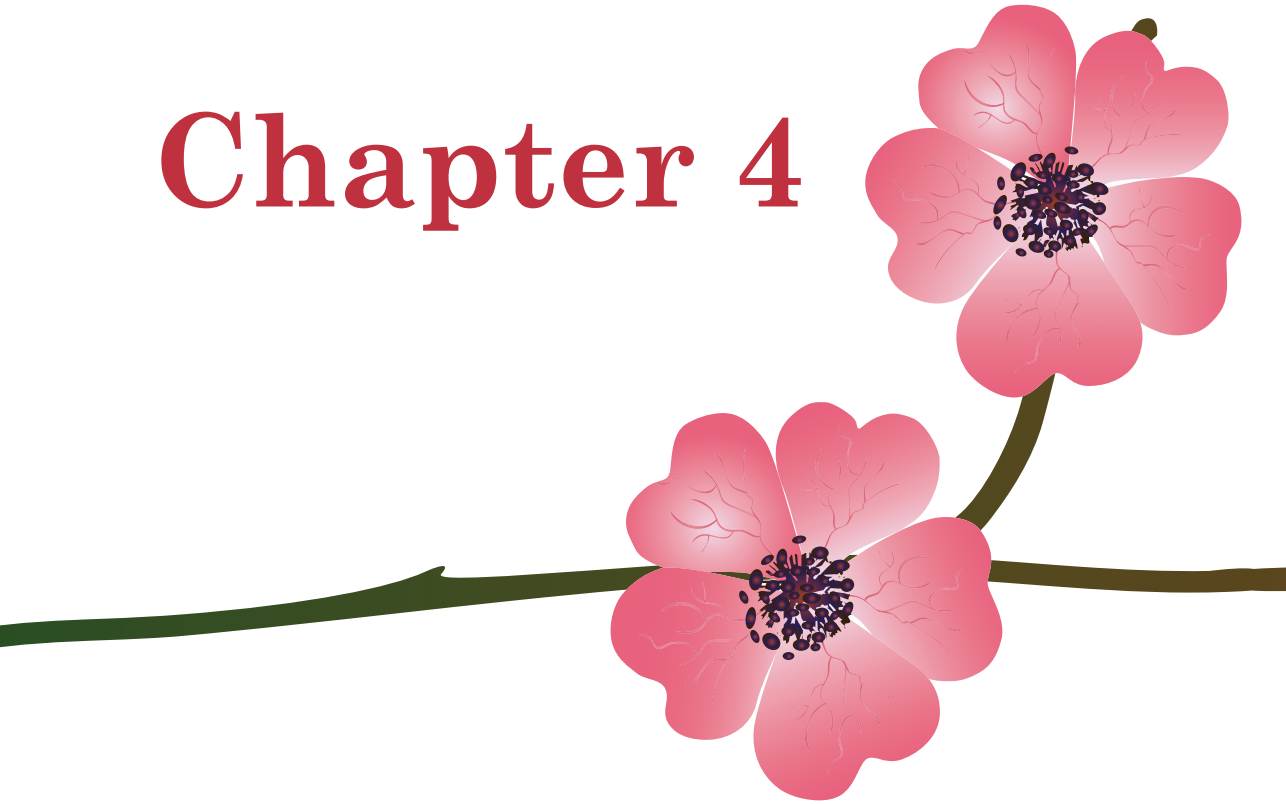
Chapter 4



Carmen D. Rietdijk[1], Lydia de Haan[2], Richard J. A. van Wezel[3,4], Johan Garssen[1,5], Aletta D. Kraneveld[1]

1. Div. of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, PO box 80082, 30508 TB Utrecht, the Netherlands
2. Div. of Pharmacoepidemiology & Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, PO box 80082, 30508 TB Utrecht, the Netherlands
3. Dept. of Biomedical Signals and Systems, MIRA, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, the Netherlands
4. Dept. of Biophysics, Donders Institute of Brain, Cognition, and Behaviour, Radboud University Nijmegen, 6525 EZ, Nijmegen, the Netherlands
5. Nutricia Research, Utrecht Science Park, Uppsalalaan 12, 3584 CT Utrecht, the Netherlands

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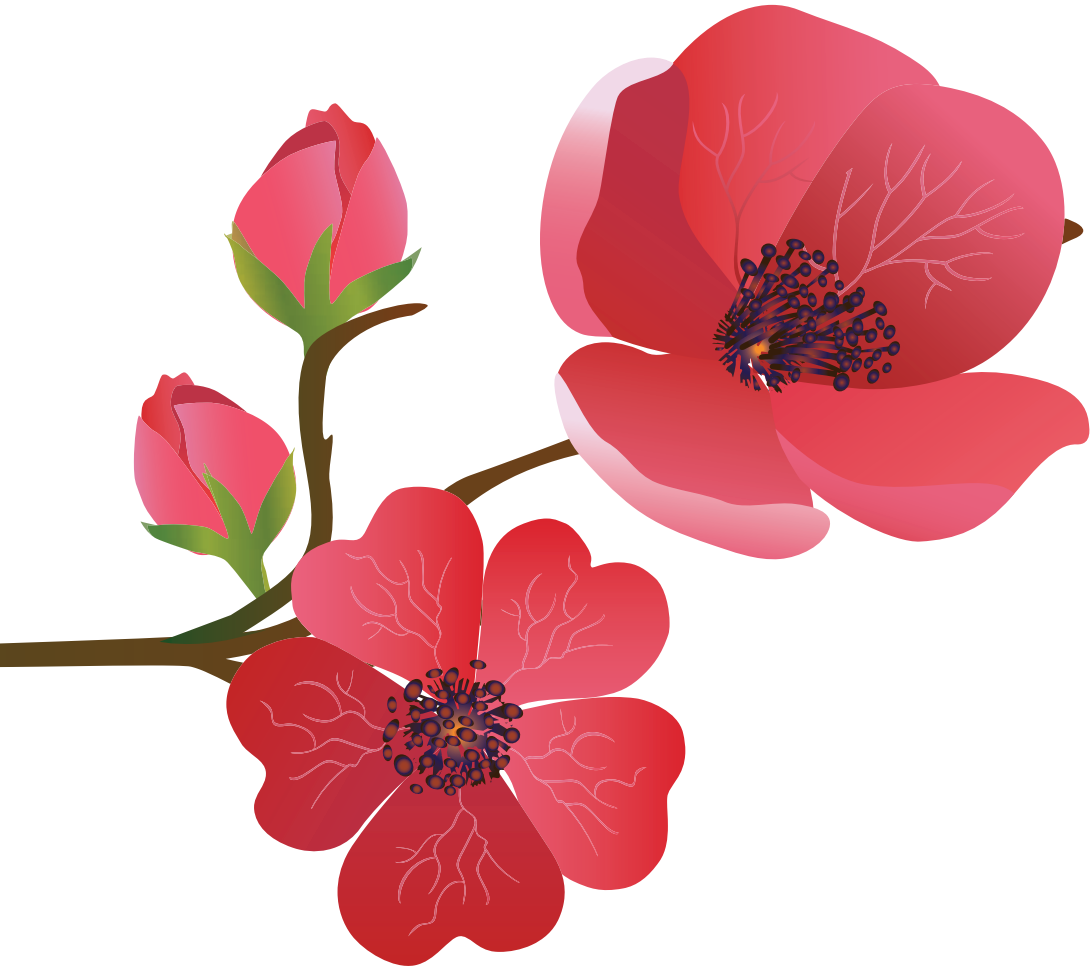

Best practice for passaging murine embryonic enteric neuronal cell line before differentiation

Abstract

The enteric nervous system (ENS) is a complex network of neurons in the gut, regulating many local, vital functions of the gastro-intestinal tract. The ENS is also part of the bidirectional gut-brain axis. The murine immorto fetal enteric neuronal (IM-FEN) cell line was chosen as a model to study enteric neurons. This cell line can be differentiated into cells with a neuronal phenotype, although they do not produce action potentials in vitro. It was concluded that the differentiation process in our laboratory was successful, based on positive staining for neuronal proteins. Proliferating IM-FEN cells have an unstable growth rate in our laboratory. An indicator of growth rate was calculated, and this indicator was found to be related to seeding density and number of days in culture, and was unrelated to person culturing, previous overconfluency or passage number. The indicator of growth rate was also unrelated to successful use of differentiated cells in follow-up experiments. We recommend the following conditions for optimal culture of IM-FEN cells. Keep cells in culture until 80 % confluent before passaging, seed cells at a density of 0.0133 million cells per cm^2, and anticipate on unstable growth rates and the risk for overconfluency.

Abbreviations

ENS – Enteric nervous system
IM-FEN – Murine immorto fetal enteric neuronal cell line
IFN-γ – Interferon-γ
PGP9.5 – Protein Gene Product 9.5
DMEM/F12 – Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12
GDNF – Glial cell line derived neurotrophic factor
FCS – Fetal calf serum

Introduction

The ENS is a complex autonomic network of neurons and glial cells in the gut, discovered in the nineteenth century (Furness 2005; Johnson et al. 2012). The ENS is organized in two ganglionated plexuses, the myenteric or Auerbach's plexus, and the submucuous or Meissner's plexus. The myenteric neurons lie between the inner circular and outer longitudinal smooth muscle layers, and the submucuous neurons lie in the submucosa. Together these plexuses regulate gastric acid secretion, fluid motion across the epithelium, epithelial barrier integrity, local and systemic inflammatory responses, gut motility, blood flow, and the secretion of neurotransmitters, hormones and peptides in the gastro-intestinal tract(Furness 2005; Johnson et al. 2012; de Jonge 2013). The ENS communicates in a bidirectional fashion with the central nervous system through vagal and spinal autonomic nervous system pathways, the hypothalamic-pituitary-adrenal axis, and in the central nervous system the nucleus of the solitary tract and the dorsal motor nucleus of the vagus(de Jonge 2013). This connection is known as the gut-brain axis and it integrates neural, hormonal and immunological signaling.

The ENS can be studied in vitro through enteric neuronal cell culture. Primary enteric neuronal cell culture requires the sacrifice of laboratory animals, is time consuming and yields only few cells. Therefore, a cell line of enteric neurons was selected to conduct experiments, known as the IM-FEN cell line as described by Anita M. et al(Anitha et al. 2008). The IM-FEN cell line was developed at Emory University using H-2Kb-tsA58 transgenic mice(Jat et al. 1991). These mice and the derived cells have stably integrated the thermolabile strain of simian virus 40 large tumor antigen gene. This is an immortalizing gene under the control of an interferon-γ (IFN-γ)-inducible H-2Kb promotor. This gene produces conditionally immortalized cells. When IM-FEN cells are cultured at 33°C and in the presence of IFN-γ, the activity of the promotor is increased above basal levels and the gene product is active, resulting in cell proliferation(Anitha et al. 2008). Once the desired amount of cells has been reached and differentiation of the cells to a neuronal phenotype is desired, the IM-FEN cells are cultured at 39°C without IFN-γ(Anitha et al. 2008). In the absence of IFN-γ the promotor is not stimulated and at 39°C the gene product is inactive, inhibiting cell proliferation and supporting differentiation into neuronal cells.

The neuronal phenotype of differentiated IM-FEN cells has been thoroughly studied(Anitha et al. 2008; Anitha et al. 2010). Differentiated IM-FEN cells express several neuronal markers(Anitha et al. 2008). Specifically they express intermediate filaments Nestin and peripherin, the neurotrophic factor receptor cRET, the serotonin receptors 2a, 3a, and 4a, the serotonin transporter SERT,

the microtubule-associated protein Tau, the synaptic marker synaptophysin, and ubiquitin carboxy-terminal hydrolase L1 known as protein gene product 9.5 (PGP9.5). Differentiated IM-FEN cells express only low amounts of markers for glial cells (GFAP, S-100β) and smooth muscle cells (αSMA). Bone morphogenetic protein 2 and low dose lipopolysaccharide are able to influence the phenotype or viability of the cells(Anitha et al. 2010; Anitha et al. 2012). There is limited knowledge on the electrophysiological properties of the IM-FEN cell line. The firing of action potentials is the main characteristic of neurons, and to fire action potentials neurons need ion channels. Differentiated IM-FEN cells express mRNA for sodium, potassium and chloride ion channels, but unfortunately the IM-FEN cells do not seem to be able to fire action potentials in vitro(Hawkins et al. 2013). In this context it is interesting to consider that fact that IM-FEN cells were able to survive and function properly in three different in vivo experiments(Anitha et al. 2008; Raghavan et al. 2011). Mice with a reduced number of enteric neurons in the colon (Piebald heterozygous mice) were shown to have improved colonic neuronal function after IM-FEN transplantation(Anitha et al. 2008). Mice known to have impaired relaxation in the lower esophageal sphincter, pyloric sphincter and the ileum (nNOS knock out mice) were shown to have improved relaxation of the longitudinal muscle of the proximal colon after IM-FEN transplantation(Anitha et al. 2008). And when IM-FEN cells were combined with smooth muscle cells to construct an artificial internal anal sphincter , the internal anal sphincters were shown to have proper neuronal functioning when implanted in immune suppressed recombination-activation gene 1 knock out mice(Raghavan et al. 2011). Additionally, the constructed internal anal sphincters expressed markers for both excitatory and inhibitory motor neurons, and expressed neuronal β-III tubulin. These in vivo results have demonstrated that the IM-FEN cells can develop into functional neurons, and they seem to hold the promise that there are circumstances that allow the IM-FEN cells to become mature, electrically active neurons. Unfortunately at this moment it is not yet possible to culture electrically active IM-FEN cells in vitro. Successful differentiation of IM-FEN cells was achieved in our laboratory, which was confirmed by positive staining for neuronal proteins. In our hands IM-FEN cells have an unstable growth rate during the proliferation phase. In the past this problem has also occurred in the laboratory in Atlanta where these cells were developed (personal communication). Because the cells have been growing at an unstable rate it is has been hard to plan experiments or to build up a bank of frozen cells. It has been impossible to use the growth rate to judge whether the cells are healthy or not, and the unstable growth rate has increased the risk of overconfluency since unexpected spurts in growth rate have happened. In an effort to explain and find a solution to the variability in growth rate and predict growth

rate in future cultures, a calculated indicator for growth rate was combined with information on the passage number, previous overconfluency (since the moment of thawing), number of days in culture (seeding to harvesting), the person culturing, and seeding density in a preliminary study. The relationship between the indicator for growth rate and the use of differentiated cells in experiments has also been examined, to determine whether the growth rate is related to suitability of the cells for experiments.

Material and methods

Reagents Cell Culture

Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (DMEM/F12) medium (Invitrogen, Bleiswijk, the Netherlands; 11330-032), glial cell line-derived neurotrophic factor (GDNF, R&D Systems Europe, Abingdon, UK; 512-GF), fetal calf serum (FCS, Bodinco BV, Alkmaar, the Netherlands), IFN-γ (Millipore, Amsterdam, the Netherlands; IF005), Penicillin/Streptomycin (Sigma Aldrich, Zwijndrecht, the Netherlands; P0781), Neurobasal A medium (Invitrogen, Bleiswijk, the Netherlands; 10888-022), B27 supplement (Invitrogen, Bleiswijk, the Netherlands; 17504-044), glutamine (Invitrogen, Bleiswijk, the Netherlands; 25030-032), Trypsin/EDTA (Gibco, Bleiswijk, the Netherlands; 25300-062), selenium (Sigma Aldrich, Zwijndrecht, the Netherlands; S5261), putrescine (Sigma Aldrich, Zwijndrecht, the Netherlands; P7505), progesterone (Sigma Aldrich, Zwijndrecht, the Netherlands; P6149), insulin (Sigma Aldrich, Zwijndrecht, the Netherlands; I6634), transferrin (Sigma Aldrich, Zwijndrecht, the Netherlands; T5391), fetuin (Sigma Aldrich, Zwijndrecht, the Netherlands; F3385), BSA (Sigma Aldrich, Zwijndrecht, the Netherlands; A8806).

Antibodies: PGP9.5 (Millipore, Amsterdam, the Netherlands; AB5925), Tubulin (Covance, Rotterdam, the Netherlands; MRB-435P), HuD (Millipore, Amsterdam, the Netherlands; AB5971), peripherin (Millipore, Amsterdam, the Netherlands; AB1530). Secondary antibody: Alexa fluor 594 conjugated (LifeTechnologies, Bleiswijk, the Netherlands; A-21207).

In vitro culture of IM-FEN cells

The IM-FEN cell line was established and characterized as described previously(Anitha et al. 2008). For proliferation cells were plated onto plastic 75-cm2 or 175-cm2 flasks in modified N2 medium(Heuckeroth et al. 1998) containing GDNF (100ng/ml), FCS (10%), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin/ml) and recombinant mouse IFN-γ (20 units/ml). The cells were cultured in a humidified incubator containing 10% CO2 at

the permissive temperature 33°C. The cells were observed regularly for signs of proliferation and were passaged when the flask became 80% or more confluent, using trypsin/EDTA.
When cells were approximately 60% confluent and proliferation was desired the medium of the cells was changed. Differentiating cells were cultured in Neurobasal-A medium containing GDNF (100ng/mL), FCS (1%), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin/ml), B27 (1:50 dilution), and glutamine (1mmol/liter), and were placed in a humidified incubator containing 5% CO2 at the non-permissive temperature 39°C.
The calculation of the indicator of growth rate was performed on data of proliferating cells, because during proliferation the cells are growing, regularly passaged and counted. Once cells are differentiating they barely grow, and they cannot be passaged or counted anymore. Therefore analysis of growth rate during differentiation was not performed. For neuronal protein staining the cells were differentiated for 7 days.

Immunocytochemistry neuronal proteins

Differentiated IM-FEN cells were stained for four different neuronal proteins: postmitotic neuronal marker HuD, peripheral nervous system cytoskeletal protein peripherin, neuronal microtubule protein β III tubulin, and the ubiquitin-protein hydrolase PGP9.5. The cells were washed, fixed, permeated and blocked before overnight incubation with primary antibody at 4°C. Concentrations of the antibodies were PGP9.5 1:500, Tubulin 1:100, HuD 1:100 and peripherin 1:100. The secondary antibody was conjugated to Alexa Fluor 594. After immunofluorescent staining the samples were counterstained with Hoechst (Thermo Fisher Scientific, Landsmeer, the Netherlands; 62249) and preserved in ProLong Gold (LifeTechnologies, Bleiswijk, the Netherlands; P36930). These experiments were performed once, using the first differentiated IM-FEN cells, to determine the success of our culturing techniques.

Data analysis

The indicator for growth rate of the cells was calculated based on cell count numbers obtained when the proliferating cells were passaged, and the number of cells that were initially seeded in the flask(s), and expressed as increase or decrease of cells per day of culture.
The indicator for cell growth rate was calculated using equation 1, where r = indicator for growth rate, #1 = number of cells at seeding, #2 = number of cells at harvesting, t = number of days between seeding and harvest (time), ln = natural logarithm.

$r = \ln(\#2/\#1)/t$ Equation 1

These data were combined with information on the passage number of the cells at seeding, overconfluency of the cells in previous cultures (continuous cultures, not separated by a freeze-thaw cycle), the number of days between seeding and harvesting, the person culturing the cells, and the seeding density using Excel and prepared for analysis in SPSS version 21.

The range of the passage number was 33 to 56 (mean 43.5778), the range of number of days between seeding and harvesting was 1 to 5 (mean 2.7556), and the range of seeding density was $0.4286*10^{-2}$ to $2.2000*10^{-2}$ (mean $1.140644*10^{-2}$) million cells per square centimeter. The range of growth indicator was -0.649641 to 0.910212 (mean 0.22676354).

Cells in the first cycle after thawing and infected cells were excluded from the analysis. Each case consists of one flask of cells from the moment the cells are inserted in the flasks, until the moment the cells are removed from the flask. Incomplete datasets were deleted listwise, leaving 42 complete datasets suitable for analysis.

An exploratory linear regression model was built to examine the relationship between passage number, previous overconfluency, number of days in culture, person culturing, and seeding density with the indicator for growth rate of the IM-FEN cells during proliferation. Dummy variables were made for 'person culturing' and 'previous overconfluency'. For 'person culturing' the values 0 and 1 were used to represent two individual researchers who cultured the cells independently (example: '0' represents Marc, '1' represents Emily), neither represent the same cells being cultured by more than one person at the same time. For 'previous overconfluency' the value 0 represented cells that had not been overconfluent in a continuous culture from the moment the cells were thawed, while the value 1 represented cells that had been overconfluent.

Effect sizes were expressed as standardized β coefficients, including significance and 95% confidence intervals. Partial regression plots were made to visualize the correlation between each individual predictors and the indicator of growth rate.

For the 42 included cases, data were also collected on the final fate of the cells. Possible outcomes were proliferation, differentiation, and freezing. We were interested in the differentiated IM-FEN cells, which were 9 cases in total (21.4% of all 42 cases). Differentiated IM-FEN cells were either used in an experiment (5 cases; 11.9% of all cases) or discarded because of premature death (4 cases; 9.5% of all cases), as determined by entries in laboratory journals. The relationship between the indicator of growth rate and the final fate of differentiated cells was analyzed using a Mann-Whitney U test.

All statistical tests were two-sided. Effects with $p<0.05$ and confidence intervals that did not contain zero were considered significant.

Results

Differentiated IM-FEN cells were immunoreactive to four neuronal proteins (fig. 1A-D). The strongest reactivity was found for postmitotic neuronal marker HuD and peripheral nervous system cytoskeletal protein peripherin (fig. 1A, 1B). Staining for neuronal microtubule protein beta III tubulin was weak and diffuse, but detectable (fig. 1C). Staining for the ubiquitin-protein hydrolase PGP9.5 was detectable, but the image was blurry (fig. 1D). No staining was detected without primary antibody (fig. 1E).

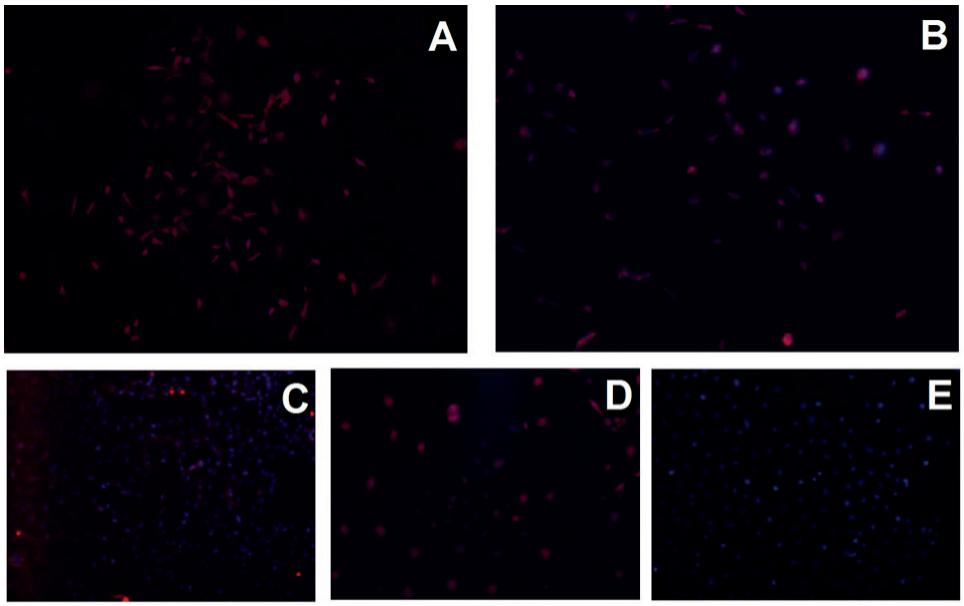

Figure 1. Immmunocytochemical staining for neuronal proteins (red) of differentiated IM-FEN cells. With Hoechst counterstaining for nuclei (blue). A HuD, B Peripherin, C β III tubulin, D PGP9.5, E negative control (no antibody against neuronal protein). Cells express all four neuronal proteins, while the negative control does not show staining.

The overall model accounted for 21.1% of the variability in the indicator of growth rate in the sample tested, as indicated by the R2 value 0.211 in table 1. The adjusted R2 value was 0.102, therefore the model only accounts for 10.2% of variability of the indicator of growth rate of the entire population of IM-FEN cells. The model only marginally improves the prediction of the indicator of growth rate compared to the mean, indicated by the F-ratio 1.931. This improvement was not significant (p=0.113).

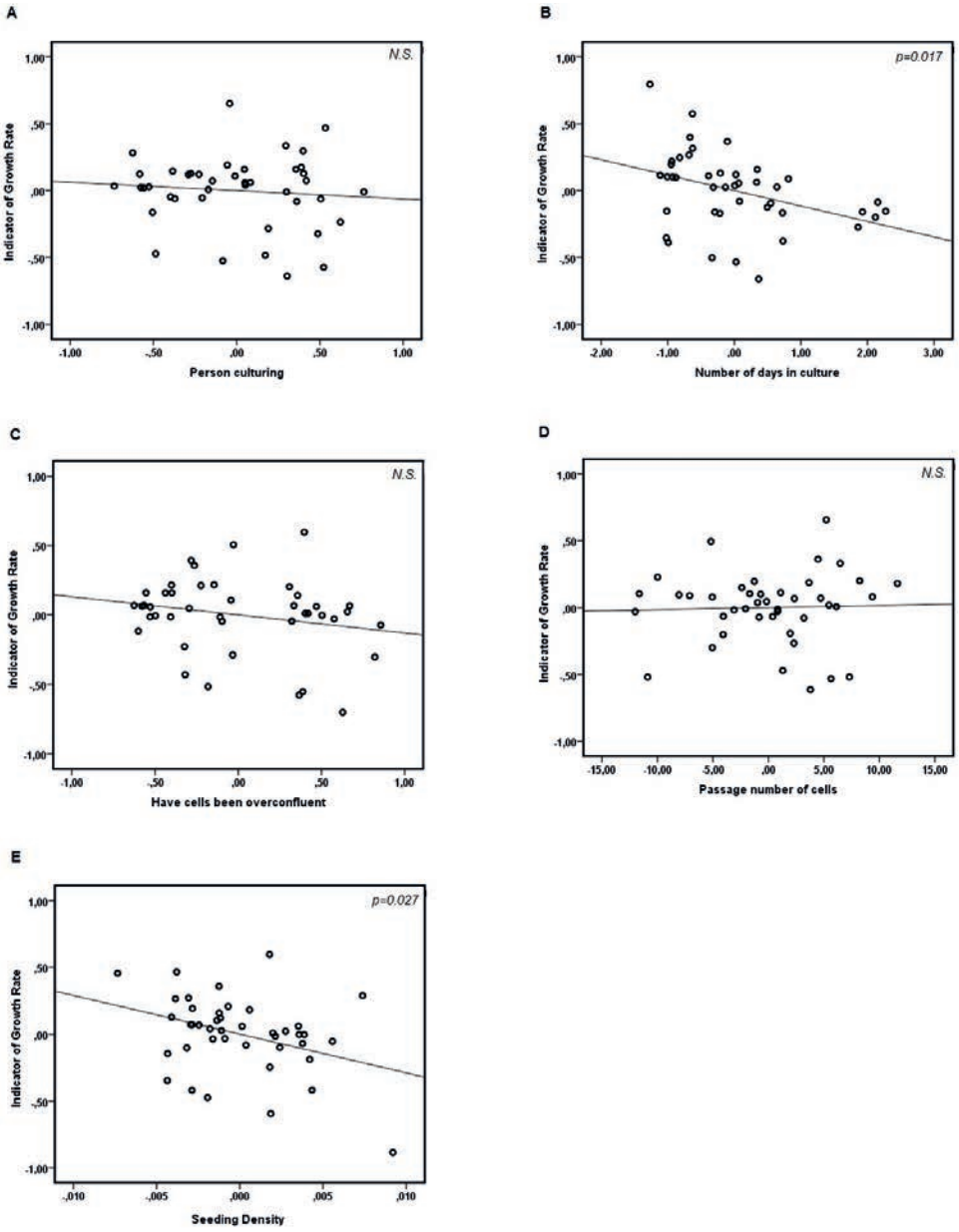

Figure 2. Partial regression plots of all five predictors in the exploratory linear regression model. In these plots the gradient of the regression line is equivalent to the standardized β coefficient of the predictor in the model, and signifies the correlation between cell growth and each predictor, when all other predictors are held constant. The axis display the residuals of the indicator of growth rate and the predictors. The significant negative correlations between indicator of growth rate and both number of days in culture (b) and seeding density (e) is visible, while no clear correlation is visible between indicator of growth rate and person culturing (a), previous overconfluency (c) and passage number (d)

However, there were two predictors that had a significant negative relationship with the indicator of growth rate, being 'Number of days in culture' and 'Seeding density' (table 1, fig. 2B,2E). 'Number of days in culture' had a significant negative standardized β coefficient of -0.407 (p=0.017) and a confidence interval not containing zero, meaning the negative relationship between the indicator of growth rate and 'number of days in culture' is statistically significant when all other variables are held constant (table 1).

Table 1. $R^2 = 0.211$, adjusted $R^2 = 0.102$, *F-ratio* = 1.931, sig. *F-ratio* = 0.113

Variable	Unstandardized B Coefficient	Standardized β Coefficient	Significance	Lower bound 95% CI	Upper bound 95% CI
Passage number	0.002	0.032	0.841	-0.014	0.017
Previous overconfluency	-0.131	-0.217	0.188	-0.329	0.067
Number of days in culture	-0.116	-0.407	***0.017***	-0.209	-0.022
Person	-0.063	-0.103	0.572	-0.285	0.160
Seeding density	-28.951	-0.393	***0.027***	-54.486	-3.417

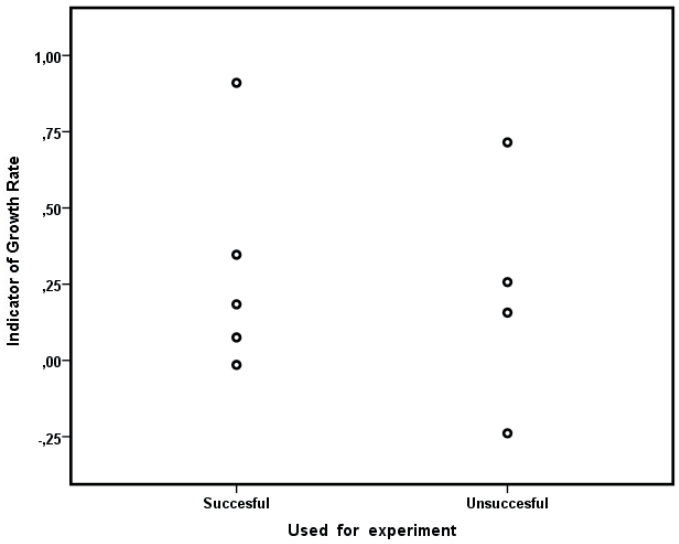

Figure 3. The indicator of growth rate of IM-FEN cells during proliferation does not affect the final fate of the differentiated cells. The spread of cell growth rates in both groups is very similar, and the difference between groups is not significant (p=0.905)

'Seeding density' had a significant negative standardized β coefficient of -0.393 (p=0.027), and a confidence interval not containing zero, meaning the negative relationship between the indicator of growth rate and 'seeding density' is statistically significant when all other variables are held constant (table 1). The correlations between the indication of growth rate and each individual predictor is visualized in fig. 2A-E.

There was no difference in the indicator of growth rate during proliferation between differentiated cells that were either successfully used in an experiment, or cells that died prematurely during differentiation (p=0.905) (fig. 3).

Discussion

Upon introduction of the ENS cell line in our laboratory, differentiated IM-FEN cells were tested for the expression of the four neuronal proteins HuD, peripherin, tubulin, and PGP9.5 (Anitha et al. 2008); the expression of all four proteins was confirmed. Two proteins, HuD and peripherin, were expressed strongly in the samples. Tubulin was also expressed, but at low levels. PGP9.5 staining was also present in the samples, unfortunately it could not be determined if this staining was strong because the image was blurry. These results provided evidence that differentiated IM-FEN cells express a neuronal phenotype, and gave the confidence that future experiments with these cells would be performed on properly differentiated neuron-like cells.

We did not stain undifferentiated cells for neuronal markers. However, in the original publication (Anitha et al. 2008) it was shown that the expression of neuronal proteins changes gradually over time between the proliferating (33° C) and differentiating (39° C) conditions. Some of the parent IM-FEN cells do express neuronal markers in proliferating conditions. It has not yet been determined whether these individual cells are still proliferating. In future studies this could be determined using tracer studies. At this moment it can be stated that in a cell population of IM-FEN cells proliferation decreases as differentiation increases.

Enrichment of proliferating cells could potentially be achieved through immunoselection for proliferation markers, but this has not yet been done. There has been no need for the enrichment of IM-FEN cells, since the proliferation is high at 33° C, when the cells are cultured in N2-medium in the presence of IFN-γ.

We have struggled with unstable growth rates of proliferating IM-FEN cells, and in this paper we have tried to find a solution. Being able to predict the growth rate of the cells would help to plan future experiments more efficiently, to build up a bank of frozen cells, to judge the health of the cells by their growth rate, and

to prevent overconfluency. The analysis of five potential predictors yielded a non-significant model for the prediction of an indicator of growth rate. However, two of the chosen predictors were useful for the prediction of the indicator of growth rate; days in culture and seeding density were significant negative predictors of indicator of growth rate, meaning that more days in culture or a higher seeding density were significantly related to a lower indicator of growth rate.

Number of days in culture showed the strongest negative correlation with the indicator of growth rate. The most likely explanation for the negative correlation is that slow growing cells are culture in the same flask for a longer time, because it takes the cells longer to reach a high level of confluency. However, the causality of this relationship was not clear. Therefore, it is not clear whether culturing in the same flask for more than the average number of days (3-4 days) should be avoided. This can be tested experimentally by passaging cells with different growth rates at different schedules, before any final conclusion can be drawn concerning causality.

Seeding density also showed a significant negative correlation with the indicator of growth rate. The most likely explanation is that slow growing cells get passaged at a higher density, to support their growth. Another explanation could be that a drop in reactive oxygen species reduces growth rate at higher densities(Limoli et al. 2004). However, the causality of this relationship is unclear as well. The effect of seeding density on growth rate should again be determined empirically, including measurements of the level of reactive oxygen species, to differentiate between both explanations. However, if a lower growth rate of the previous culture indeed entices the researcher to seed at a higher density and to culture for a longer time, from these data it seems clear that these approaches do not resolve the problem of unstable cell growth. Therefore culturing slow growing cells at higher density than average (the average being about 1 million cells per 75cm^2 flask, or 0.0133 million cells per cm^2) is not recommended, since it does not seem to improve growth rate. Leaving slow growing cells for many days in the same culture flask (until they become 80% confluent, and will be passaged) does not seem to speed up the growth rate either, but is nonetheless recommended, because passaging the cells at an earlier moment will only increase their passage number and will not increase their growth rate, therefore patience is the best course.

Increasing passage number did not affect the indicator of growth rate of IM-FEN cells, making it possible to keep them in culture for a long time. Regardless, it is generally advisable to work with cells as young as possible, since over time cells potentially accumulate deviations from the original source(Hughes et al. 2007; Balls et al. 2006) and this may influence experimental results. Passage number is an indicator for the 'age' of the cells, their distance from the original source. A more accurate estimate is the 'population doubling number', however we did not

have these data available. Therefore 'passage number' has been used in our model. Previous overconfluency in the same continuous culture did not affect the indicator of growth rate, although the growth rate in the first culture after overconfluency is usually low (personal observation). It appears that the cells were able to recover from overconfluency, which allowed subcultures of cells from flasks that grew faster than expected and became overconfluent as a result. Nonetheless, it is good cell culture practice to passage the cells when they have not yet reached overconfluency, but are rather in the logarithmic phase of growth(Phelan 2007), so the growth of the cells is not inhibited by cell-cell contact. Additionally, overconfluent cells underwent an unintended and uncontrolled selection process where some cells died off during the overconfluency, while more sturdy cells were still alive and able to proliferate in a new flask. Therefore it is advisable to prevent overconfluency. Finally, the person culturing did not predict the indicator of growth rate, showing that experience or subtle differences in culturing techniques did not affect growth rate. Although two variables relevant to growth rate were found, three variables were irrelevant. To improve the model, the three irrelevant variables can be removed, and other potential variables involved in growth rate can be added. Variables linked to the culture conditions of the cells (for example the culture medium) are interesting to include in the model(Phelan 2007; Balls et al. 2006). Testing these variables was not possible in our model, because we did not have data on these factors detailed in the laboratory journals. The general practice for these factors is known, and can be used to make predictions for their potential as predictors for growth rate. The culture medium was always prepared according to protocol, using fresh ingredients; therefore the expected variability in the medium is limited. Nonetheless, in future experiments the batch and freshness/age of the medium and all its ingredients should be listed since no batch is exactly the same and medium deteriorates over time(Phelan 2007; Balls et al. 2006). When these data are collected they can be tested as predictors for cell growth. The culture flasks in our laboratory are bought from Greiner Bio-One and are used for the culture of different cell lines and primary cells, without problems. This is an unlikely source of variation in cell culture and is therefore not interesting as a potential predictor in future experiments. It should be noted that this was a preliminary study, and the sample size was only 42. Therefore the current model should be interpreted with caution, and results should be confirmed in other, larger samples. Although the sample size was small, our preliminary study does offer insight into the potential factors influencing the growth rate of IM-FEN cells. Researchers using these cells in future experiments can build on these results with new analyses, to be determine which (if any) additional factors can predict growth rate in proliferating IM-FEN cells. It could be an inherent feature of these cells to have changeable growth rates. The unstable

growth rate has also been noted in the laboratory where the cells were originally developed (personal communication, Prof. Dr. Shanthi Srinivasan); therefore it is possible that these cells are unstable by nature, resulting in an unknown amount of variability of growth rate. It is noteworthy that in this other laboratory two adaptations have been made to the culture conditions: the CO^2 concentration at 33°C during proliferation has been reduced from 10% to 5%, and the flasks and plates containing cells are placed close to the walls of the incubators to keep the temperature as stable as possible. Since these adaptations were introduced in the laboratory, the growth rate of the cells has been more stable. Although causality cannot be proven, it could be that these changes are beneficial to IM-FEN cells. Therefore, these changes are recommended for other laboratories culturing these cells. On the other hand, in Chinese hamster ovarian cells there was no significant difference between the growth rates of mother and daughter cells in 13 out of 23 monoclonal cultures, indicating growth rate can be inherited from mother to daughter cells(Davies et al. 2013), giving support to the idea that unstable growth rates might be an inherited trait in the IM-FEN cell line.

For the 42 cases used to analyze growth rate we also analyzed the fate of the cells. Twenty-one percent was used for differentiation, as most cells were used for continuous proliferation or freezing for storage and future use. Of the 42 cases 11.9% was eventually used for experiments, while 9.5% died in the differentiation process of unknown causes. No relationship was found between the indicator of growth rate during proliferation and cell fate during differentiation. This means that cells should not be judged on their usefulness for differentiation based on their growth rate during proliferation. However, when interpreting these results it should be kept in mind that no causal relationship was tested in this analysis, and only 9 cases were included. More data should be collected on this topic to gain more confidence in the conclusion that there is no relationship between growth rate and cell fate during differentiation.

Conclusion

The culture of IM-FEN cells has been successfully implemented in our laboratory, resulting in properly differentiated neuron-like cells. These cells are considered valuable and suitable for further experiments based on the results presented here. The culture of IM-FEN cells has not been without problems. Especially the proliferation stage of the IM-FEN cells has been challenging, with unexpected increases and decreases in growth rate. Two factors significantly predicting the indicator of growth rate in our statistical model were days of culture and seeding density. The causality between these predictors and growth rate remains to be

determined, however a recommendation is already made not to seed slow growing cells at an increased or decreased density, and to wait until 80% confluency before passaging cells, even if this take many days for slow growing cells. Three factors were unrelated to indicator of growth rate (person culturing, passage number, previous overconfluency) and can be excluded from future models, while factors related to the culture medium could be added in an effort to predict growth rate of IM-FEN cells in the future.

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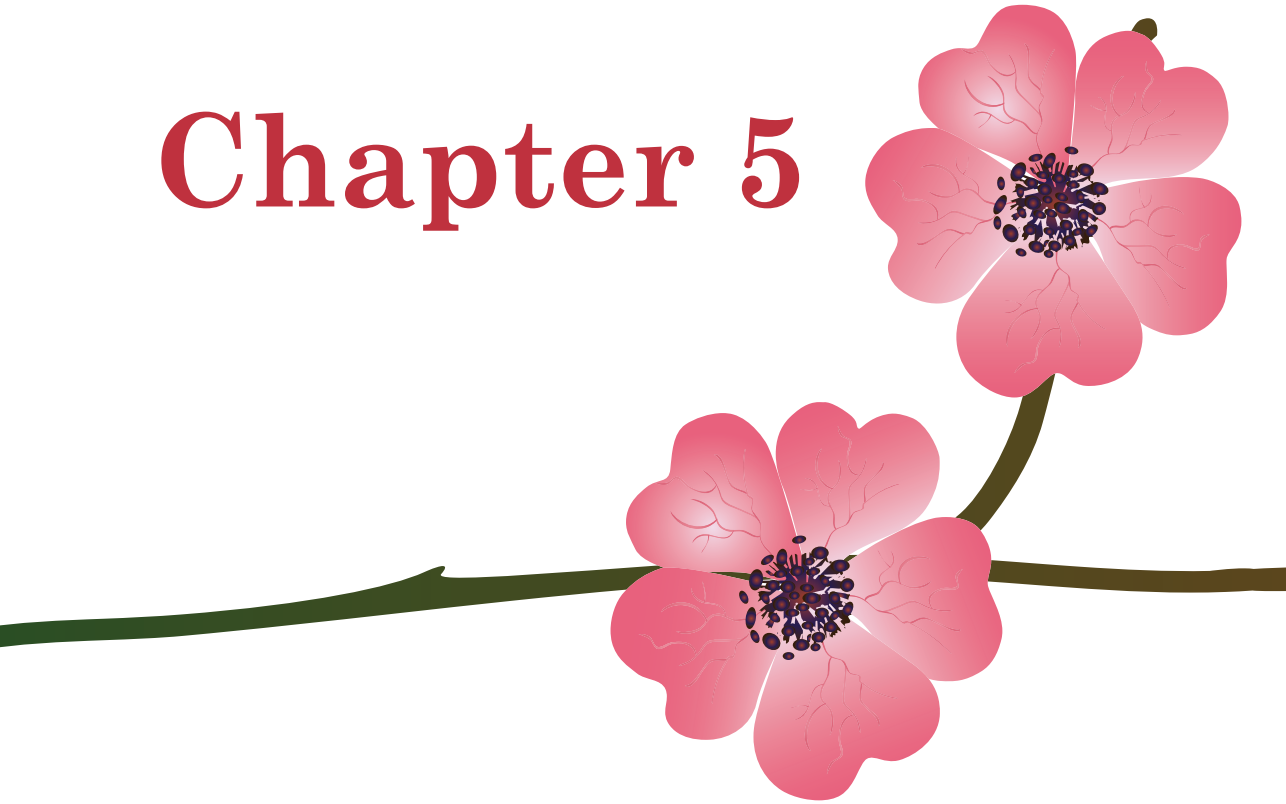
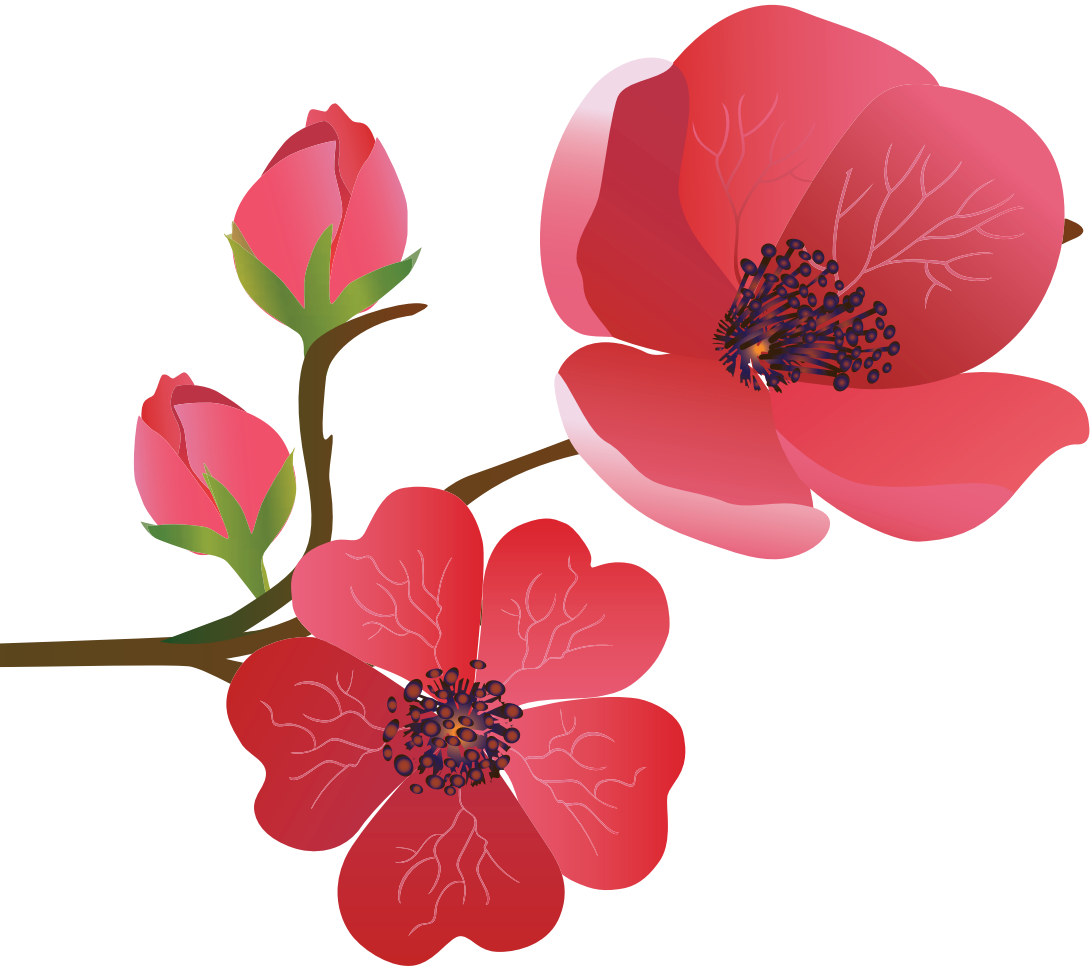
Chapter 5



C.D. Rietdijk[1], P. Perez-Pardo[1], K. Szklany[1], R.J.A. van Wezel[1,2,3], S. Srinivasan[4], J. Garssen[1,5], A.D. Kraneveld[1]

1. Div. of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, the Netherlands
2. Dept. of Biomedical Signals and Systems, MIRA, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, the Netherlands
3. Dept. of Biophysics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, PO box 9010, 6500 GL, Nijmegen, the Netherlands
4. Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, USA
5. Nutricia Research, Utrecht Science Park, Uppsalalaan 12, 3584 CT Utrecht

To be submitted



Expression of toll-like receptors on enteric neurons and the association with α-Synuclein aggregates in the gut

Abstract

Toll-like receptors (TLRs) are innate immune receptors that respond to both exogenous and endogenous danger signals, such as pathogens. These receptors are expressed by neurons as well, and have been linked to neurodegenerative diseases, like Parkinson's disease (PD). Pathogen-induced inflammation in the gut, as suggested by Braak, could trigger PD with neurons of the enteric nervous system (ENS) being the site of onset for the disease. Little is known about the expression of TLRs on neurons of the ENS, or their role in PD initiation and development. Therefore expression of TLRs on the murine immorto fetal enteric neuronal (IM-FEN) cell line, on murine primary enteric neurons, and in the murine ENS in ileum and colonic tissue has been investigated, to determine the usability of these models for future PD research. IM-FEN cells express TLR2-4 and TLR7. Murine primary enteric neurons and the murine ileum and colonic ENS both express TLR2 and TLR4. Expression of TLR4 on neurons in the ENS was significantly reduced in a mitochondrial dysfunction-associated murine PD model. IM-FEN cells express α-Synuclein (α-Syn) , and co-expression with α-Syn was found in the ENS for both TLR2 and TLR4. Therefore enteric neuronal TLRs provide a valuable research topic for future PD research, both on a cellular and an *in vivo* level. A more thorough understanding of enteric neuronal TLRs could help identify new biomarkers for PD, focus the search for pathogenic triggers for PD, and help to find new intestinal-related targets for future therapies.

Abbreviations

TLR – Toll-like receptor
PD – Parkinson's Disease
ENS – Enteric nervous system
α-Syn – α-Synuclein
IM-FEN – Murine immorto fetal enteric neuronal cell line
ICC – Immunocytochemistry
IHC – Immunohistochemistry
PGP9.5 – Ubiquitin carboxy-terminal hydrolase protein gene product 9.5
CNS – Central nervous system

Introduction

Toll-like receptors (TLRs) are pattern recognition receptors of the innate immune system. They recognize exogenous small molecular motifs known as pathogen-associated molecular patterns and endogenous molecules produced during inflammation and tissue damage known as damage-associated molecular patterns[1–4]. These innate receptors are not only expressed by immune cells and epithelial cells, but also by neurons[5–11] and glial cells[12–17] in the central nervous system as well as in the peripheral nervous system. Neuronal TLRs are suspected to be important during neuroinflammation and might be a novel target for treatment in neurodegenerative disorders, like Parkinson's disease (PD)[18–25]. Moreover, studying enteric nervous system (ENS) –associated TLRs is becoming more relevant since alteration of the composition of the intestinal microbiome has recently been reported in PD[26,27].

PD is an incurable neurodegenerative disease hallmarked by damage to the dopaminergic neurons of the substantia nigra, and αSynuclein (α-Syn) containing inclusion bodies (Lewy pathology[28]) in the surviving neurons. Misfolding and aggregation of α-Syn seem to be paramount processes in the development of PD[29–35]. These phenomena are associated with the disease characteristics such as tremor and motor impairments[36]. Besides these hallmark motor problems, PD patients also suffer from non-motor symptoms such as gastrointestinal deficits, which usually present themselves long before the diagnosis of PD[37–40]. In vitro and in vivo studies have shown that neuron-released oligomers of α-Syn can activate microglia via TLR2[20], and an *in vitro* study has shown that TLR4 is important for the α-Syn induced inflammatory response in astrocytes[41]. In addition, the activation of microglia by PD-associated misfolded α-Syn results in mRNA upregulation for TLR1-3 and TLR7, and downregulation of expression of mRNA for TLR4[42,43]. Clinical evidence also links both TLR2 and TLR4 to PD; the brains of PD patients show increased levels of TLR2 in microglia[44] and TLR2 and TLR4 overall[45], and a TLR2 polymorphism that results in lower TLR2 expression might increase the risk of PD[46]. It could be that TLR activity in the brain of PD patients is associated with α-Syn-induced, microglia-mediated neuroinflammation and oxidative stress signals[42,43], affecting nearby neurons.

In PD the ENS is regarded as an early pathogenic site[39,40,47–49]. Braak and co-workers state that the ENS is a possible site of initiation of the disease, that PD is triggered by pathogen-induced intestinal inflammation, and postulate a staging system describing the spread of Lewy pathology from the peripheral to the central nervous system[50,51]. Very recently, TLR2 and TLR4 upregulation has been found in the sigmoid mucosa of a subset of PD patients, which was associated with enhanced intestinal permeability and an increase of pro-, and decrease of anti-inflammatory cytokines (Keshavarzian, personal communication). Until now no data are available

on the expression of TLRs in the ENS in PD patients. Despite the importance of both the ENS and TLRs in the context of PD, the specific relationship between enteric neurons and TLRs in PD has yet to be determined. To determine whether enteric neurons could be a viable model for future PD research, we have studied mRNA and protein expression of different TLRs by the murine immorto fetal enteric neuronal (IM-FEN) cell line[52], by primary murine enteric neurons, and by the ENS of intestinal tissue samples collected from a mitochondrial dysfunction-induced murine PD-model .

Materials & methods

Culture of IM-FEN cells

The IM-FEN cell line was established and characterized as described previously[52]. For proliferation cells were plated onto plastic 75-cm^2 or 175-cm^2 flasks in modified N2 medium[53] ; Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 medium (Invitrogen, Bleiswijk, the Netherlands; 11330-032) containing selenium (5 ug/ml; Sigma Aldrich, Zwijndrecht, the Netherlands; S5261), putrescine (16,11 ug/ml; Sigma Aldrich; P7505), progesterone (6.35 ng/ml; Sigma Aldrich; P6149), insulin (5ug/ml; Sigma Aldrich; I6634), transferrin (1ug/ml; Sigma Aldrich; T5391), fetuin (100ug/ml; Sigma Aldrich; F3385), Bovine Serum Albumin (1mg/ml; Sigma A8806), glial cell line-derived neurotrophic factor (100ng/ml; R&D Systems Europe, Abingdon, UK; 512-GF), fetal calf serum (10%; Bodinco BV, Alkmaar, the Netherlands), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin per mL; Sigma Aldrich; P0781) and recombinant mouse interferon gamma (20 units/mL; Millipore, Amsterdam, the Netherlands; IF005). The cells were cultured in a humidified incubator containing 10% CO_2 at the permissive temperature of 33°C. The cells were observed regularly for signs of proliferation and were passaged when the flask became 80% or more confluent, using trypsin/ethylenediaminetetraacetic acid (Gibco, Bleiswijk, the Netherlands; 25300-062).
When cells were approximately 60% confluent and differentiation was desired the medium of the cells was changed. For differentiation into neuronal cells, cells were cultured in Neurobasal-A medium (Invitrogen; 10888-022) containing glial cell line-derived neurotrophic factor (100ng/mL; R&D Systems; 512-GF), fetal calf serum (1%; Bodinco 3000), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin per mL; Sigma Aldrich; P0781), B27 (1:50; Invitrogen; 17504-044), and glutamine (1mmol/L; Invitrogen; 25030-032), and placed in a humidified incubator containing 5% CO_2 at the non-permissive temperature 39°C. Differentiation was continued for 4-7 days, either in flasks or culture plates, on glass bottom 8-well culture slides or pre-coated glass cover slips for immunocytochemistry (ICC).

Mitochondrial dysfunction-induced murine model for PD

For the mitochondrial dysfunction-induced murine PD model, the mice were treated with oral rotenone (Sigma Aldrich, 128875-1G). Seven week-old C57BL/6J mice (Charles River, The Netherlands) were housed at room temperature under 12h light/dark cycle. Food and water was provided ad *libidum*. All animals procedures were conducted according to governmental guidelines and approved by the Ethical Committee of Animal Research of Utrecht University, Utrecht, The Netherlands (DEC2013.I.01.007). Mice received 10mg per kg rotenone once a day for 28 days by oral gavage. Sham-treated animals received vehicle (4% carboxymethylcellulose, Sigma Aldrich, 419273-100G) and 1.25% chloroform (EMSURE, Merck-Millipore) at the same volume. On day 28, mice were sacrificed by decapitation to collect brain and intestinal tissue. Oral administration of rotenone induced PD-like symptoms: from day 7 onwards motor deficits developed. After 28 days of daily rotenone gavages dopaminergic cell loss, delayed intestinal transit time, colonic inflammation, and α-Syn accumulation in the ENS was observed[54].
Ileal and colonic tissue samples from rotenone- and sham-treated mice were collected to perform immunohistochemistry (IHC) and for the isolation of primary enteric neurons.

Primary enteric neurons

A cell suspension was prepared from fresh ileum or colon tissue derived from control or oral rotenone-treated C57BL/J6 mice, using PBS and a cell strainer (Thermo Fisher Scientific, Landsmeer, the Netherlands; 352350). This cell suspension contained all cells from the tissue, including the neurons, which would later be labeled in the flow cytometry experiment, as described below.

RNA isolation and cDNA preparation

Cell culture samples were lysed in TRIzol reagent (LifeTechnologies, Bleiswijk, the Netherlands; 15596026), after which total RNA was extracted and purified using a Nucleospin RNA-clean-up kit (Macherey-Nagel, Dueren, Germany; 740903). cDNA was prepared using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands; 170-8890).

PCR & qPCR

PCR was performed using Taq DNA Polymerase (LifeTechnologies; 10342-020), qPCR was performed using iQ SYBR Green (Bio-Rad; 170-8880). Gene-specific primers for TLR1-9 and RPS13 were commercially purchased (Qiagen, Venlo, the Netherlands) and their efficiency was validated (data not shown). RPS13 was used

as a housekeeping gene. Relative target mRNA abundance was calculated using relative mRNA abundance = 100x $2^{Ct[RPS13]-Ct[target]}$

Immunocytochemistry

IM-FEN cell culture samples were washed three times in PBS, fixed in 4% paraformaldehyde, permeabilized using Triton X-100 (Sigma, X100), and blocked. The primary antibodies against TLR2 or TLR4, and ubiquitin carboxy-terminal hydrolase protein gene product 9.5 (PGP9.5) (see table 1) were incubated overnight at 4°C. The samples were washed three times and incubated with the secondary antibody in the dark for 1 hour at room temperature. Subsequently samples were washed and stained with DAPI (Thermo Fisher Scientific; 62284). Images where captured on a Zeiss Axioskop 2 plus microscope. In another set of experiments, the primary antibody against α-Syn (see table 1) was incubated for 2 hours at room temperature, due to practical considerations. The samples were washed three times, incubated with secondary antibody in the dark for 1 hour at room temperature. Subsequently the samples were washed three times, stained with Hoechst (Thermo Fisher Scientific; 62249), washed twice, and mounted with ProLong gold (Life Technologies; P36930). Images where captured on an Olympus BX60 microscope (Olympus Optical Co., Hamburg, Germany) and Leica DFC425C camera (Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry of intestinal tissue

Colon tissue samples from healthy C57BL/6J mice, were embedded in paraffin and cut in 5µm sections. Subsequently they were put on glass slides, deparaffinized in an ethanol gradient solution, and subjected to antigen retrieval using citrate buffer. Then the slides were washed once, blocked, and incubated with primary antibody (see table 1) overnight in a moist and dark environment at 4°C. The slides were washed four times, incubated with secondary antibodies for 1 hour at room temperature, washed twice, dried, and mounted with ProLong gold containing DAPI (Life Technologies; P-36931). Images where captured on a Keyence BZ-900 microscope (Keyence International, Mechelen, Belgium).

Flow cytometry IM-FEN cells and murine primary intestinal cells

IM-FEN cell culture samples were collected, counted, resuspended in blocking buffer (5% fetal calf serum (Bodinco 3000)), 1% bovine serum albumin (Sigma Aldrich; A2153) in PBS at a suitable concentration, divided over 96-wells plates and incubated for 30 minutes at room temperature. For intracellular staining a double amount of cells was used compared to extracellular staining; 200,000 vs

100,000 cells per well resp. Plates included an a well containing unstained cells, compensation controls, and isotype controls. For extracellular staining (TLR2, TLR4, TLR5) the antibody was incubated for 30 minutes at room temperature protected from light, and the cells were washed. For the intracellular staining (TLR3, TLR7) the cells were permeabilized using Foxp3/Transcription Factor Buffer Staining Set (Affymetrix, High Wycombe, UK; 00-5523), blocked, the antibody was incubated for 30 minutes at 4°C protected from light, and washed. The staining was measured using a FACS Canto II (BD Biosciences, Breda, the Netherlands) flow cytometer. Antibodies are presented in table 1. Primary intestinal cells from mice were counted and divided over a 96-wells plate, using 100,000 cells per well. Cells were treated as described above for intracellular staining using Foxp3/Transcription Factor Buffer Staining Set (Affymetrix; 00-5523). Plates included a well containing unstained cells, compensation controls, and isotype controls. Only samples containing more than 20 neurons were used for analysis, as determined by the number of cells positive for the neuron specific β-III tubulin antibody. Antibodies are presented in table 1. The staining was measured using a FACS Canto II (BD Biosciences) flow cytometer.

Western blot

IM-FEN and J774 mouse monocyte macrophage cell line[55] cell culture samples were lysed in Laemmli Sample Buffer (Bio-Rad; 161-0737) with Phosphostop (Roche, Indianapolis, IN, USA; 04 906 845 001) and protease (Roche; 11 836 170 001) inhibitor cocktails, and sonicated. Protein concentration of the samples was measured using the DC Protein assay Lowry method (Bio-Rad; 5000112) and measured at 750nm using a Molecular Devices VERSAmax Tunable Microplate Reader (Molecular Devices, Inc., Sunnyvale, CA, USA). Fifteen μg of each sample was denatured; samples and protein markers were run together in a 12% Mini-PROTEAN TGZ Precast gel (Bio-Rad; 456-1044). Samples were blotted on a Poly Vinyllidene Fluoride membrane (Bio-Rad; 162-0177), the membrane was washed and blocked, after which the primary antibody (see table 1) was incubated overnight at 4°C. The housekeeping protein β-Actin was used. The membrane was washed and incubated with the secondary antibody for 1 hour at room temperature. The membrane was washed again and stained using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Wauwatosa, WI, USA; RPN2132). The staining was visualized using an RP X-OMAT Processor, Model M6B (Kodak, Rochester, NY, USA).

Primary antibodies

Table 1. Primary antibodies used in different experimental settings.

Technique	Figure	Target	Company	Product #
Immunocytochemistry	4A	TLR2	Millipore	06-1119
	4A & 5	PGP9.5	Abcam	AB72911
	5	TLR4	Santa Cruz Biotechnology	sc-30002
	6	α-Syn	Sigma Aldrich	SAB4300529
Immunohistochemistry	7	TLR2	Acris Antibodies	AP30897PU-N
		α-Syn	GeneTex, Irvine, CA, USA	GTX21903
Flow Cytometry	3	TLR2	LifeSpan Biosciences, Seattle, WA, USA	LS-C107234
		TLR3	Novus Biologicals, Abingdon, UK	NBP2-24875
		TLR4	Abcam, Cambridge, UK	ab45104
		TLR5	Acris Antibodies, Herford, Germany	SM7102AF647
		TLR7	Acris Antibodies	SP7198F
	8 & 9	TLR2	LifeSpan Bioscience	LS-C107234
		TLR4	Abcam	ab45104
		β-III Tubulin	Abcam	ab25770
Western Blot	3B	TLR2	Millipore	06-1119
		β-Actin	Sigma Aldrich	A5441

TLR: Toll like receptor; α-Syn: α-Synuclein

Statistics

Means and medians were calculated using Microsoft Excel or GraphPad Prism 5. Mann-Whitney tests were performed to determine differences between treatment groups, using GraphPad Prism 6. All tests were two-sided. Effects with $p < 0.05$ were considered significant.

Results

IM-FEN cells express TLR mRNA

The expression of TLR1-13 on IM-FEN cells was examined by PCR and qPCR experiments. TLR1-6 mRNA expression was found in both undifferentiated and differentiated cells by PCR (fig. 1), TLR7-13 were undetectable by PCR (data not shown). The expression of TLR2-4 was high in both undifferentiated and differentiated cells, while TLR5 expression was low, and TLR6 expression was moderate. The expression of TLR1 was high in undifferentiated cells, but low in differentiated cells.

The expression of TLR2-5 mRNA in differentiated cells was confirmed by qPCR, and in addition TLR7 mRNA was detected by qPCR (fig. 2). Expression of TLR1 and TLR6 mRNA could not be confirmed by qPCR, while absence of TLR8 and TLR9 mRNA was confirmed.

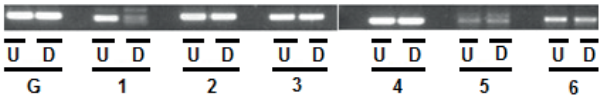


Figure 1. mRNA expression profile of Toll-like receptors in undifferentiated (indicated by U) and differentiated (indicated by D) IM-FEN cells determined by PCR. Expression of TLR1-6 was found (indicated by numerals). GAPDH was used as reference (indicated by G).

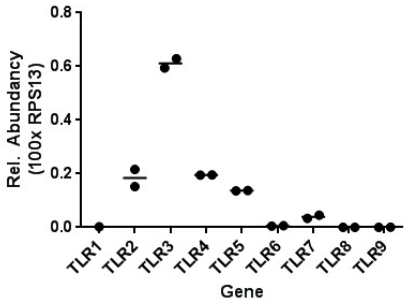


Figure 2. mRNA expression profile of Toll-like receptors in differentiated IM-FEN cells determined by qPCR. TLR expression is shown as the ratio of TLR expression to expression of housekeeping gene RPS13 (x100). n=2 for TLR2-TLR9.

IM-FEN cells express TLR and α-Syn proteins

Following the qPCR data, protein expression of TLR2-5 and TLR7 in differentiated IM-FEN cells was determined by flow cytometry, ICC, and western blot analysis. Flow cytometry analysis was used to determine the expression of TLR2-5 and TLR7 on differentiated IM-FEN cells. TLR2 was detected by extracellular staining on the membrane of 37.0%-37.6% of cells (fig. 3D-F). TLR3 and TLR7 were detected using an intracellular staining, in which 51.0%-88.1% of the cells were double positive for both TLRs (fig. 3A-C). TLR4 and TLR5 could not be detected by flow cytometry using extracellular staining (fig. 3D-I).

A-C Cells were stained with an APC-conjugated antibody against TLR3 and a FITC-conjugated antibody against TLR7. Per cell the intensity of FITC staining is plotted against intensity of APC staining. An unstained sample (A), and two double stained samples (B,C) are shown. Double stained samples show 51.0% and 88.1% double positive cells, while the unstained sample does not show any false positive double staining. *D-F* Cells were stained with an PE-conjugated

antibody against TLR4 and a PE-Cy7-conjugated antibody against TLR2. Per cell the intensity of PE staining is plotted against intensity of PE-Cy7 staining. An unstained sample (D), and two double stained samples (E,F) are shown. Double stained samples show 37.0% and 37.6% of TLR2 positive cells, while almost no cells were positive for TLR4. The unstained sample showed 3.2% of TLR2 positive cells. *G-I* Cells were stained with an APC-conjugated antibody against TLR5. Per cell the intensity of APC staining is plotted against cell size/complexity (side scatter). An unstained sample (G), and two stained samples (H,I) are shown. Stained samples show almost no positive cells, and the unstained sample does not show any false positive double staining.

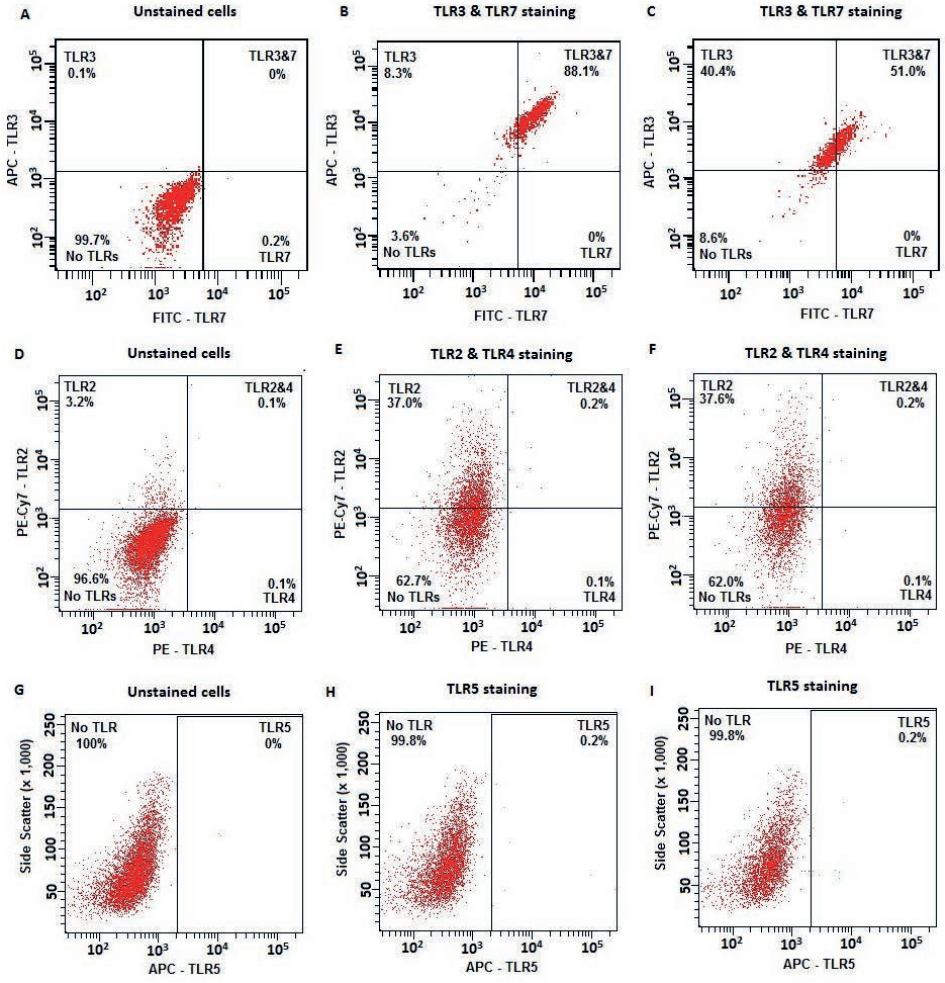


Figure 3. Protein expression of TLR2-5 and TLR7 in differentiated IM-FEN cells determined by flow cytometry analysis.

PCR and qPCR results demonstrated that both TLR2 and TLR4 had high expression levels in differentiated IM-FEN cells. Therefore we focused more on TLR2 and TLR4. IHC showed that TLR2 protein expression was found to be located in both the nucleus and the membrane (fig. 4A). Western blot analysis demonstrated that TLR2 expression was decreased approximately 11 times in younger (passage 35, P35) differentiated cells compared to undifferentiated cells. In contrast, in older (passage 46, P46) differentiated cells an approximately 3 times increased expression of TLR2 was observed compared to undifferentiated cells (fig. 4B). Despite the absence of TLR4 protein staining as measured with flow cytometry, ICC staining showed that differentiated IM-FEN cells express TLR4 on their membrane, without specific nuclear staining (fig. 5).

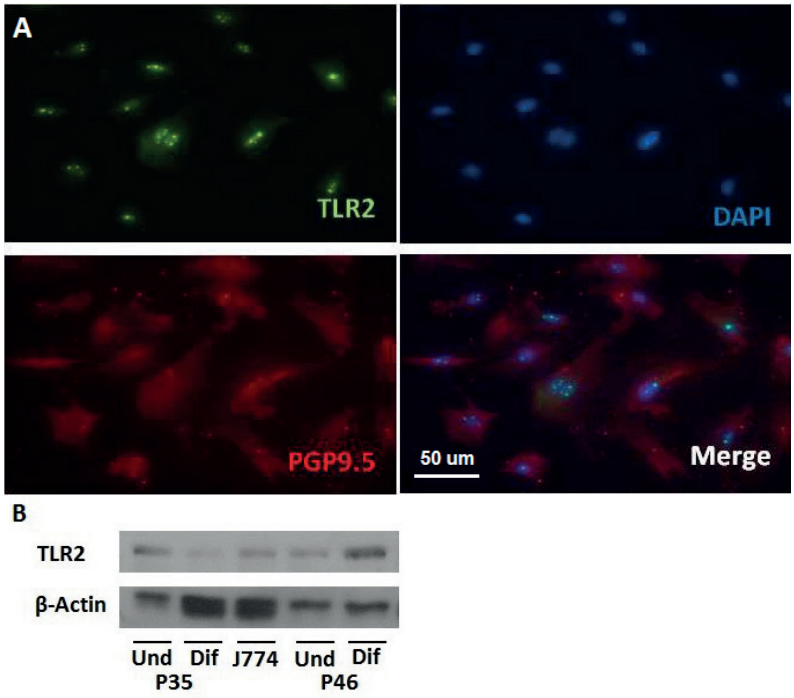


Figure 4. Protein expression of TLR2 in differentiated IM-FEN cells. *A* ICC staining for TLR2 in differentiated cells, including neuronal protein PGP9.5 staining, and DAPI staining for nuclei (scale bar represents 50μm). *B* Representative WB of TLR2 protein expression in undifferentiated (Und) and differentiated (Dif) cells, both younger (P35) and older (P46) cells are shown. Expression in IM-FEN cells is shown next to positive control J774 murine macrophage cells. For all samples expression of TLR2 is compared to housekeeping protein β-actin.

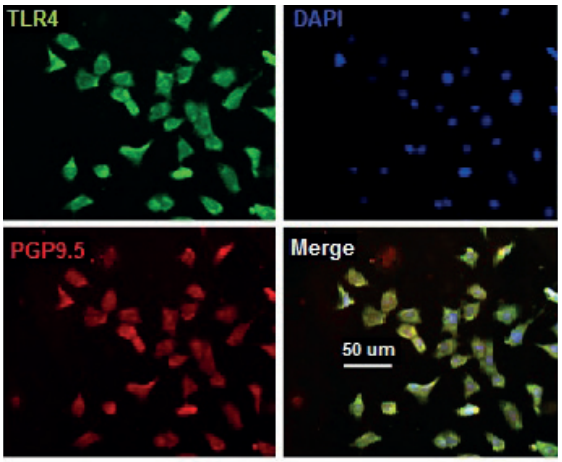


Figure 5. Protein expression of TLR4 in differentiated IM-FEN cells. ICC staining for TLR4 in differentiated cells, including neuronal protein PGP9.5 staining, and DAPI staining for nuclei (scale bar represents 50μm).

Expression of α-Syn protein was examined using an intracellular cytological staining in differentiated IM-FEN cells. Most IM-FEN cells expressed α-Syn, predominantly nuclear staining was observed, and large differences in intensity of staining were observed between the cells (fig. 6).

Protein expression of TLRs and α-Synuclein in murine primary enteric neurons

Protein expression of TLRs in the ENS and on primary enteric neurons was investigated using IHC and flow cytometry. The myenteric neuronal plexus in the colon of mice expressed TLR2 (fig. 7B) and TLR4 (fig. 8B), and co-localization of TLR2 or TLR4 and α-Syn (fig. 7D) was detected. However, the cell type in the neuronal plexus responsible for this TLR positive staining was unknown.

To determine whether the TLR expression could be located on neurons, flow cytometry analysis of cell suspensions of whole colonic and ileum tissue was performed. Neurons in the ileum and the colon of mice showed expression of both TLR2 and TLR4 (fig. 9&10). Neurons in the colon of PD mice expressed significantly less TLR4 than neurons in the colon of control mice ($p = 0.0292$, fig. 10F), no other differences were found in neuronal TLR expression between both groups (fig. 10A, B, E, I, J). When analyzing non-neuronal cells, cells derived from the colon of PD mice expressed significantly less TLR2 than the control sample ($p = 0.0434$, fig. 10D), no other differences were found in non-neuronal TLR expression between both groups (fig. 10C, G, H, K, L). Additionally, there were no significant

differences in the percentage of neurons or other cells expressing neither TLR2 nor TLR4 (data not shown).

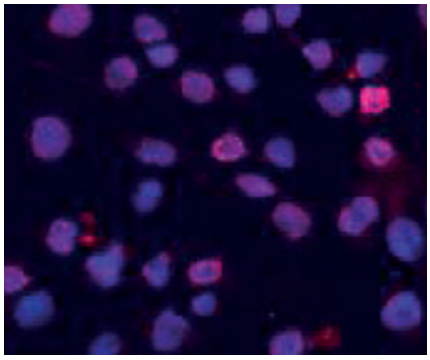


Figure 6. Expression of α-Syn protein (red) in differentiated IM-FEN cells, nuclei are stained with Hoechst (blue).

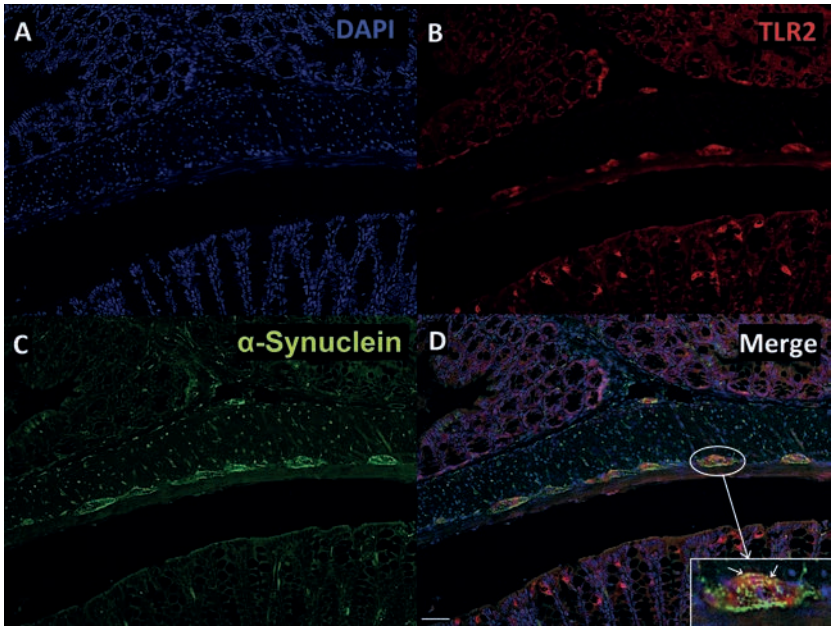


Figure 7. Protein expression of TLR2, and α-Syn on the enteric neuronal plexus of the colon of healthy C57BL/6J mice, measured by IHC. A DAPI staining. B TLR2 staining. C α-Syn staining. D Overlay of DAPI (blue), TLR2 (red) and α-Syn (green) staining. Overlap between TLR2 and α-Synuclein can be seen as yellow staining in the plexus, indicated in a magnified ganglion by white arrows (see inset in D). (Scale bar represents 50 μm).

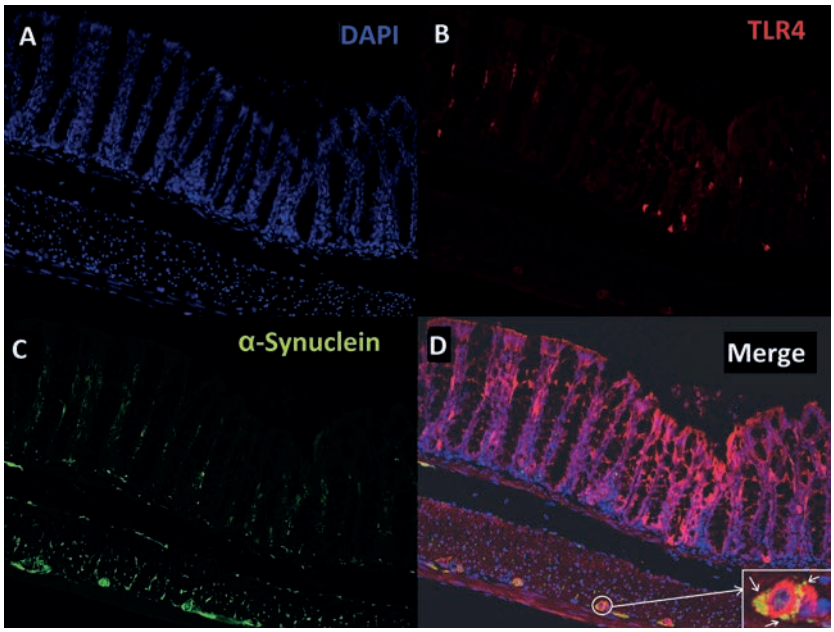


Figure 8. Protein expression of TLR4, and α-Syn on the enteric neuronal plexus of the colon of healthy C57BL/6J mice, measured by IHC. A DAPI staining. B TLR4 staining. C α-Syn staining. D Overlay of DAPI (blue), TLR4 (red) and α-Syn (green) staining. Overlap between TLR4 and α-Syn (yellow) was not found (see inset in D).

To determine whether the TLR expression could be located on neurons, flow cytometry analysis of cell suspensions of whole colonic and ileum tissue was performed. Neurons in the ileum and the colon of mice showed expression of both TLR2 and TLR4 (fig. 9&10). Neurons in the colon of PD mice expressed significantly less TLR4 than neurons in the colon of control mice ($p = 0.0292$, fig. 10F), no other differences were found in neuronal TLR expression between both groups (fig. 10A, B, E, I, J). When analyzing non-neuronal cells, cells derived from the colon of PD mice expressed significantly less TLR2 than the control sample ($p = 0.0434$, fig. 10D), no other differences were found in non-neuronal TLR expression between both groups (fig. 10C, G, H, K, L). Additionally, there were no significant differences in the percentage of neurons or other cells expressing neither TLR2 nor TLR4 (data not shown).

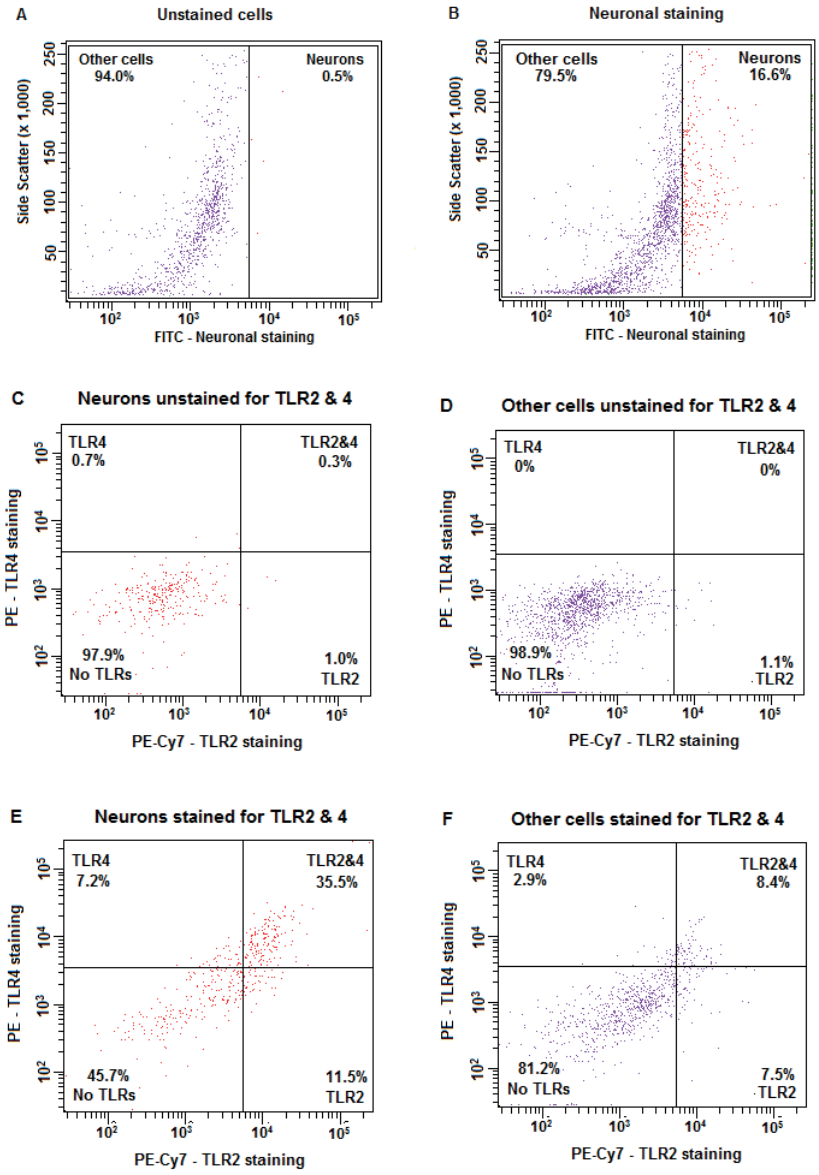


Figure 9. Gating strategy for neuronal, TLR2 and TLR4 staining. Intensity of FITC neuronal staining is plotted against cell size/complexity (side scatter) (A&B), or intensity of PE-Cy7 TLR2 staining is plotted against intensity of PE TLR4 staining (C-F). Neurons are shown in red, non-neuronal cells are shown in purple. A Gate for neurons set in an unstained sample, showing 0.5% false positive staining. B Gate for neurons shown in a β-III tubulin stained sample, showing 16.6% of the cells are neurons. C&D Gate for TLR2 and TLR4 staining set in a sample stained for neurons, but unstained for TLRs (same sample as B), showing 0.3% false positive double staining in neurons (C) and 0% false positive double staining in other cells (D). E&F Gate for TLR2 and TLR4 shown for neurons (E) and for other cells (F) in a representative sample, showing 35.5% double positive staining in neurons and 8.4% positive staining in other cells.

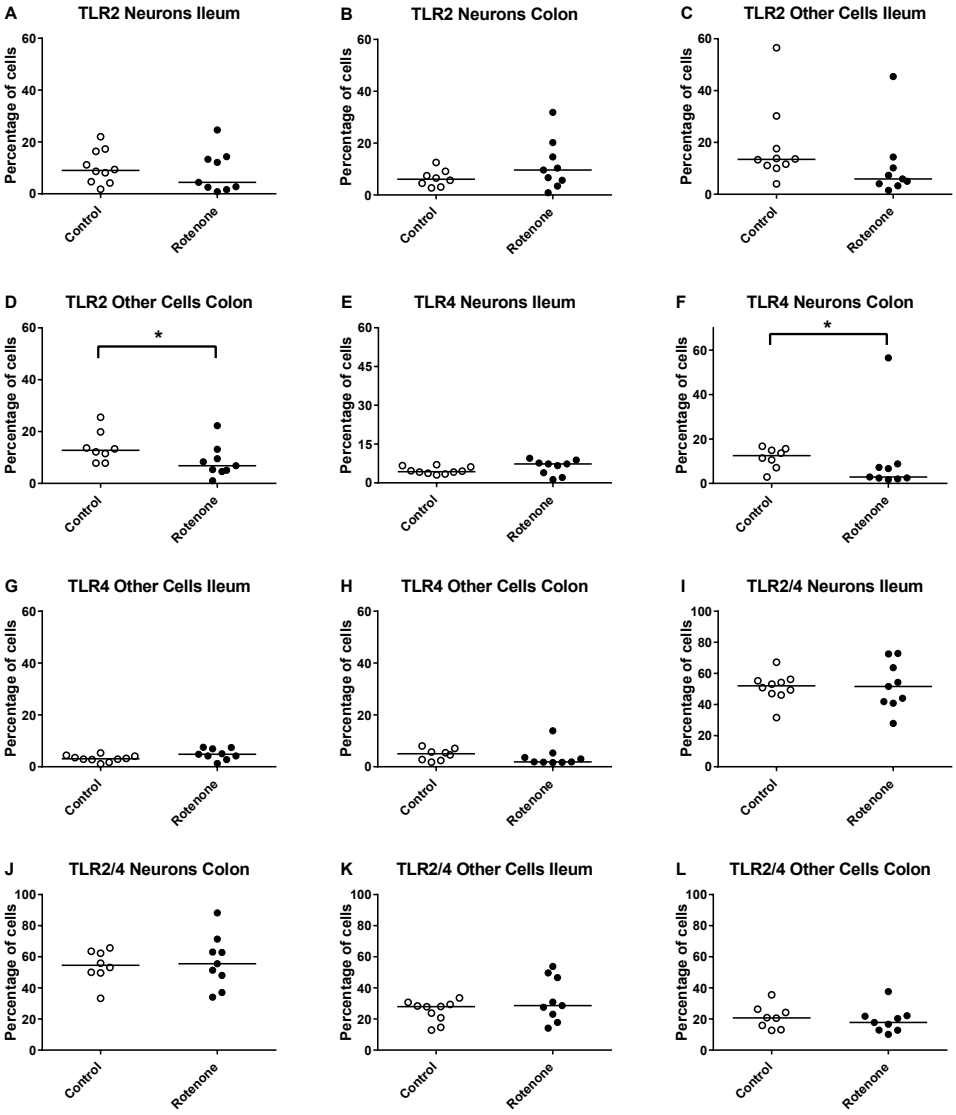


Figure 10. Expression of TLR2 and TLR4 in the ileum and colon of control and induced mitochondrial dysfunction PD mice, using flow cytometry. Expression was determined in neurons and in other cells. Differences were statistically determined by Mann-Whitney test, with statistical significance set at p=0.05, indicated by *. The horizontal bars represent the median. A-C, E, G-L No significant differences were found in the percentages of neurons expressing TLR2, TLR4 and TLR2/4 in the ileum, neurons expressing TLR2 and TLR2/4 in the colon, other cells expressing TLR2, TLR4 and TLR2/4 in the ileum, and other cells expressing TLR4 and TLR2/4 in the colon. D Significantly less non-neuronal cells express only TLR2 in rotenone treated colon compared to control colon (p = 0.0434). F Significantly less neurons express only TLR4 in rotenone treated colon compared to control colon (p = 0.0292). Representative dot plots of the flow cytometry experiment can be found in figure 9.

Discussion

The ENS is an early pathogenic site in a significant portion of PD patients[39,40,47–49], and is potentially involved in the initiation of this neurodegenerative disease[50,51]. Neuroinflammation, and specifically TLRs, play an important role in the disease process of PD[45]. It is known that PD patients have an altered microbiome which is correlated with motor symptoms[26], and these altered microbes are hypothesized to trigger α-Syn misfolding in the nervous system in the gut via inflammation[27] and/or molecular mimicry[56]. Since TLRs are very important in recognizing microbes, it is not surprising that an upregulation of TLR2 and TLR4 has been found in the sigmoid mucosa of a subset of PD patients (Keshavarzian, personal communication). Despite the importance of both the ENS and TLRs in the context of PD, the relationship between enteric neurons and TLRs in PD has not yet been extensively studied. Therefore we have examined the IM-FEN cell line[52] and murine primary enteric neurons as potentially valuable models to study the involvement of TLRs of the enteric nervous system in PD. Our results show IM-FEN cells and murine primary enteric neurons express TLRs. Differentiated IM-FEN cells express high levels of mRNA for TLR2-4, and also express mRNA for TLR1, and TLR5-7. These results were obtained by PCR and qPCR, providing solid evidence that enteric neurons might have the machinery necessary to respond to pathogen associated molecular patterns and damage-associated molecular patterns. Protein expression of TLR2-5 and TLR7 was subsequently determined by flow cytometry, ICC, and western blot. Differentiated IM-FEN cells were found to express TLR2-4 and TLR7, but TLR5 protein expression was not detectable. IHC and western blot analysis showed that TLR2 expression differed between undifferentiated and differentiated IM-FEN cells, showing a decrease in expression after differentiation in younger cells, and an increase in expression after differentiation in older cells. Why this difference between younger and older cells occurs is not known, but it is known that cell lines change as they get to higher passage numbers[57]. Since TLR expression seems to change as IM-FEN cells get older, future TLR research using IM-FEN cells would be well advised to track TLR expression over time. TLR2 was present on the cell surface of 37.0%-37.6% of differentiated cells but was also found to be localized to the nucleus. TLR2 is known to respond to gut bacteria-associated danger signals[58], which are located outside the cell, so the surface staining of neuronal cells was expected. However, the nuclear staining was unexpected. Nuclear staining was also found for α-Syn, which corresponds to earlier findings of nuclear localization of α-Syn in olfactory and central nervous system (CNS) neurons during mouse embryonic development[59]. It could be that the nuclear localization of both α-Syn

and TLR2 is due to the embryonic origin of the cell line, although to our knowledge nuclear expression of TLRs has not been previously reported. The similarity between the expression patterns of TLR2 and α-Syn is in line with previous results, which showed that TLR2 interacts with microglial α-Syn *in vitro* and *in vivo*[20], Therefore it can be hypothesized that our results indicate an interaction between both proteins in the IM-FEN cell line. Thus the presence of α-Syn makes the IM-FEN cells not only useful for future studies of enteric neuronal TLRs in general, but makes them specifically suitable for the study of PD related questions. TLR4 was only detectable using ICC. The staining was localized at the membrane, and not at the nucleus. Responses of differentiated IM-FEN cells to the classic TLR4 ligand lipopolysaccharide has already been reported[60] and observed in our own laboratory (unpublished results). Since TLR4 is known to be involved in PD and in α-Syn induced inflammation[41,45], the presence of functional TLR4 adds to the suitability of IM-FEN cells for further research into the role of the ENS in (early) PD.

The expression of TLR2 and TLR4 on primary intestinal cells and tissue was studied, both to verify the results obtained from the IM-FEN cell line, and to determine the suitability of these methods to study enteric neuronal TLRs for future PD research. The focus was on TLR2 and TLR4, because clinical research has shown these TLRs to be important in PD[44–46]. As previously reported[61] TLR4 expression was observed in the ENS in mouse neuronal plexus of colon, and here we report TLR2 positive staining using IHC in the myenteric neuronal plexus in the colon of mice. Additionally, we have demonstrated that the tissue staining for TLR2 in the myenteric plexus co-localizes with staining for α-Syn, and this was also observed for TLR4[61]. Although this co-localization cannot be traced back to any specific cell type in the myenteric plexus it is of interest to note that this co-localization indicates a potential interaction between TLRs and α-Syn in the ENS. Using flow cytometry we have demonstrated that a significant portion of primary enteric neurons in both the ileum and the colon are positive for both TLR2 and TLR4. We hypothesize that an interaction between TLRs and αSyn in the ENS could occur specifically in neurons, and this could be further studied in mouse models of PD. A significant decrease in TLR4 expression was found in neurons in the colon of PD mice compared to control mice, while no difference was found for non-neuronal cells. A significant decrease in TLR2 expression was found in non-neuronal cells in the colon of PD mice compared to control, while no difference was found in the neurons. These results show that the most pronounced effects of the mitochondrial dysfunction-associated murine PD model were found in the colon, and that effects on TLR expression of neurons do not mirror the effects seen in non-neuronal cells.

Therefore it is of great interest to further study TLR expression in the ENS of PD models on neurons, because these cells are specifically affected by the disease and they show differential TLR expression compared to other cells. The differential expression of TLR2 and TLR4 in control and PD mice is of great interest in light of the altered microbiome in PD patients[26], offering two potential receptors that could mediate the consequences of the altered microbiome for motor symptoms in patients[26] and the potential role of the microbiome on α-Syn misfolding in the ENS[26,27].

The positive staining of IM-FEN cells for TLR3 and TLR7 is interesting because these are viral sensing TLRs. A pathogenic infection in the ENS could potentially trigger PD, and a virus is a likely candidate as suggested by the Braak hypothesis[51]. It is already known that neuronal TLR3 is important for neuronal development[62,63]. Our results, combined with knowledge from literature, raise the question whether viral sensing TLRs trigger viral induced neuroinflammation and neurodegeneration in the context of (early) PD. It is yet unknown whether TLR3 and TLR7 are differentially expressed in the ENS or brain of PD patients compared to controls, or whether this is related to disease initiation or progression. Future pre-clinical and clinical studies together can determine the importance and role of viral sensing TLRs in PD. Taken together, these results show that IM-FEN cells, primary enteric neurons, and the ENS in intestinal tissue derived from mice are attractive models for future PD research into the role of enteric neuronal TLR functions, because both the neuronal TLRs and the PD-related protein α-Syn are present in these intestinal cells and tissues, and changes in TLR expression occur in a mitochondrial dysfunction-associated murine PD model. Future experiments studying neuronal TLRs in the intestinal tract in the context of PD have many questions to answer, and will ultimately be aimed at determining the protective or detrimental role of these receptors. Potential research topics include the response of IM-FEN or primary ENS neurons to TLR stimulation, for instance pro-/anti-inflammatory effects, induction or prevention of apoptosis, the development of α-Syn aggregation and Lewy pathology, release of neurotransmitters/-peptides and immunological messenger molecules, changes in electrical activity, differential responses to signaling molecules after TLR stimulation, interaction between neuronal TLRs and α-Syn in the gut of murine PD models, and most importantly changes in neuronal TLR expression, and interactions between neuronal TLRs and α-Syn in the gut of patients. Furthermore, it would be interesting to study the effects of TLR blocking or stimulation in the ENS in murine PD models; and the effects of complete or enteric neuron-specific TLR knock-out in murine PD models. Differential effects between different TLRs are to be expected in all these experimental settings, since the receptors respond to different types of stimuli,

and initiate different types of responses as a result[64]. The most compelling evidence for a role of TLRs in PD so far has been found for TLR2 and TLR4, therefore these TLRs should be studied first, and the runner-up is TLR3. Other TLRs should be studied too, since their importance for PD is not yet clear and should be elucidated. The relevance of future experiments to better understand enteric neuronal TLRs in PD cannot be overstated. These receptors could prove to be viable biomarkers for the diagnosis of early PD, since digestive function and neurons in the ENS are known to be affected early in PD[39,40,47–49], and upregulated TLR2 and TLR4 have already been found in the sigmoid mucosa of PD patients (Keshavarzian, personal communication). Knowledge of TLRs in the ENS of PD patients could also help answer the question whether a pathogen, potentially a bacterium related to the intestinal microbiome[26,27] or a virus[51], is indeed responsible for the initiation of PD via the gut. If certain TLRs are differentially expressed on neurons in the intestinal tract of PD patients, this could help focus the search for an invader to a specific group of pathogens known to interact with those specific TLRs. Finally, TLRs could also be important targets for future PD therapy. If pathogens are indeed the initial trigger for PD in enteric neurons, TLRs might be the very first gatekeepers to interact with this dangerous microbe, offering a potential target to prevent PD in people exposed to these pathogens. It is known that α-Syn can spread between neurons[30], and can act as a damage-associated molecular pattern by stimulating TLR2[20]. Therefore, if toxic α-Syn spreads between neurons and acts as a damage-associated molecular pattern during the development of PD, TLRs could also offer a potential target for treatment of patients at any stage of the disease.

Conclusion

Previous research has implicated TLRs as well as the ENS in the disease process of PD. The presence of TLR2-4, TLR7 and α-Syn on the IM-FEN cell line, the presence of TLR2 and TLR4 on primary enteric neurons, and the co-localization of α-Syn and TLR2 and TLR4 in the ENS strengthens our believe that enteric neuronal TLRs provide a valuable topic for future PD research. Several avenues for new experiments have been described, showing a wide range of options for researchers in this field. A more thorough understanding of enteric neuronal TLRs could help identify biomarkers for early diagnosis of PD, it could focus the search for pathogenic triggers for PD, and to find new targets for future therapies.

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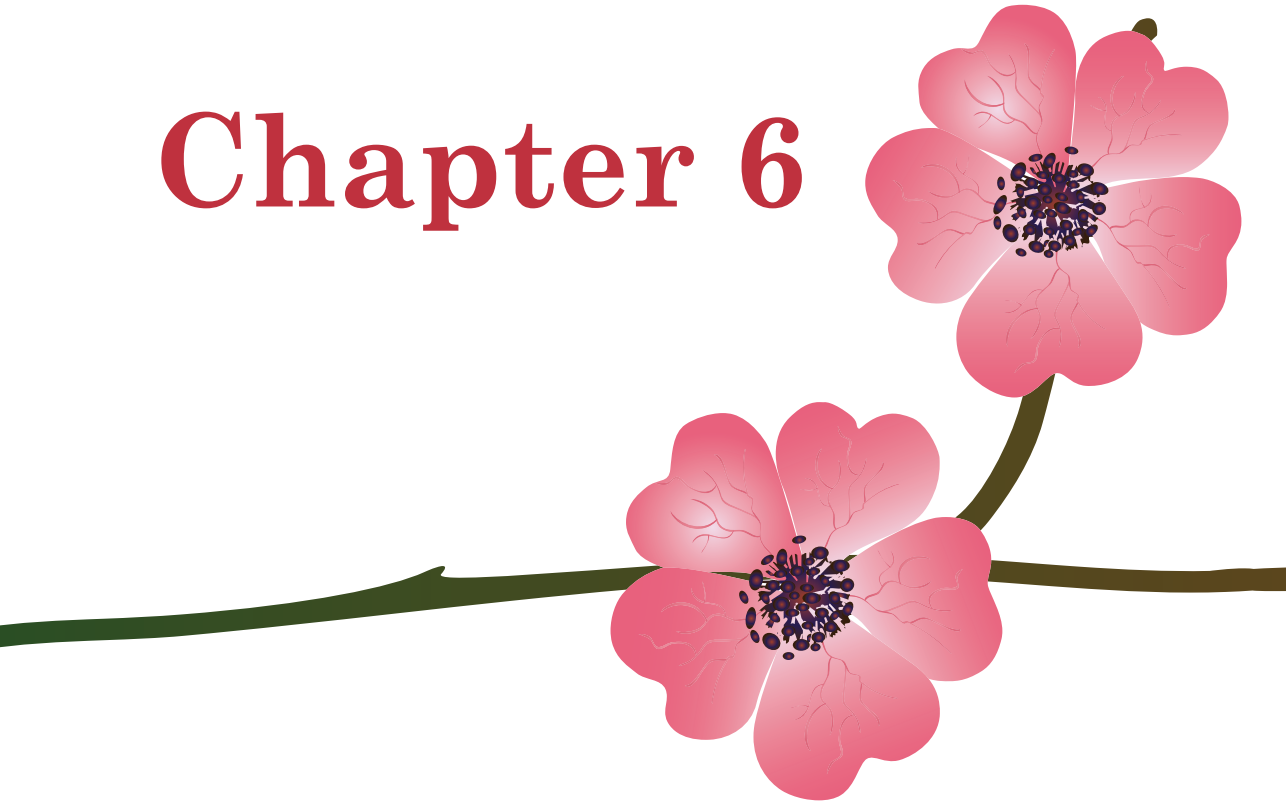
Chapter 6



C.D. Rietdijk[1], D.J. Doorduijn[1], M.M.A.E. Claessens[2], J. Garssen[1,3], R.J.A. van Wezel[4,5,] S. Srinivasan[6], A.D. Kraneveld[1]

1. Div. of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Universiteitsweg 99, 3584 CG Utrecht, the Netherlands
2. Nanobiophysics, MESA+ and MIRA, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, the Netherlands
3. Nutricia Research, Utrecht Science Park, Uppsalalaan 12, 3584 CT Utrecht
4. Biomedical Signals and Systems, MIRA, University of Twente, Drienerlolaan 5, 7522 NB, Enschede, the Netherlands
5. Dept. of Biophysics, Donders Institute for Brain, Cognition and Behaviour, Radboud University Nijmegen, PO box 9010, 6500 GL, Nijmegen, the Netherlands
6. Dept. of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, USA

To be submitted

Neuroinflammatory effects of lipopolysaccharide and α-Synuclein monomers and fibrils in the enteric nervous system

Abstract

The enteric nervous system (ENS) is an early pathogenic site in Parkinson's disease (PD). Early in the disease patients develop hallmark α-Synuclein (α-Syn) aggregates in the ENS. The appearance of these α-Syn aggregates positively correlates with increased epithelial (gut)barrier permeability. At the same time PD patients show increased colonic inflammation, and have a different gut microbiome composition. To determine how enteric neurons contribute to inflammation in the intestinal tract in PD, murine immorto fetal enteric neuronal cell line (IM-FEN) cells were exposed to the Toll-like receptor (TLR)-4 ligand lipopolysaccharide (LPS), and monomers and fibrils of recombinantly produced α-Syn. Aggregated α-Syn might contribute to inflammation in the ENS, and α-Syn fibrils might have the ability to seed α-Syn aggregation within neurons, and aggregated α-Syn is thought to be neurotoxic. α-Syn fibrils mimic the toxic α-Syn aggregates found in PD. Therefore we hypothesize that α-Syn fibrils and LPS contribute to ENS-related inflammation.

LPS and α-Syn monomers (separately) increased interleukin (IL)-1β mRNA expression, which we suggest is a neuroprotective or neurotropic effect. This effect was not present after co-incubation of LPS and α-Syn monomers, while at the same time a reduction in tumor necrosis factor (TNF)-α mRNA was observed. We interpret this as a shift towards an anti-inflammatory response. Co-incubation of α-Syn fibrils and LPS triggered a pro-inflammatory response through the increase of TNF-α and IL-6 mRNA expression, and a reduction in the release of anti-inflammatory neuropeptide vasoactive intestinal peptide (VIP). α-Syn fibrils alone also acted pro-inflammatory through increased release of pro-inflammatory neuropeptide Substance P (Sub P). LPS alone also increased the release of Sub P.

Overall, the results support our hypothesis that α-Syn fibrils and LPS can cause an inflammatory response that might be involved in gastrointestinal pathologies seen in PD patients. Therefore cytokines, Sub P and VIP in the ENS provide interesting targets for future PD research and the search for new treatment options for PD symptoms.

Abbreviations

ENS – Enteric nervous system
PD – Parkinson's disease
α-Syn - α-Synuclein
IM-FEN – Murine immorto fetal enteric neuronal cell line
TLR – Toll-like receptor
LPS – Lipopolysaccharide
IL – Interleukin
TNF – Tumor necrosis factor
VIP – Vasoactive intestinal peptide
Sub P – Substance P
LP – Lewy Pathology
CNS – Central nervous system
GDNF – Glial cell derived neurotrophic factor
EDTA - ethylenediaminetetraacetic acid
PBS – Phosphate-buffered saline
GABA – Gamma- aminobutyric acid

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder with an incidence rate between 5 and 346 per 100,000 per year in Europe[1]. PD is idiopathic in most cases, while a small proportion is linked to genetic mutations located to the gene for α-Synuclein (α-Syn)[2–11]. Hallmarks of the disease are dopaminergic cell loss in the substantia nigra, and presence of α-Syn-containing aggregates in the surviving neurons, known as Lewy Pathology (LP)[12,13]. These aggregates contain fibrils of misfolded α-Syn monomers, which are considered to be toxic[13–19].

The enteric nervous system (ENS) is an early pathogenic site in PD[20–24]. Patients develop LP in the ENS early in the disease[20,21,23], and this pathology remains during later stages of the disease[23,25,26]. The ENS is suggested to be the site of initiation of PD pathology through pathogen/microbe-induced intestinal inflammation[27,28]. From the ENS, the disease is hypothesized to spread to the central nervous system (CNS) via the vagus nerve and the dorsal motor nucleus of the vagus[27–34].

The theory that PD is triggered in the ENS is supported by clinical studies that show that PD patients have a different microbiome[35], have increased colonic inflammation[36] and enhanced permeability of the epithelial barrier[37], and an increased serum lipopolysaccharide (LPS) binding protein expression which is indicative for bacterial translocation[37]. Increased epithelial barrier permeability is positively correlated with LP in the ENS, higher load of bacterial products in the deeper layers of the gut (near the ENS), and oxidative stress[37]. It has also been suggested that misfolded α-Syn, which is more prone to aggregate, induces inflammation in the ENS[38]. Therefore it is likely that changes in the microbiome, translocation of gram-negative bacteria, and increased inflammation directly affect the neurons of the ENS in the context of PD.

Toll-like receptors (TLRs) are a family of so called 'innate immune receptors' that recognize both endogenous and exogenous danger signals[39–42]. These receptors are not only expressed by epithelial cells and immune cells, but also by neurons[43–49] and glial cells[50–55]. Their expression seems to be increased in the brain of PD patients[56,57], and denatured human α-Syn has been shown to elicit a TLR4-dependent inflammatory response in primary murine astrocytes[58]. Recently, we have demonstrated an increased intestinal TLR4 expression in a murine model of PD[59], which was confirmed in biopsies of PD patients (Prof Dr Ali Keshavarzian, personal observations). Additionally, PD patients show increased levels of *E. coli* in the intestinal mucosa and LPS binding protein in the blood [37], and in mice systemic administered LPS leads to colonic barrier disruption, and an increase in α-Syn immunoreactivity in the colonic ENS and the dorsal motor nucleus of the vagus[60]. Based on the literature, we hypothesize that exposure of immorto fetal enteric

neuronal cell line (IM-FEN) cells[61] to α-Syn monomers will not yield an inflammatory response, while exposure to α-Syn fibrils or the TLR4 ligand LPS will result in a pro-inflammatory response. The exposure to LPS is a model for the pro-inflammatory milieu caused by translocation of bacteria from the intestinal lumen to the ENS, while α-Syn fibrils are a model for the toxic α-Syn species in the ENS during PD, and α-Syn monomers are a model for the non-PD affected state[62–64]. To test this hypothesis, IM-FEN cells were exposed to TLR4 ligand LPS and monomers and fibrils of recombinantly produced α-Syn. During and after exposure, the expression of cytokines, neurotransmitters and neuropeptides has been measured in both cell lysate as well as culture supernatants.

Materials & Methods

Cell culture

The IM-FEN cell line was established and characterized as described previously[61]. Cells were plated onto plastic 75-cm^2 or 175-cm^2 flasks in modified N2 medium[65] for proliferation. Modified N2 medium contains Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 medium (Invitrogen 11330-032)containing selenium (5 ug/ml; Sigma S5261), putrescine (16,11 ug/ml; Sigma P7505), progesterone (6.35 ng/ml; Sigma P6149), insulin (5ug/ml; Sigma I6634), transferrin (1ug/ml; Sigma T5391), fetuin (100ug/ml; Sigma F3385), Bovine Serum Albumin (1mg/ml; Sigma A8806), Glial cell line derived neurotrophic factor (GDNF) (100ng/ml; R&D Systems 512-GF), FCS (10%; Bodinco 3000), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin per mL; Sigma P0781) and recombinantly produced mouse interferon-γ (20 units/mL; Millipore IF005). The cells were cultured in a humidified incubator containing 10% CO_2 at the permissive temperature 33°C. The cells were observed regularly for signs of proliferation and were passaged when the flask became 80% or more confluent, using trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco 25300-062).

When cells were approximately 60% confluent and differentiation into neuron-like cells was desired the medium of the cells was changed. Differentiating cells were cultured in flasks in Neurobasal-A medium (Invitrogen 10888-022) containing GDNF (100ng/mL; R&D Systems 512-GF), FCS (1%; Bodinco 3000), penicillin/streptomycin (1% of stock; 100 units penicillin and 100ug streptomycin per mL; Sigma P0781), B27 (1:50; Invitrogen 17504-044), and glutamine (1mmol/L; Invitrogen 25030-032), and placed in a humidified incubator containing 5% CO_2 at the non-permissive temperature 39°C. Differentiation was continued for 5-7 days.

Recombinant production of α-Syn monomers and fibrils

Expression and purification of human wild-type αSyn was performed as previously described by van Raaij *et al.* with some modifications[66]. Instead of the dialysis step described in[66] the protein was concentrated using a Vivaspin 20, 10 kDa cutoff ultrafiltration unit and subsequently desalted on a HiPrep 26/10 column. The final buffer concentration of the protein solution was 10 mM Tris-HCl, pH 7.4. The protein concentration was determined by measuring the absorbance on a Shimadzu spectrophotometer at 276 nm, using molar extinction coefficients of 5600 $M^{-1}cm^{-}$. The successful production of αSyn fibrils was analysed using atomic force microscopy, as previously described by Sidhu et al[67]. The protein was stored at -80 °C in the buffer mentioned above at a concentration of 250 µM.

To induce aggregation of monomeric αS into fibrils the protein was thawed and diluted to a concentration of 100 µM αS in 10mM NaCl, 100 µM Na_2EDTA, 10 mM Tris-HCL, pH 7.4. This solution was sterilized by filtration over a 0.2 µm CA-membrane (Sartorius) using a syringe and transferred to 1.5 ml Eppendorf tubes. The thus obtained samples were incubated for 8 days in a thermomixer at 37 °C at 750 rpm orbital shaking. This resulted in the formation of amyloid fibrils. To remove residual monomers the fibril containing samples were dialyzed against PBS using Biotech CE tubing with a molecular weight cutoff of 100 kD (Spectrum Labs). The fibrils were stored at 4˚C until further use.

Cell treatment

For all experiments IM-FEN cells were stimulated after 5-7 days of differentiation. LPS (Sigma 0127:B8) was dissolved in phosphate-buffered saline (PBS) (Lonza BE17-516F) and added to the culture medium in various concentrations (5, 10, 50, 100 and 500 ng/mL). Wild-type recombinantly produced α-Syn monomers and fibrils were dialyzed against PBS, and added at a concentration of 1 µM. Co-stimulation was performed at concentrations of 10 ng /mL for LPS and 1 µM for α-Syn monomers or fibrils. PBS was used as negative control. Medium was sampled at 1, 6 and 24 hours after stimulation. Cell lysate was collected after 24 hours of stimulation; trypsinization was performed using trypsin/EDTA (Gibco 25300-062), samples were spun down at 130 rcf at 4 °C for 5 minutes and resuspended in PBS. Cells were lysed by snap freezing in liquid nitrogen.

qPCR

Cell culture samples were lysed in TRIzol reagent (LifeTechnologies 15596026), after which total RNA was extracted and purified using a Nucleospin RNA-clean-up kit (Macherey-Nagel 740903). cDNA was prepared using iScript cDNA

synthesis kit (Bio-Rad 170-8890). qPCR was performed using iQ SYBR Green (Bio-Rad 170-8880). Gene-specific primers were commercially purchased (Qiagen) and their efficiency was validated for the comparative Ct method (data not shown). The comparative Ct method was used to determine relative mRNA abundance between control group and treatment group for the genes of interest normalized against housekeeping gene RPS13, using equation:

$2^{(Ct[\text{Target Control}]-Ct[\text{RPS13 Control}])-(Ct[\text{Target Treatment}]-Ct[\text{RPS13 Treatment}]}$.

ELISA

Competitive ELISA kits were used for Substance P (Sub P) (R&D Systems KGE007) and vasoactive intestinal peptide (VIP) (Bachem S-1183). All samples were thawed and spun down at 20.000 x g for 10 minutes to remove particulates. Pre-coated plates with a catching antibody were used. ELISAs were performed according to manufacturers' instructions. The optical density of non-specific binding wells (in which no primary antibody was used) was subtracted from each value found in the samples and standards and standard curves ($R2 > 0.999$) were calculated with four parameter analyses (MPM 6.0).

For Sub P, a primary antibody was added, which was caught by the catching antibody. Subsequently and simultaneously horseradish peroxidase conjugated Sub P and sample were added to the plate; conjugated Sub P competed with the sample Sub P for binding sites on the primary antibody. After incubation the plate was washed, horseradish peroxidase substrate (TMB) was added, and after incubation the reaction was stopped. Absorbance was read at 450 nm using a microplate reader (BioRad iMark).

For VIP, after adding the primary antibody, sample and biotinylated-VIP were added to the wells. After incubation the plate was washed and streptavidin-conjugated horseradish peroxidase was added. After incubation and plate washing, TMB was added, and after incubation the reaction was stopped. Absorbance was read at 450 nm using a microplate reader (BioRad iMark).

HPLC

Samples were stored at –70 °C until analysis. Lysate and supernatant samples were pre-treated in the following manner: to precipitate proteins, samples were mixed with 2.0 M perchloric acid ($HClO_4$) (Acros Organics 22331). Subsequently samples were centrifuged for 10 minutes at 15.000 x g at 4°C. The resulting supernatants were mixed with 1.0 M NaOH (Acros Organics 38021) to stabilize pH (pH > 8). To remove any particulate matter samples were filtered through a 0.2 µm filter (Millex-GV, Millipore) using a centrifuge. Gamma-aminobutyric

acid (GABA) was detected by UHPLC with electrochemical detection using an Alexys 110 LC-EC analyzer (Antec, The Netherlands). The system consisted of two pumps, one autosampler with a 1.5µl loop, a column (Acquity UPLC HSS T3 1.0 x 50 mm, 1.8 µm particle size, Waters, Milford USA), a flow cell detector with glassy carbon working electrode and ISAAC reference electrode (potential setting +0.70 V). The column and detector cell were kept at 40°C in a column oven. During analysis the samples were kept at 4°C in the autosampler. The chromatogram was recorded and analyzed using a Clarity data system (Antec). Primary amino acids in the sample were derivatized pre-column[59] using a reagent consisting of 37.5 mM o-Phtalaldehyde (OPA)(Pickering Laboratories, USA O120), 50 mM Sodium sulphite (Sigma S4672), 90 mM Sodium borate (Boric Acid Merck 1.00165 & NaOH) buffer pH 10.4. This reagent was prepared by mixing a 0.75M OPA solution (prepared in methanol) with a 1M Sodium sulphite solution (in water) and a 0.1 M Sodium borate buffer pH 10.4 (mixing ratio 1:1:18). The derivatisation was performed automatically in-line using the autosampler. Nine µl sample was mixed with 0.5 µl reagent just prior to the analysis. Separation was achieved using mobile phase A (50 mM phosphoric acid (Acros Organics 42404), 50 mM citric acid (Acros Organics 12491), 8 mM KCl (Acros Organics 19677), 0.1 mM EDTA (Acros Organics 40997) pH 3.25, 2% Acetonitril (Biosolve BV 012007), 4% Methanol (Biosolve BV 136806)). As soon as the compounds of interest were completely detected a step gradient using mobile phase B (50 mM phosphoric acid, 50 mM citric acid, 8 mM KCl, 0.1 mM EDTA pH 3.25, 60% Acetonitril) was applied to rinse the column from any late eluting compounds. The flow rate was set at 0.2 ml/min.

Statistics

Data from qPCR, ELISA and HPLC were statistically analyzed using a Kruskal-Wallis test combined with a Dunn's multiple comparisons test using an alpha value of 0.05, after removal of outliers found by ROUT analysis with a Q value of 1.0. Outliers were only removed when a source for their abnormal value was found that made the data points untrustworthy, other outliers were maintained in the datasets. The following groups were compared in the Kruskal-Wallis test: control was compared to all other groups; LPS10 was compared to LPS10 + α-Syn monomer, and LPS10 + α-Syn fibril; α-Syn monomer was compared to α-Syn fibril, and LPS10 + α-Syn monomer; LPS10 + α-Syn fibril was compared to α-Syn fibril, and LPS10 + α-Syn monomer. No other comparisons were made, to increase the power of the analysis. Data were analyzed using Graphpad Prism 6.0.

Results

LPS, and α-Syn monomers and fibrils differentially induced cytokine mRNA expression

Interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α are expressed on mRNA level by differentiated IM-FEN cells (fig. 1A-C). Exposure of differentiated IM-FEN cells to LPS for 24 hours seems to induce a dose dependent increase in IL-1β mRNA expression (fig. 1A), though this did not reach statistical significance. α-Syn monomers induced a similar response (fig. 1A).

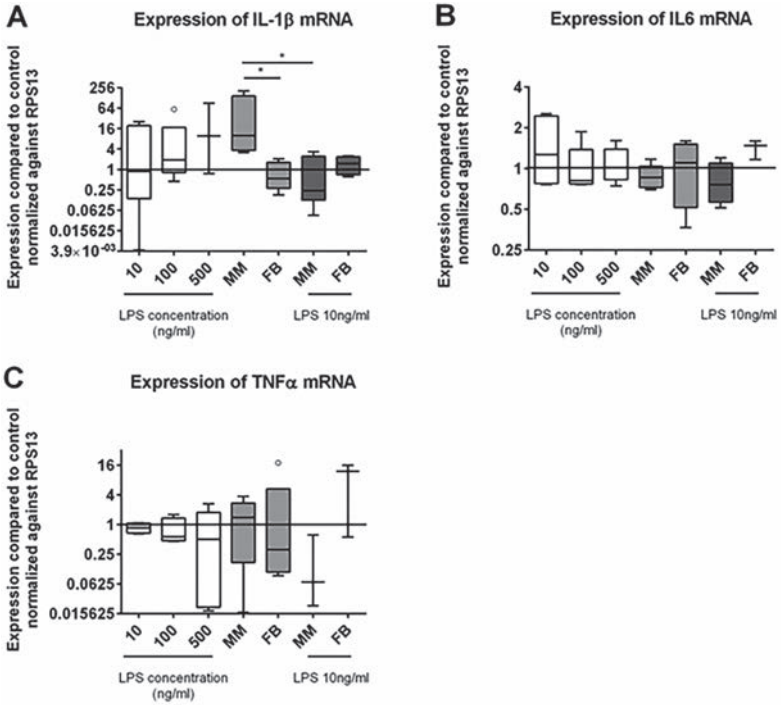


Figure 1. Expression of cytokine mRNA in differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (5-500 ng/mL), α-Syn monomers (1 μM), α-Syn fibrils (1 μM), or a combination (LPS 10 ng/mL and α-Syn monomers or α-Syn fibrils 1 μM). Differences were statistically determined by Kruskal-Wallis test and Dunn's multiple comparisons tests, graphs show quartiles. * = $p < 0.05$

The Y-axis are logarithmic, since the data were analyzed using the ΔΔ-Ct method, and multiplication of gene products (as measured by their Ct-values) is a logarithmic process. Genes of interest were normalized against housekeeping gene RPS13, and treated groups were compared to the control group. The control group is indicated by the lines at value '1'.

IL-1β n = 3 (LPS 500 ng/mL), 4 (LPS 10 ng/mL, LPS 10 ng/mL + Fibril), 5 (Monomer, LPS 10 ng/mL + Monomer, fibril), 6 (LPS 100 ng/mL).

IL6 n =3 (LPS 10 ng/mL + Fibril), 4 (LPS 10 ng/mL + Monomer, Fibril), 5 (LPS 500 ng/mL, Monomer), 6 (LPS 10 ng/mL, LPS 100 ng/mL).

TNFα n = 3 (LPS 10 ng/mL + Monomer, LPS 10 ng/mL + Fibril), 4 (LPS 10 ng/mL, LPS 100 ng/mL), 5 (LPS 500 ng/mL, Monomer), 6 (Fibril).

Co-incubation with LPS (10 ng/ml) abolished the α-Syn monomer enhanced IL-1β mRNA expression (fig. 1A). α-Syn fibrils did not affect IL-1β mRNA expression. No significant differences were induced by LPS, α-Syn monomers or α-Syn fibrils for IL-6 and TNF-α mRNA expression (fig. 1B, 1C). However, co-incubation with LPS (10 ng/ml) and fibrils increased IL-6 and TNF-α mRNA expression (fig. 1B, 1C), while co-incubation with LPS (10 ng/ml) and monomers reduced TNF-α mRNA expression (fig. 1C), compared to the control group.

IM-FEN cells release increased levels of Sub P in response to a combination of LPS and α-Syn fibrils

The neuropeptide Sub P was found in both the supernatant and the cell lysate of differentiated IM-FEN cells (fig. 2 & 3). Incubation of differentiated IM-FEN cells with LPS 5 to 50 ng/ml for 6 to 24 hours induced the release of Sub P as assessed by increased levels in the supernatant and reduced levels in the cell lysate (fig. 2A-C), which seems to be a dose-dependent response.

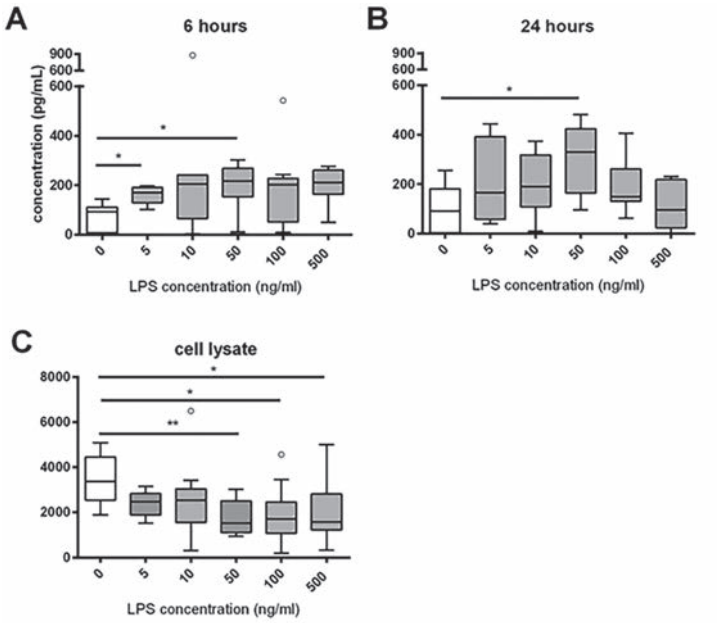


Figure 2. Sub P in supernatant and cell lysate of differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (5-500 ng/mL).
Differences between the control group and other groups were statistically determined by Kruskal-Wallis and Dunn's multiple comparisons tests, graphs show quartiles.
* = $p < 0.05$ ** = $p < 0.01$
Supernatant 6h n= 6 (LPS 5 ng/mL), 7 (LPS 10 ng/mL), 8 (Others), 9 (LPS 100 ng/mL)
Supernatant 24h n = 6 (LPS 50 ng/mL), 8 (Others), 10 (LPS 100 ng/mL).
Cell lysate n = 10.

After 24 hour incubation with the higher LPS doses (100 and 500 ng/ml) a decreased release of Sub P was observed in the supernatant (fig. 2B). Incubation with α-Syn fibrils, but not monomers, resulted in a significant release of Sub P in the supernatant when compared to control after 6 hours (fig. 3A), and shows a trend at 24 hours (fig. 3B). At 24 hours this α-Syn fibril response was significantly inhibited by co-incubation with LPS (fig. 3B). α-Syn fibril incubation alone induced a significant reduction of Sub P in the cell lysate when compared to control cells, which mirrored the Sub P release observed after 24 hours (fig. 3C). In contrast to the effect measured in the supernatant, co-incubation with LPS (10 ng/ml) and α-Syn fibril reduced the Sub P levels in the cell lysate even further (fig. 3C).

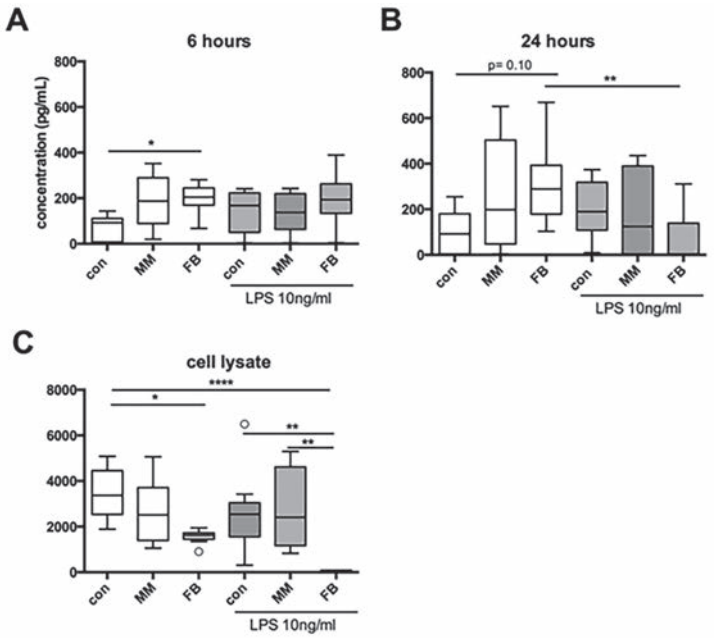


Figure 3. Sub P in supernatant and cell lysate of differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (10 ng/mL), α-Syn monomers (1 μM), α-Syn fibrils (1 μM), or a combination (LPS 10 ng/mL and α-Syn monomers or α-Syn fibrils 1 μM).
Differences were statistically determined by Kruskal-Wallis test and Dunn's multiple comparisons tests, graphs show quartiles.
* = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$ **** = $p < 0.0001$
Supernatant 6h n = 7 (LPS 10 ng/mL), 8 (Others), 10 (Fibril), 12 (LPS 10 ng/mL + Fibril).
Supernatant 24h n = 8 (Others), 10 (Fibril), 12 (LPS 10 ng/mL + Fibril).
Cell lysate n = 10.

IM-FEN cells release less VIP upon stimulation with α-Syn fibrils

VIP was detected in both the supernatant and the cell lysate of differentiated IM-FEN cells (fig. 4 & 5). LPS and α-Syn monomers did not significantly change the release of VIP by neuronal cells after 6 hours (fig. 4A & 5A). After 24 hours of treatment, the concentration of VIP was reduced to almost undetectable levels (data not shown).

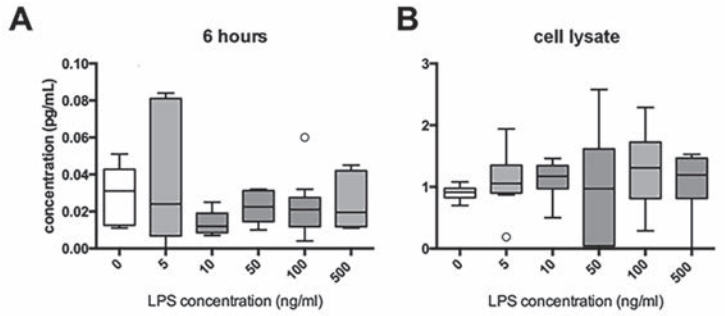


Figure 4. Vasoactive intestinal peptide in supernatant and cell lysate of differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (5-500 ng/mL).
Differences between the control group and other groups were statistically determined by Kruskal-Wallis test and Dunn's multiple comparisons tests, graphs show quartiles.
Supernatant 6h n=6 (Others), 8 (LPS 10 ng/mL), 10 (LPS 100 ng/mL).
Supernatant 24h n = 6 (Others), 8 (LPS 10 ng/mL) 12 (LPS 100 ng/mL).
Lysate n = 6 (control), 8 (LPS 10 ng/mL) 10 (Others), 14 (LPS 50 ng/mL).

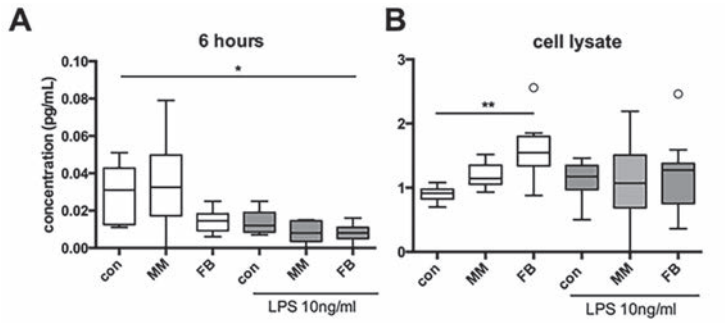


Figure 5. Vasoactive intestinal peptide in supernatant and cell lysate of differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (10 ng/mL), α-Syn monomers (1 μM), α-Syn fibrils (1 μM), or a combination (LPS 10 ng/mL and α-Syn α-Syn monomers or α-Syn fibrils 1 μM).
Differences were statistically determined by Kruskal-Wallis test and Dunn's multiple comparisons tests, graphs show quartiles.
* = $p < 0.05$, ** = $p < 0.01$
Supernatant 6h n=6 (Others), 8 (LPS 10 ng/mL), 10 (Fibril), 12 (LPS 10 ng/mL + Fibril).
Supernatant 24h n = 6 (Others), 8 (LPS 10 ng/mL) 12 (Fibril), 14 (LPS 10 ng/mL + Fibril).
Lysate n = 6 (control), 8 (LPS 10 ng/mL) 10 (Others), 12 (LPS 10 ng/mL + Fibril).

The supernatant of neuronal cells incubated for 6 hours with LPS 10 ng/ml + fibrils contained significantly less VIP than control (fig. 5A). In the cell lysate of the α-Syn fibril-treated differentiated IM-FEN cells the concentration of VIP was significantly higher than in controls (fig. 5B).

GABA concentration in supernatant increases in response to a combination of LPS and α-Syn fibrils

GABA was detectable in both the supernatant and the cell lysate of differentiated IM-FEN cells (fig. 6 & 7). Exposure of neuronal cells to different doses of LPS did not affect GABA content or release (fig. 6A-D).

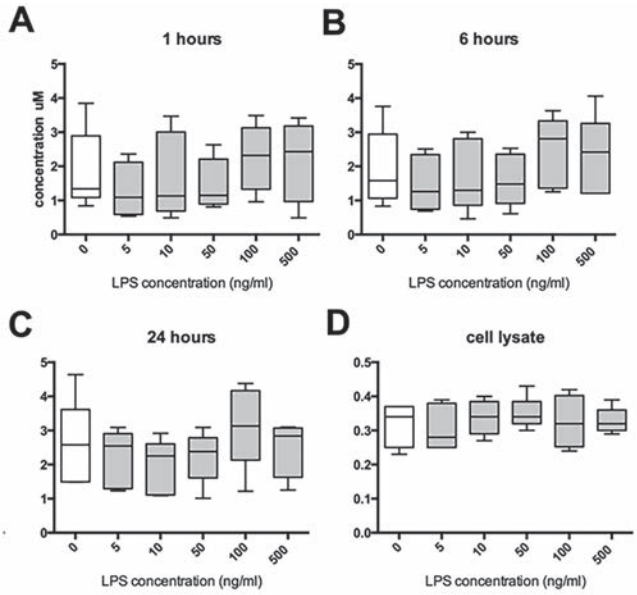


Figure 6. Gamma-aminobutyric acid (GABA) in supernatant and cell lysate of differentiated IM-FEN cells after stimulation with TLR4 ligand LPS (5-500 ng/mL).
Differences between the control group and other groups were statistically determined by Kruskal-Wallis test and Dunn's multiple comparisons tests, graphs show quartiles.
Supernatant 1h n = 4 (LPS 500 ng/mL), 5 (Others).
Supernatant 6h, and 24h n = 5.
Lysate n = 4 (LPS 100 ng/mL), 5 (Others).

However, with increasing incubation time GABA concentration in the supernatant was increased when cells were stimulated with a combination of LPS 10 ng/ml and α-Syn fibrils, when compared to cells stimulated with LPS 10 ng/ml alone. This was a weak trend at 1 hour (p=0.13) and a strong trend at 24 hours (p=0.07) (fig. 7A-C). A concomitant decrease of GABA in the cell lysate was also found (n.s.)(fig. 7D).

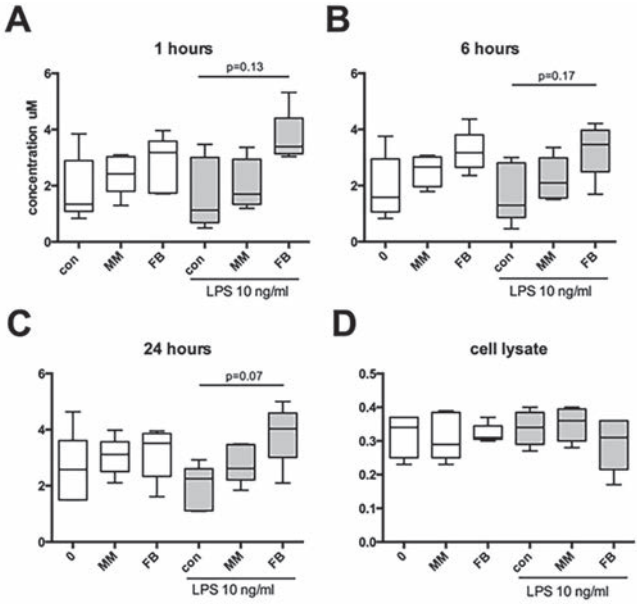


Figure 7. Gamma-aminobutyric acid (GABA) in supernatant and cell lysate of differentiated IMFEN cells after stimulation with TLR4 ligand LPS (10 ng/mL), α-Syn monomers (1 μM), α-Syn fibrils (1 μM), or a combination (LPS 10 ng/mL and α-Syn monomers or α-Syn fibrils 1 μM). Differences were statistically determined by Kruskal-Wallis test, graphs show and Dunn's multiple comparisons tests, graphs show quartiles. Trends (p-values) are presented above the columns. Supernatant 1h, 24h, and lysate n = 5. Supernatant 6h n = 4 (Monomer), 5 (Others).

Discussion

PD patients are known to suffer from intestinal inflammation and gastrointestinal dysfunction[36,68,69]. In addition to this, their microbiome differs from healthy controls[35], and the permeability of their epithelial barrier in the intestines seems to be increased, which is associated with translocation of gram-negative bacteria[37]. Therefore it is not surprising that the ENS is an early pathogenic site in PD[20–24], including the occurrence of Lewy Pathology (LP) in enteric neurons early in the disease[20,21,23], forming lesions that remain later on in the disease[23,25,26]. It has been hypothesized that this pathology is triggered by pathogen/microbe-induced intestinal inflammation[27,28].

The response of neurons to pathogen-associated molecular patterns in the context of intestinal inflammation is a crucial missing link, since neurons are the cells that eventually become affected by LP via the toxic aggregation of misfolded α-Syn monomers[13–19,62–64], potentially after exposure to a pathogen or as a result of inflammatory conditions. Therefore the relationship between pathogen-associated molecular patterns and enteric neurons is potentially paramount to disease

initiation and disease progression in PD. Our results show that differentiated neurons of the IM-FEN cell line are able to respond to the TLR4 ligand LPS, and to α-Syn monomers and fibrils, with distinct immune-related signals. A broad overview of all the results can be found in table 1.

Changes in the expression of cytokines IL-1β, IL-6 and TNF-α were measured, because these cytokines are known to be important during neuroinflammation and neurodegeneration[70–72]. The mRNA expression patterns of the cytokines support our hypothesis that α-Syn fibrils act pro-inflammatory, while α-Syn monomers do not. However, the effect of LPS is more complex than the hypothesized pro-inflammatory action.

The increase in IL-1β mRNA in response to both LPS and α-Syn monomers, but not α-Syn fibrils, is an unexpected result. IL-1β is generally considered to be pro-inflammatory[73–75], and to be harmful in rodent PD models[76–79] and in PD patients[36,80–84]. At the same time α-Syn fibrils seem to be more toxic to neurons than monomers[13,15,16,62] and are thought to seed LP[63,64,85]. Therefore an increase in IL-1β mRNA was expected as a result of exposure of IM-FEN cells to the pro-inflammatory molecules LPS and α-Syn fibrils, but not as a result of exposure to the α-Syn monomers. We therefore hypothesize that in our experiments IL-1β is not a pro-inflammatory signal[86]. It has been shown that in the intestines IL-1β stimulates intestinal smooth muscle cells to release GDNF, which stimulates neurite outgrowth[87]. In other parts of the nervous system it has been shown that IL-1β is neuroprotective in the context of excitotoxicity[88], an effect that is dependent on timing and concentration[89]. IL-1β is also important for the plasticity of the brain, and acts as a neurotrophic factor during prenatal development[86], an interesting fact considering that the IM-FEN cell line is derived from embryos. Thus, IL-1β is likely a neuroprotective or neurotropic signal, and not pro-inflammatory. Additionally, LPS has previously been shown to have a positive effect on enteric neuronal survival in primary mouse enteric neurons at concentrations similar to our experiments (10 ng/mL to 1000 ng/mL)[90], giving credence to the non-inflammatory action of LPS in these experiments. There is no correlation between enteric LP and IL-1β expression in the colon of patients[36], which supports the lack of IL-1β-response to fibrils in these experiments. Together these results indicate that the increase of IL-1β in response to either LPS or α-Syn monomers could be a neuroprotective or neurotrophic signal, instead of pro-inflammatory.

Co-incubation of IM-FEN cells with LPS and α-Syn monomers completely inhibited the neuroprotective IL-1β response, which could indicate an initiation of an inflammation under influence of a combination of both substances. However, it seems more likely that this reduction in the IL-1β-response is part of a shift towards an anti-inflammatory response, since exposure of IM-FEN cells to a combination of

LPS and α-Syn monomers also reduced the expression of TNF-α. TNF-α promotes intestinal inflammation through epithelial barrier disruption[91,92], a process which is important in PD[37], therefore the decrease in TNF-α mRNA after co-incubation of LPS and α-Syn monomers indicates an anti-inflammatory effect.

Table 1. An overview of the combined results.

	LPS	α-Syn		α-Syn + LPS	
		MM	FB	MM	FB
	mRNA				
IL-1β	↑ a	↑ a	↓ b, p<0.05	↓ b, p<0.05	–
IL6	–	–	–	–	↑ a
TNF α	–	–	–	↓ a	↑ a
	Substance P				
supernatant	↑ a, p<0.05	–	↑ a, p<0.05	–	↓ c, p<0.01
cell lysate	↓ a, p<0.05	–	↓ a, p<0.05	–	↓ a, p<0.0001
	Vasoactive intestinal peptide				
supernatant	–	–	–	–	↓ a, p<0.05
cell lysate	–	–	↑ a, p=0.01	–	–
	Gamma-aminobutyric acid				
supernatant	–	–	–	–	↑ d, p=0.07
cell lysate	–	–	–	–	–

↑: enhanced mRNA or protein expression; ↓: reduced mRNA or protein expression; MM: monomeric α-synuclein; FB: α-synuclein fibrils.
a: compared to control or baseline; b: compared to MM alone; c: compared to FB alone; d: compared to LPS alone, e: compared to LPS and monomers combined, f: compared to LPS and fibrils combined

At the same time, co-incubation of IM-FEN cells with LPS and α-Syn fibrils increased TNF-α mRNA expression, which indicates a pro-inflammatory action. This pro-inflammatory action of co-incubation with LPS and α-Syn fibrils was also observed by an increase in IL-6 mRNA, as IL-6 has pro-inflammatory properties[93]. However, IL-6 is also involved in epithelial barrier repair and considered to be [94], in which case the increase in IL-6 could be interpreted as a repair response to the damage done to the epithelial barrier through the increase in TNF-α as a result of co-incubation with LPS and α-Syn fibrils.

Based on the cytokine mRNA expression patterns we conclude that LPS and α-Syn monomers (separately) potentially induced a neuroprotective signal in the IM-FEN cells, by increasing IL-1β. It seems that when enteric neuronal cells are exposed to both LPS and α-Syn monomers, this potentially neuroprotective IL-1β response no longer occurs, which might be the start of a pro-inflammatory condition of the

ENS. On the other hand, a reduction of TNF-α occurs at the same time, which could indicate a shift towards an anti-inflammatory response. In addition, α-Syn fibrils combined with LPS triggered an increase in TNF-α and IL-6, which could be a pro-inflammatory signal, a repair response to tissue damage in the intestines, or both. We speculate that these changes in IL-1β, IL-6 and TNF-α expression could affect neurotransmitter or neuropeptide release patterns in the intestine, and could alter gastrointestinal functioning through alterations in secretomotor neuron functioning. PD patients are known to suffer from gastrointestinal problems throughout the course of the disease[22,24,68,69], and based on our results this could be (partially) due to an increased exposure to LPS and α-Syn monomers and fibrils resulting in neuronal IL-1β, IL-6 and TNF-α responses in the ENS.

The release of the pro-inflammatory neuropeptide Sub P[95] by differentiated IM-FEN cells was also studied after exposure to LPS, α-Syn monomers, α-Syn fibrils, or a combination of LPS and α-Syn monomers or fibrils. Sub P was significantly increased in the supernatant of α-Syn fibril or LPS treated cells, while it was reduced in the cell lysate of α-Syn fibril and LPS treated cells. It seems that α-Syn fibrils and LPS separately stimulated the cells to release Sub P, which supports our hypothesis that LPS and α-Syn fibrils act pro-inflammatory in ENS neurons. Additionally, co-incubation with both LPS (10 ng/ml) and α-Syn fibrils for 24 hours resulted in a decrease in both the supernatant and the cell lysate, which could reflect cell death rather than a lack of Sub P release in response to the co-incubation. Sub P is known to be involved in murine colitis[96], is neurotoxic to dopaminergic neurons when co-cultured with microglia[97], and increases toxin-induced neuroinflammation and dopaminergic neuronal death in the substantia nigra of a PD rat model[98]. Sub P knock-out mice are partially protected from toxin-induced dopaminergic neuronal cell death[99]. Therefore we hypothesize that both LPS- and fibril-induced release of Sub P could be involved in the intestinal inflammation as well as in the ENS pathology found in a PD mouse model[100] and in the colon of PD patients[36,101].

The release of the anti-inflammatory neuropeptide VIP[102,103] by IM-FEN cells was studied after exposure to LPS, α-Syn monomers, α-Syn fibrils, or a combination. VIP was significantly increased in the lysate of fibril treated cells, while it was significantly decreased in the supernatant of LPS 10 ng/ml + fibril treated cell. It seems that α-Syn fibril treatment reduces the release of VIP, with or without LPS, which supports our hypothesis that α-Syn fibrils and LPS support a pro-inflammatory environment in the ENS, in this case by reducing anti-inflammatory signals. VIP has a direct neuroprotective effect on neurons in the context of PD: it increases survival of primary enteric neurons in culture[104], and protects neuronal cell cultures against dopamine and 6-hydroxydopamine toxicity[105]. VIP is even suggested as a potential target to treat PD[106]. VIP acts neuroprotective

by reducing microglial activation[107,108] and TLR2 and TLR4 activation on T lymphocytes[109]. VIP also increases the release of activity-dependent neurotrophic factor by astrocytes[110,111] and the number of NGF positive mast cells in the CNS[112]. Furthermore, VIP is known to be anti-inflammatory in the intestines since it reduces intestinal inflammation in the 2,4,6-trinitrobenzenesulfonic acid mouse model of Crohn's disease[113], and is suggested as a potential target to treat Crohn's disease[102]. Therefore we hypothesize that both LPS- and α-Syn fibril-induced reduction of VIP release could precipitate the intestinal inflammation in mice[100] and humans[36] suffering from PD, and enhance enteric dopaminergic neuronal loss in PD[101]. The changes observed in both Sub P and VIP release align mostly with the results observed in the cytokine mRNA expression. Overall α-Syn fibrils alone or together with LPS seem to act pro-inflammatory, while α-Syn monomers alone or together with LPS act neuroprotective or anti-inflammatory.

The most unexpected and interesting result is the effect of LPS alone. The increase in IL-1β mRNA in response to LPS is interpreted as a neuroprotective effect, while LPS also increased the release of pro-inflammatory neuropeptide Sub P. It seems that neuroprotection and inflammation are not mutually exclusive, and they can both be elicited by the same stimulus, in this case LPS. The release of neurotransmitter GABA by IM-FEN cells was studied after exposure to LPS, α-Syn monomers, α-Syn fibrils, or a combination. There was a consistent trend for increase of GABA in the supernatant of LPS 10 ng/ml + α-Syn fibril group compared to LPS alone. The role of GABA in the ENS is still not yet completely understood, but it has been suggested that GABA has anti-inflammatory properties and affects gut motility[114]. Additionally, GABA potentially reduces enteric neuronal excitability, resulting in decreased basal tone of the smooth muscle, decreased force of spontaneous contractions, and increased frequency of spontaneous contractions[115]. In the biological environment of the whole intestine the combined LPS and a-syn fibril induced release of GABA could therefore result in reduced contractility of the colon and associated reduced transit time and constipation observed in PD patients[116,117]. However, since the effects of LPS and α-Syn fibrils on GABA release were not significant, it could also be concluded that neuronal GABA does not play a major role in the neuroinflammatory milieu of the PD intestine. Changes in the release of GABA by other sources, like endocrine cells[118] or the microbiome[119,120], could easily overrule the subtle changes in neuronal GABA. In this context it is of interest to note again that PD patients have an altered microbiome[35], which could result in a different GABA content of the gut. Of interest is the recent report on positive association of the abundance of lactic acid producers Enterobacteriaceae with PD symptoms[35], because lactic acid producing bacteria in the intestinal tract are known to be able to produce GABA[121].

To determine the effect of the changed IL-1β, IL-6 and TNF-α expression, and the changed Sub P and VIP concentrations on neuroinflammation, LP development and intestinal neuronal loss in PD, it would be interesting to develop mouse models with selective knock-out of IL-1β, IL-6, TNF-α, Sub P and / or VIP in enteric neurons. Gut inflammation and disease development could then be monitored after orally exposing these mice to α-Syn fibrils, PD-related toxins like rotenone or MPTP that induce mitochondrial dysfunction, or to different sets of PD-associated bacteria. Intestinal barrier function, infiltration of pro-inflammatory cells, intestinal tract function, neuron loss and development of LP in the ENS and the CNS, and motor function are some of the parameters that could potentially be affected differentially in the suggested knock-out animals. It would also be interesting to examine different cell types obtained from intestinal biopsies of PD patients and healthy controls in relation to neuro-immune interactions in the intestinal tract. Based on these results it might be possible to target the intestinal neuro-immune dysfunction for future therapies for early or progressed PD, aimed at reducing neuroinflammation and intestinal problems in patients. This could potentially even be used as an early intervention, aimed at slowing down or preventing PD disease progression from the gut to the CNS[27,28].

Conclusion

Intestinal inflammation and dysfunction are important features in PD, and point to an important role for the ENS in this disease. The results of this study show that neurons actively contribute to the neuro-inflammation, through changes in IL-1β, IL-6 and TNF-α expression, and the release of Sub P and VIP in a response to α-Syn monomers, α-Syn fibrils and LPS. Cytokines and neuropeptides in the ENS offer new targets for future PD research, and may eventually offer targets for the treatment of PD symptoms, like gastrointestinal problems and the spread of LP from the ENS to the CNS.

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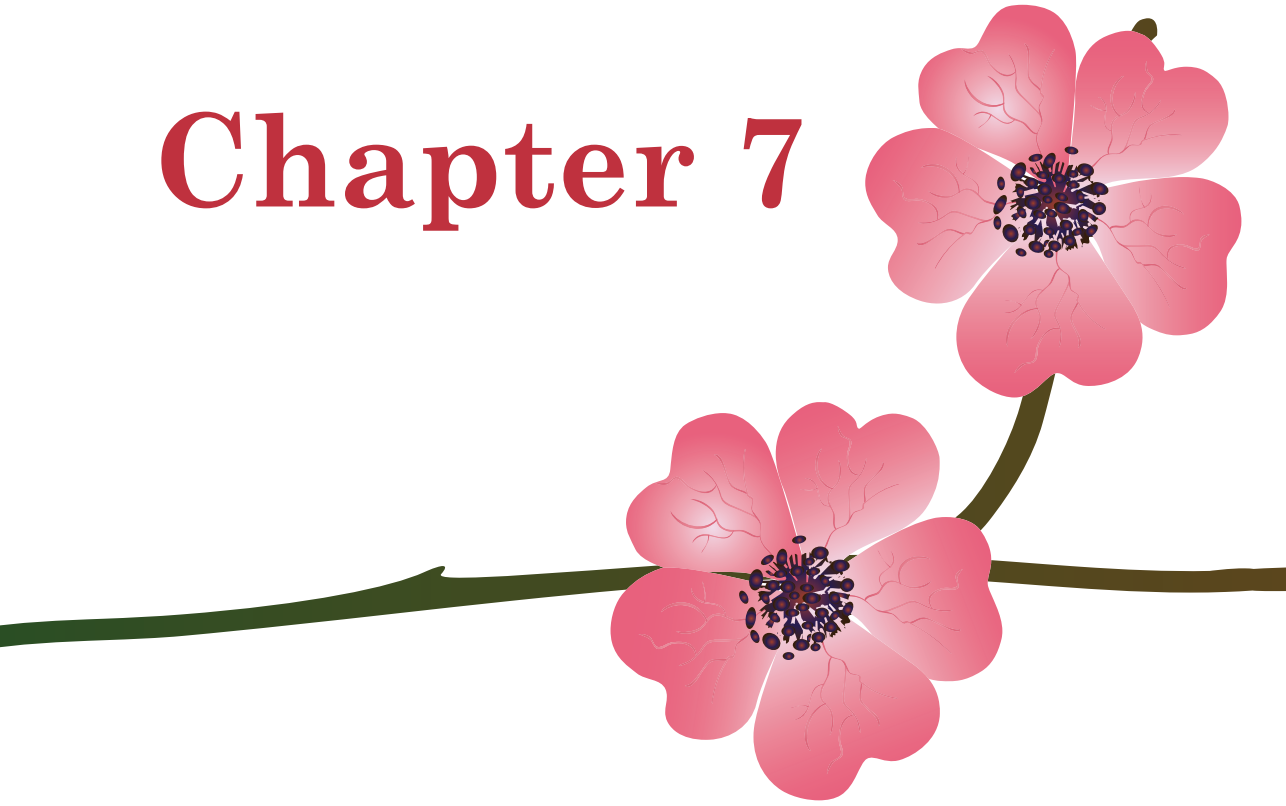
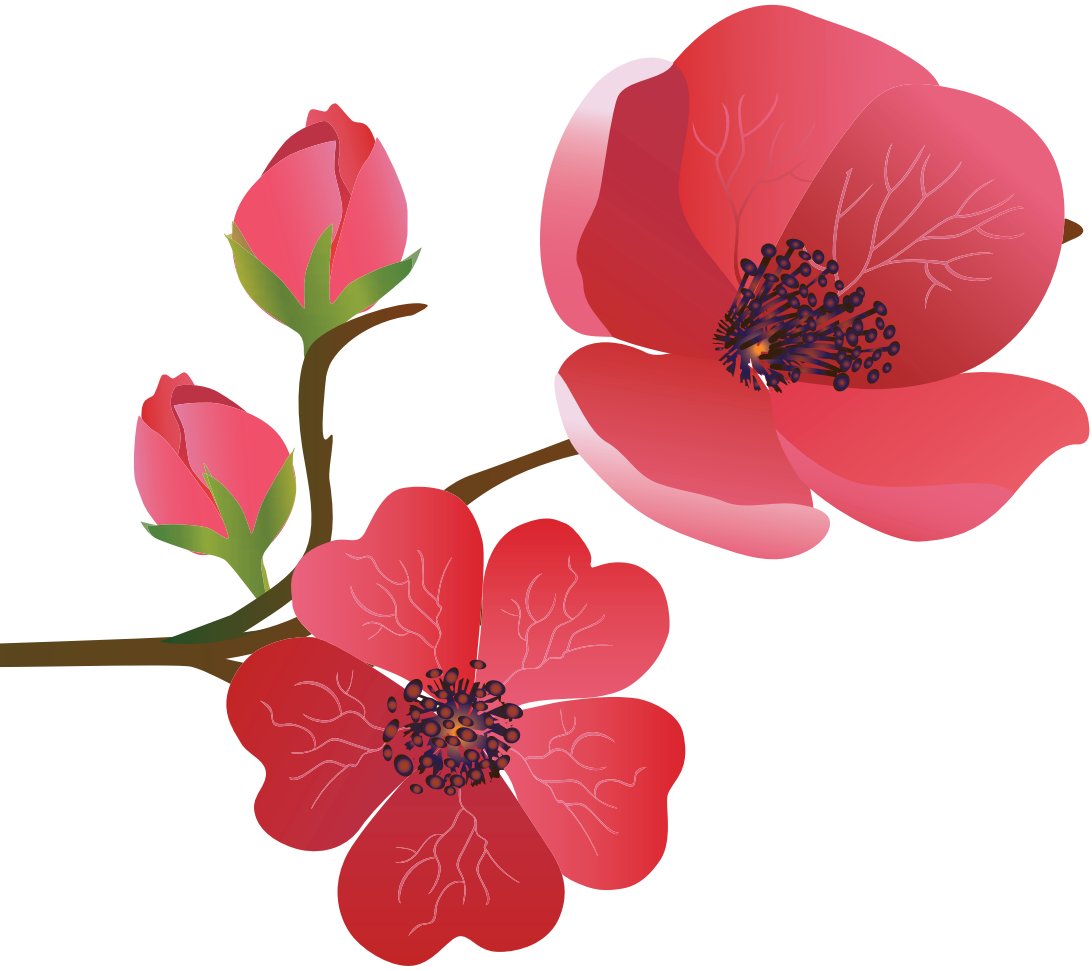
Chapter 7





Summary and general discussion

Abbreviations

PD – Parkinson's disease
α-Syn - α-Synuclein
LP – Lewy pathology
ENS – Enteric nervous system
CNS – Central nervous system
TLR – Toll-like receptor
IM-FEN – Murine immorto fetal enteric neuronal cell line
LPS – Lipopolysaccharide
IL – Interleukin
TNF – Tumor necrosis factor
SubP – Substance P
VIP – vasoactive intestinal peptide

Introduction

Parkinson's disease (PD) is a neurodegenerative disease which is characterized by primary motor symptoms, the loss of dopaminergic neurons in the substantia nigra, and aggregates of misfolded α-Synuclein (α-Syn) in the remaining neurons, known as Lewy pathology (LP)[1,2]. Between 5 and 346 new cases of PD are diagnosed per 100,000 people in Europe every year[3]. The current medicinal and surgical treatment options offer relief of the motor symptoms, but are not curative, unfortunately[4,5]. Most patients suffer from sporadic PD, for which no specific cause is known. It is potentially triggered by a combination of genetic and environmental factors[6-8]. Neuroinflammation, oxidative stress, and α-Syn misfolding and aggregation in the central and peripheral nervous system are known to contribute to disease development[9-16].

The enteric nervous system (ENS) of PD patients is also affected by characteristic dopaminergic neuron loss[17] and LP[18-20], often leading to gastrointestinal problems years before the disease is diagnosed[21,22]. Pathogen-induced inflammation has been suggested as the initial event causing PD via the olfactory tract and the ENS[23,24]. In this thesis the ENS route in the pathophysiology of PD has been studied. The involvement of the ENS in PD initiation is supported by evidence that colonic inflammation and epithelial barrier permeability are increased in patients[25,26]. The microbiome of patients is different from controls[27], which is suggested to cause α-Syn misfolding in the ENS through the induction of inflammation[28]. The ENS and the central nervous system (CNS) are connected and communicate via vagal and spinal pathways, a connection known as the gut-brain axis[29]. Once PD is initiated in the ENS, it is suggested to spread via the gut-brain axis to the central nervous system, where it eventually leads to LP and neuron loss in the substantia nigra and other parts of the brain[23,24].

Toll-like receptors (TLRs) are innate immune receptors that respond to both exogenous pathogen derived danger signals, and endogenous danger signals[30-33]. They are expressed by both neurons and glial cells[34-40], amongst other cell types. TLRs contribute to neuroinflammation in PD[9], and TLR2 and -4 are upregulated in the sigmoid mucosa of the intestines and in the brain of patients (Perez-Pardo, Keshavarzian, et al, manuscript inpreparation) [41,42].

In light of these findings, the study of the potential role of TLRs on enteric neurons in the context of PD using *in vitro* techniques has been pursued in this thesis.

IM-FEN model

In order to study enteric neurons, the murine immorto fetal enteric neuronal cell line (IM-FEN) was introduced in our laboratory, as described in **chapter 4**. IM-FEN cells are a validated model for enteric neurons. This cell line was developed at Emory University from H-2K^{b}-tsA58 transgenic mice[43,44]. IM-FEN cells proliferate at the permissive temperature of 33°C in the presence of interferon-γ, while they differentiate into neuron-like cells at the non-permissive temperature of 39°C in the absence of interferon-γ. Differentiated IM-FEN cells express several neuronal markers, and they express only low amounts of markers for glial cells and smooth muscle cells[43]. Little is known about the electrophysiological properties of differentiated IM-FEN cells. They seem unable to fire action potentials *in vitro*, but survived when implanted into mice, where they improved colonic and anal sphincter function[43,45,46]. Therefore it seems that IM-FEN cells have the potential to become fully functional neurons in the biological context of the gastrointestinal tract, but that current culturing techniques do not mimic these conditions close enough for the neurons to become electrically active *in vitro*.

Although the proliferating cells showed unpredictable increases and decreases in growth rate, which posed certain practical problems, we were able to successfully culture and differentiate IM-FEN cells. Through an exploratory linear regression model the relationship between a calculated indicator of growth rate and five culture-related factors was determined, which showed that both 'days in culture' and 'seeding density' were significant predictors for the indicator of growth rate, as described in **chapter 4**. However, the precise causality between these factors and growth rate remains to be determined. We make the recommendations to not increase or decrease the seeding density of slow growing cells, and to wait until the cells are 80% confluent before passaging.

Based on these results, future models of the prediction of growth rate of proliferating IM-FEN cells are advised to exclude the factors 'person culturing', 'passage number' and 'previous overconfluency', since these did not predict the indicator of growth rate. Furthermore, these future models should include other relevant factors in their analysis, especially factors related to the culture medium, in an effort to increase the predictive power of the model.

Expression and function of TLRs on enteric neurons in PD

As a first step in the study of enteric neuronal TLRs in PD, the expression of these receptors by differentiated IM-FEN cells was determined in **chapter 5**. IM-FEN cells express TLR2 -5, and -7 on mRNA level, and TLR2-4 and -7 at the protein level.

Clinical evidence links both TLR2 and TLR4 to PD; both TLRs are upregulated in the brain of patients[41], and TLR2 is shown to be specifically upregulated in microglia and not astrocytes[42]. Additionally, a TLR2 polymorphism that leads to a lower TLR2 expression, as measured in whole blood samples, shows a trend for increasing the risk for PD as well[47,48]. At first this may seem contradictory, since both an increase and a decrease of TLR2 is linked to PD. However, the polymorphism might make a healthy person more susceptible to the development of PD *before* the onset of PD, while after the onset of PD the expression of TLR2 might be increased as a result of the disease process. Therefore these results might be surprising, but they are not mutually exclusive. Furthermore, the expression of both TLRs are increased in the sigmoid mucosa of patients (Perez-Pardo, Keshavarzian, et al, manuscript in preparation), indicating the relevance of TLR2 and -4 for the ENS in particular. In **chapter 5** it was shown that primary enteric neurons of rotenone-treated mice (a PD animal model) expressed less TLR4 compared to neurons of control animals, a finding that is in agreement with immunohistochemical staining of intestinal tissue of these animals, which showed a decreased staining for TLR4 in the ENS and an increase in TLR4 staining in the epithelium, and co-localization of TLR4 and αSyn (Perez-Pardo, et al, manuscript in preparation). The co-localization of TLR4 and αSyn could indicate that TLR4 increases the vulnerability of these neurons to toxic αSyn, which would explain that the expression of TLR4 is downregulated by these cells upon initiation of αSyn pathology through rotenone treatment. Therefore, the response of IM-FEN cells to TLR4 stimulation in the context of PD was further studied in **chapter 6**.

Differentiated IM-FEN cells were exposed to the TLR4 ligand lipopolysaccharide (LPS) , and the PD-related protein α-Syn as monomers or fibrils. The cells were either exposed to each substance separately, or co-incubated with LPS and either α-Syn monomers or α-Syn fibrils. The α-Syn monomers alone had a neuroprotective or neurotropic effect, which was apparent through an increase of interleukin (IL)-1β mRNA expression[49-51]. LPS exposure elicited a similar response, while co-incubation of α-Syn monomers and LPS abolished the effect. Furthermore, the co-incubation of α-Syn monomers and LPS reduced the expression of tumor necrosis factor (TNF)-α mRNA, which is interpreted as an anti-inflammatory reaction[52,53]. Therefore the co-incubation of α-Syn monomers and LPS seems to shift the response of the IM-FEN cells from neuroprotective to anti-inflammatory.

As expected, α-Syn fibrils exposure induced a pro-inflammatory response through increased release of the pro-inflammatory neuropeptide Substance P (SubP)[54]. Co-incubation of LPS and α-Syn fibrils induced a pro-inflammatory response through the increase in mRNA expression of TNF-α and IL-6[55], and reduced the release of anti-inflammatory neuropeptide vasoactive intestinal peptide (VIP)[56,57].

LPS alone acted both neuroprotective, through the increase of IL-1β mRNA expression, and pro-inflammatory, through increased release of SubP. This is an unexpected result, but these effects are not mutually exclusive.
There was no statistically significant effect of the different treatment conditions on GABA release, however a trend was observed for increased GABA release after co-incubation of α-Syn fibrils and LPS, compared to LPS alone. GABA is known to affect gut motility, and the increased release of GABA could be associated with constipation observed in PD patients[58-61].
Overall, the α-Syn fibrils (alone or combined with LPS) seem to be an interesting candidate in the search for a trigger for intestinal inflammation in PD patients. Cytokines, SubP and VIP in the ENS offer interesting markers and potential targets for the treatment of PD-related intestinal inflammation.
Concluding, the IM-FEN cells express several TLRs (**chapter 5**) and TLR4 seems involved in the pro-inflammatory response of enteric neurons in the context of the PD-related protein α-Syn (**chapter 6**). Using IM-FEN cells as a model for enteric neurons, these results show that enteric neurons are differentially affected by α-Syn monomers and fibrils. In the intestines of PD patients, this could mean that the specific combination of a changed microbiome and mucosal barrier dysfunction, that allows these microbes to reach the enteric neurons, spurs α-Syn misfolding and neurogenic inflammation in the ENS. This effect could be mediated via altered cytokine expression and neuropeptide release, potentially harming the ENS and leading to neuronal death, resulting in gastrointestinal complaints and the initiation or progression of PD pathology towards the CNS.

Limitations

The aim of the research presented in this thesis is to better understand the relationship between TLRs on neurons of the enteric nervous system and the development of PD. It is important to consider what has been discovered by these experiments, which conclusions we can draw, and what remains uncertain or unknown.
The IM-FEN cell line, as described in chapter 4, has limitations in several ways as a model for neurons in the intestines of PD patients. Foremost, this is a cell line with a genetic modification to make continuous cell culture possible, an important difference with genetically unaltered, primary cells. Secondly, these cells are of murine origin, requiring an important translational step to human cells before these results can be accurately interpreted for human patients. A third limitation is the embryonic origin of the cell line, which places them at the opposite end of life from the mostly elderly patients who suffer from PD. A final limitation is the fact that these cells were cultured outside of the biological environment of the intestines,

which removes all possible interactions between neurons and other types of cells. A limitation with regards to the expression of TLRs in chapter 5 is the lack of information about the functionality of the different TLRs that were detected. Although information about the functionality of TLR4 has been presented in chapter 6, and has been published before[62], little is known about the functionality of TLR2, -3 and -7. In relation to TLR2 preliminary data show increase in apoptosis of differentiated IM-FEN cells after 15 hours exposure to the TLR1/TLR2 ligand, Pam3CSK4. Cells showed an dose-dependent increase in cleaved caspase 3, an apoptosis marker, and a dose-dependent decrease in PGP9.5, a neuronal marker (preliminary data, fig. 1). This indicates a potential involvement of TLR2 in PD-related pathogen-induced neuronal cell loss in the ENS, a finding which invites further studying.

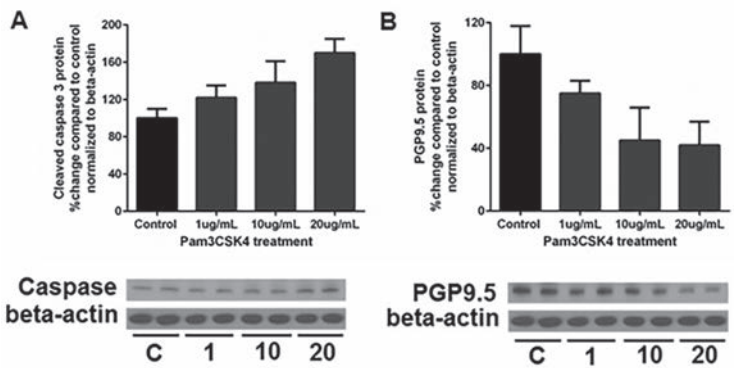


Figure 1. Preliminary data on function of TLR2 on enteric neurons. Dose-dependent apoptosis response of IM-FEN cells after 15 hours of Pam3CSK4 exposure, as determined by Western Blot. Cells exposed to different concentrations of the TLR1/2 ligand Pam3CSK4 show an increase in apoptosis signaling molecule cleaved caspase 3 (n=3) and a decrease in neuronal protein PGP9.5 (n=2), indicated as mean and SD. Two representative Western Blots are included.

Since PD is suggested to originate in enteric neurons and to be caused by a pathogen that enters the digestive tract, it is unavoidable to study the functionality of TLRs on these neurons, and to determine if, when and how these receptors contribute to or impede the development of PD, before a general conclusion can be drawn about the relationship between pathogen exposure of the ENS, intestinal inflammation and PD development or progression. Another important limitation to the results presented in chapter 6 is the limited amount of inflammation-related mediators that were measured, and specifically the lack of data on the changes in cytokine expression on a protein level, since it is uncertain that the results found on the mRNA level will translate to changes in protein expression. The lack of a more extensive overview of changes in the expression of inflammation-related mediators

in our experimental setting prevents an encompassing assessment about the promotion or inhibition of inflammation through neurons of the ENS in the intestines of PD patients. We found evidence for a pro-inflammatory response in IM-FEN cells elicited by exposure to αSyn fibrils. This pro-inflammatory response consisted of an increased release of SubP and a decreased release of VIP, and increased expression of mRNA for cytokines IL-6 and TNF-α. We also observed a potential neuroprotective or neurotropic response after exposure of IM-FEN cells to αSyn monomers. This effect consisted of an increase in the expression of IL-1β mRNA. These results need to be put in a larger context to better understand their significance in relation to other cell types and other immune-modulating substances.

Clinical significance

The clinical significance of the results presented in this thesis is subject to the limitations described above. A proof-of-concept for enteric neurogenic inflammation in the context PD is presented in this thesis. The presence of innate immune receptors, the changes in inflammatory signals expressed by enteric neurons upon exposure to α-Syn monomers and fibrils, and the differential response of enteric neurons to α-Syn monomers versus fibrils are all proof of the concept that enteric neurons play a role in intestinal inflammation related to PD. This proof-of-concept can be a basis for future pre-clinical and clinical PD research, where this topic can be more thoroughly studied. A better understanding of intestinal inflammation can aid the development of better diagnostics and/or treatments for (early) PD.

Future perspectives

As always, the results presented in this thesis raise more questions than they answer, and therefore future research is needed, continuing the perpetual circle of research. The effects of TLR ligands on IM-FEN cells and primary neurons is the first avenue to be explored, including effects on pro- and anti-inflammatory signaling, apoptosis, α-Syn (Lewy) pathology, and changes in electrical activity. In PD animal models the effect of TLR stimulation on enteric neurons should be studied for the effects on neurogenic inflammation, neuronal survival, LP, and gastrointestinal functioning. Effects of specific blocking or knock-out of enteric neuronal TLRs on these outcome measures should also be determined. In patients the co-localization of LP and TLRs on enteric neurons could clarify which TLRs are the most likely to be linked to PD initiation and development. Current knowledge favors a role for TLR2 and TLR4 in PD[41,42,48], therefore these two receptors should be the first to be studied. However, other TLRs should be studied too, since their importance is unknown and should be revealed.

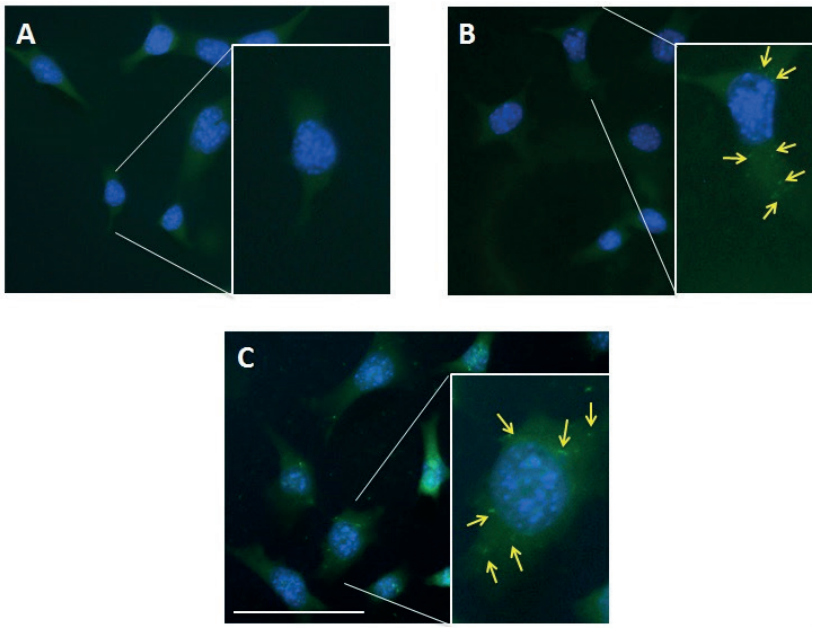


Figure 2. Preliminary data on the uptake of αSyn monomers (B) and fibrils (C), and control (A), in differentiated IM-FEN cells. FITC-labelled αSyn can be seen in green, while Hoechst-stained cell nuclei are shown in blue. Yellow arrows indicate αSyn inclusions. (Scale bar represents 50 μm)

There is a high relevance for future experiments to better understand enteric neuronal TLRs in PD. These receptors could prove to be viable biomarkers for the diagnosis of early PD, since digestive function and neurons in the ENS are known to be affected early in PD[20-22,63,64], and upregulated TLR2 and TLR4 have already been found in the sigmoid mucosa of PD patients (Perez-Pardo, Keshavarzian, et al, manuscript in preparation). Knowledge of TLRs in the ENS of PD patients could also help answer the question whether a pathogen, potentially a bacterium or virus related to the intestinal microbiome[24,27,28], is indeed responsible for the initiation of PD through the gut. Here I would like to present another preliminary result, showing uptake of α-Syn by IM-FEN cells. Differentiated IM-FEN cells exposed to 2 μM of FITC-labelled α-Syn monomers or fibrils seem to be able to take up these monomers and fibrils (preliminary data, fig. 2). This offers the potential to study the effects of TLR stimulation and blocking on the uptake of α-Syn in neurons of the ENS, using IM-FEN cells as a model.

If certain TLRs are differentially expressed on neurons in the intestinal tract of PD patients, this could help focus the search for an invader to a specific group of pathogens known to interact with those specific TLRs. Finally, TLRs could also be important targets for future PD therapy. If a pathogen is indeed the initial trigger

for PD in enteric neurons, TLRs might be the very first gatekeepers to interact with this dangerous microbe, offering a potential target to prevent PD in people exposed to this pathogen. It is known that α-Syn can spread between neurons[65], can act as a danger associated molecular pattern by stimulating TLR2[66], and can elicit astrocyte-induced inflammation[67]. Therefore, if toxic α-Syn spreads between neurons and acts as a danger associated molecular pattern during the development of PD, TLRs could even offer a potential target for treatment of patients at any stage of the disease.

Concluding remarks

This thesis has provided evidence for the presence and functionality of Toll-like receptors on enteric neurons in the context of PD. TLRs on enteric neurons are likely partially responsible for intestinal inflammation in PD patients, although their effects can also include neuroprotection or anti-inflammatory signaling. Future research can elucidate whether these receptors are involved in the initiation of PD via microbial invasion of the intestines, and whether they are important for disease progression. Enteric neuronal TLRs are potential biomarkers and targets for future PD treatment.

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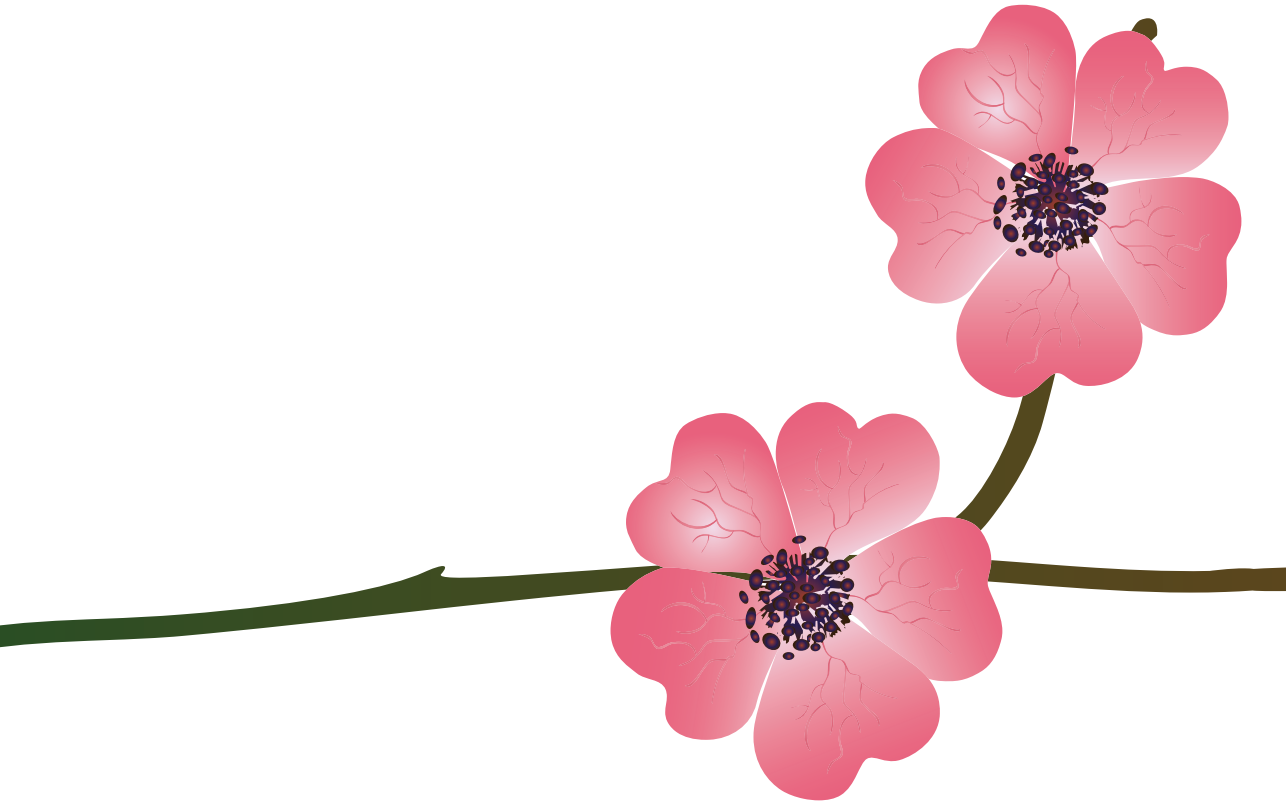
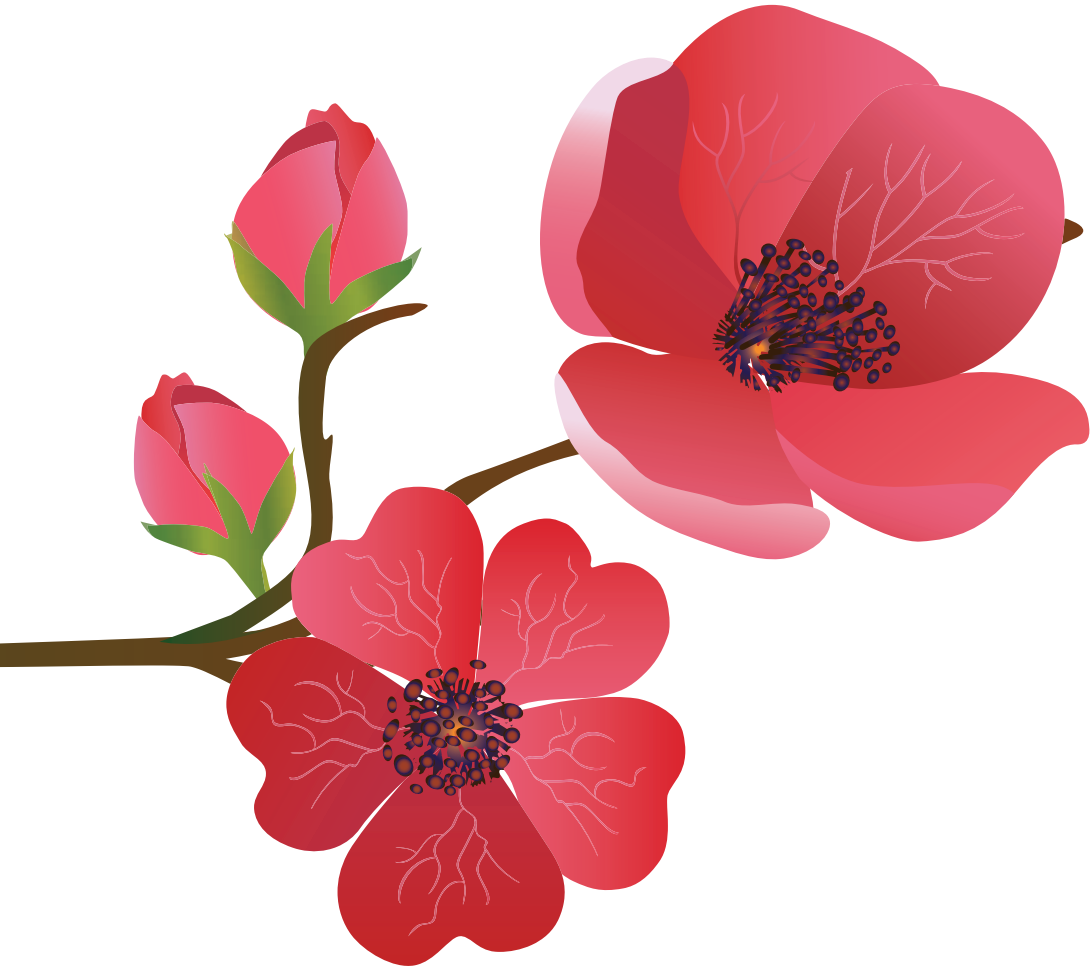




Nederlandse Samenvatting

Afkortingen

PD – Ziekte van Parkinson
SN – Substantia Nigra
α-Syn – Alfa-Synucleine
TLR – Toll-like receptor
LPS – Lipopolysaccharide

Inleiding

De ziekte van Parkinson (PD) wordt o.a. gekenmerkt door motorische problemen. Dit komt door het afsterven van zenuwcellen in een specifiek hersengebied, de Substantia Nigra (SN). Deze SN is belangrijk is voor motorische functies. De afsterving van zenuwcellen gaat gepaard met ophoping van het eiwit alfa-Synucleine (α-Syn) in verkeerd gevouwen en samengeklonterde vorm in de overgebleven zenuwcellen. Per jaar worden er in Europa per 100.000 inwoners 5 tot 346 nieuwe gevallen van de ziekte van Parkinson vastgesteld, afhankelijk van het land. De motorsymptomen kunnen worden verlicht door medicamenteuze of operatieve behandelingen, maar de ziekte kan helaas nog niet worden genezen. Veel patiënten lijden aan de *sporadische* vorm van de ziekte van Parkinson. Deze vorm van de ziekte wordt vermoedelijk veroorzaakt door een combinatie van genetische- en omgevingsfactoren. Ontstekingen van het zenuwstelsel, stofwisselingsstress in cellen, en samengeklonterd α-Syn zijn bekende factoren die bijdragen aan de ontwikkeling van de ziekte van Parkinson.

Het afsterven van zenuwcellen, de aanwezigheid van samengeklonterd α-Syn in de overgebleven zenuwcellen, en ontstekingen worden ook gevonden in het zenuwstelsel in zowel de neus als de darmen van patiënten. In dit proefschrift is specifiek onderzoek gedaan naar de zenuwcellen in de darmen. Vaak jaren vóór de definitieve diagnose worden er al darmproblemen gediagnostiseerd. Ook deze symptomen blijken gerelateerd te zijn aan de ophoping van α-Syn in de zenuwen van de darm. De Braak's hypothese stelt voor dat de ziekte van Parkinson mogelijk wordt veroorzaakt door ziektekiemen die aanleiding kunnen geven tot ontstekingen in de darmen, en samenklontering van α-Syn in de zenuwcellen in de darmen. Deze theorie wordt ondersteund door de bevinding dat het darm microbioom van patiënten met de ziekte van Parkinson afwijkt van die van gezonde vrijwilligers. Bovendien, lijden patiënten met de ziekte van Parkinson veelvuldig aan darmontstekingen die samengaan met een verhoogde doorlaatbaarheid van de darmwand. Het zenuwstelsel in de darmen is verbonden met de hersenen via de vagale zenuwbanen en via het ruggenmerg. Wanneer de ziekte van Parkinson in het zenuwstelsel van de darmen is ontstaan, kan het zich mogelijk via deze verbindingen verspreiden naar de hersenen. Zo zou de ziekte de Substantia Nigra (en andere hersengebieden) kunnen bereiken, en de kenmerkende schade veroorzaken die wordt gezien bij de ziekte van Parkinson.

Toll-like receptoren (TLRs) zijn receptoren van het aangeboren afweersysteem, en reageren zowel op lichaamsvreemde als lichaamseigen gevaarsignaalstoffen. De lichaamsvreemde stoffen waarop TLRs reageren zijn o.a. afkomstig van ziektekiemen, zoals bacteriën en virussen. Voorbeelden van moleculen die TLR

kunen triggeren zijn het DNA, RNA, of stoffen afkomstig van de buitenkant van bepaalde ziektekiemen. De lichaamseigen stoffen waarop TLRs reageren zijn bijvoorbeeld afkomstig van ontstekingsreacties of komen vrij bij weefselschade. In een gezonde omgeving komen deze doorgaans niet voor. Stimulatie van bepaalde TLRs kan leiden tot activatie van het afweersysteem en een ontstekingsreactie. Zoals gezegd kan dit een rol spelen bij de ontstekingen in de darmen zoals gezien bij veel Parkinson patiënten.
TLRs komen voor op verschillende celtypes in het lichaam, voornamelijk op cellen behorende bij het afweersysteem. De hersenen hebben een eigen uniek celtype dat verantwoordelijk is voor de afweer die veel TLRs bevatten. Dit zijn de zgn. microglia cellen. Maar ook de zenuwcellen zelf hebben TLRs, en die dragen mogelijk ook bij aan de ontstekingen in het zenuwstelsel tijdens PD. Stimulatie van deze TLRs leidt tot het vrijkomen van signaalstoffen van het afweersysteem, wat een ontstekingsreactie tot gevolg kan hebben. Met name TLRs 2 en 4 komen in verhoogde concentratie voor in de darmwand en de hersenen van PD patiënten. In het licht van deze bevindingen is in dit proefschrift de mogelijke rol van TLRs op zenuwcellen in de darmen in de context van de ziekte van Parkinson bestudeerd.

In vitro model voor enterale zenuwcellen

Om zenuwcellen uit de darmen te bestuderen is gewerkt met zogenaamde IM-FEN cellen, zoals beschreven in **hoofdstuk 4**. IM-FEN cellen zijn zenuwcellen uit de darmen van muizen, die in het laboratorium kunnen worden gekweekt. Dit is een genetisch gemodificeerd model voor zenuwcellen uit de darmen, en heeft helaas enkele technische beperkingen. De resultaten niet direct vertaalbaar naar de mens, omdat de cellen afkomstig zijn uit muizen. Hiervoor is een eerste vertaalstap naar de mens essentieel. Desondanks is dit een nuttig model, omdat het mogelijk is om in relatief korte tijd vele experimenten uit te voeren en componenten te screenen/ testen. Tevens kan het gebruik van dergelijke in vitro systemen mogelijk resulteren in vermindering van het aantal proefdieren.
De groeisnelheid van de IM-FEN cellen was niet constant, maar ondanks de praktische problemen die dit veroorzaakte was het mogelijk om IM-FEN cellen succesvol te kweken. In **hoofdstuk 4** is berekend welke factoren gerelateerd waren met de groeisnelheid van de cellen, om zodoende verbeteringen van de kweektechniek in te voeren. Belangrijkste doel was een stabielere groeisnelheid. De factoren 'days in culture' (kweekduur) en 'seeding density' (kweekdichtheid) waren significante voorspellers voor de indicator van groeisnelheid in het statistische model, maar helaas is het niet mogelijk gebleken om met dit model oorzaak en gevolg van elkaar te onderscheiden. Het is mogelijk dat een toegenomen 'days in culture', of een hogere 'seeding density' de oorzaak zijn van een lagere

groeisnelheid. Maat het is ook mogelijk dat langzaam groeiende cellen met een hogere 'seeding density' worden gekweekt, of dat ze een langere periode in kweek worden gehouden. De overige factoren waren 'person culturing' (persoon die kweekt), 'previous overconfluency' (eerdere overmatige kweekdichtheid) en 'passage number' ('leeftijd' van de cellen), en vertoonden geen significante relatie met de indicator van groeisnelheid.
Helaas konden op basis van deze methodische resultaten geen veranderingen in de kweektechniek worden voorgesteld. Wel is helder geworden dat de huidige kweektechniek kan worden gehandhaafd. In toekomstig onderzoek moeten de niet-significante factoren ('person culturing', 'previous overconfluency', 'passage number') worden verwijderd uit de berekening, en moeten nieuwe factoren worden toegevoegd, ten einde belangrijke factoren te identificeren die aanleiding kunnen geven tot verbetering van de kweektechniek. De nieuwe factoren zouden in ieder geval betrekking moeten hebben op het kweekmedium waarin de cellen groeien, want dit is een belangrijke variabele die in de huidige berekening niet is meegenomen.

Aanwezigheid en functie van Toll-like receptoren op enterale zenuwcellen

De aanwezigheid van TLRs 2-5 en TLR7 op IM-FEN cellen is bevestigd in **hoofdstuk 5**. De TLRs 2 en 4, die in verhoogde concentratie aanwezig zijn in de hersenen en darmwand van patiënten met de ziekte van Parkinson, zijn beiden aangetoond in IM-FEN cellen. Ook is hun aanwezigheid op de neuronen uit de darmen van een muizenmodel voor de ziekte van Parkinson onderzocht. De zenuwcellen in de dikke darm van deze muizen vertonen minder TLR4 dan de zenuwcellen van controle (gezonde) muizen, terwijl de niet-zenuwcellen (alle cellen behalve de zenuwcellen) juist minder TLR2 hebben. Deze verschillen worden niet gezien in de dunne darm. Ook komen zowel TLR2 als TLR4 met α-Syn samen voor in de darmen van het muismodel voor de ziekte van Parkinson, hoewel we niet zeker weten in welke cellen precies. Het is dus duidelijk dat er in dit muizenmodel voor de ziekte van Parkinson specifieke veranderingen optreden in de aanwezigheid van TLRs in de dikke darm, en dat deze veranderingen verschillen tussen zenuwcellen en andere celtypes. TLR2 en TLR4 zouden betrokken kunnen zijn bij de effecten van het veranderde microbioom in de darmen van patiënten met de ziekte van Parkinson. Ook zouden deze resultaten kunnen betekenen dat de zenuwcellen TLR4 verwijderen in een omgeving met een overschot aan α-Syn, omdat TLR4 de zenuwcellen kwetsbaar maakt voor de schadelijke gevolgen van α-Syn. Een vergelijkbaar proces lijkt te gebeuren met TLR2 in niet-zenuwcellen, hoewel we niet weten in welke cellen

precies, of waarom dit verschil tussen zenuwcellen en andere celtypes zou bestaan.
In **hoofdstuk 6** is de rol van TLR4 in de context van de ziekte van Parkinson verder bestudeerd. IM-FEN cellen werden blootgesteld aan een bacteriële stof die TLR4 activeert, genaamd lipopolysaccharide (LPS). Ook werden de cellen blootgesteld aan 'normaal' of geklonterd α-Syn, of aan combinaties van LPS en α-Syn. Er werd onderzocht of deze verschillende condities resulteerden in de vrijzetting van verschillende signaalstoffen. Er werden vier signaalstoffen van het immuunsysteem (cytokinen) bestudeerd. Ook werden drie signaalstoffen van het zenuwstelsel (neurotransmitters en neuropeptiden) bestudeerd. Zowel LPS als de normale α-Syn zorgden voor een verhoogde activiteit van het gen voor het cytokine IL-1β, waarvan bekend is dat het zenuwcellen kan beschermen in situaties van overprikkeling, en dat het de uitgroei van zenuwcellen kan stimuleren. Blootstelling van de cellen aan beide stoffen tegelijk resulteerde in een verlaagde de activiteit van het gen voor het cytokine TNF-α, wat betekent dat een ontstekingssignaal wordt geremd. Er kan dus worden gesteld dat zowel LPS als de normale α-Syn apart van elkaar leiden tot een beschermend signaal, terwijl ze in combinatie zorgen voor een ontstekingsremmend effect.
Geklonterd α-Syn en LPS zorgden zowel apart als in combinatie voor een stimulerend ontstekingssignaal.
Zowel geklonterde α-Syn als LPS apart verhoogde de afgifte van het neuropeptide Substance P, wat een ontstekingsstimulerende werking heeft. In combinatie met LPS zorgde geklonterd α-Syn voor een verhoging van de activiteit van de genen voor de ontstekingsstimulerende cytokinen TNF-α en IL-6, en een verlaging van de afgifte van het neuropeptide vasoactive intestinal peptide. Deze resultaten geven aan dat geklonterd α-Syn, potentieel in samenwerking met bacteriële producten uit de darmen, mogelijk betrokken kan zijn bij het ontstaan van ontstekingen in het zenuwstelsel in de darmen van patiënten met de ziekte van Parkinson.
LPS op zichzelf had zowel een beschermend effect als een ontstekingsstimulerend effect, respectievelijk door de verhoogde activiteit van het gen voor IL-1β en door de verhoging van de afgifte van Substance P. Het is bekend dat LPS een beschermend effect kan hebben op de neuronen in de darmen, wat het beschermende resultaat aannemelijker maakt. Dit sluit het ontstekingsstimulerende effect echter niet uit. De balans is essentieel.
Gebaseerd op de huidige wetenschappelijke literatuur en de resultaten in dit proefschrift wordt de volgende theorie voorgesteld voor het ontstaan van de ziekte van Parkinson in de zenuwcellen in de darmen. Door veranderingen in de darmflora van patiënten en een verhoging van de doorlaatbaarheid van de darmwand kunnen schadelijke bacteriën de zenuwcellen in de darmen bereiken. Hierdoor worden de zenuwcellen blootgesteld aan ontstekingen en ontstaat klontering van α-Syn,

wat kan leiden tot afsterven van de zenuwcellen. Hierbij spelen de bestudeerde signaalstoffen uit **hoofdstuk 6** mogelijk een rol. Dit proces leidt in een vroeg stadium tot darmklachten in patiënten met de ziekte van Parkinson, en is het beginstadium van waaruit de ziekte zich langzaam verspreid richting de hersenen.
Het is belangrijk om te overwegen welke conclusies kunnen worden getrokken uit de bovenstaande resultaten, en wat nog onduidelijk of onbekend is, op basis van de beperkingen waaraan de resultaten onderhevig zijn. De IM-FEN cellen zijn enkel een model voor neuronen in de darmen van patiënten met de ziekte van Parkinson, met enkele belangrijke verschillen. De IM-FEN cellen komen van oorsprong uit embryonale muizen, ze zijn genetisch gemodificeerd, en worden gekweekt buiten hun oorspronkelijke omgeving van de darmen. Dit geeft belangrijke verschillen op het vlak van levensfase (embryonaal versus ouderen), soort (muis versus mens), genetische opmaak (aangepast versus natuurlijk), en biologische omgeving (celkweek versus darmen). Deze beperkingen betekenen dat de resultaten moeten worden bevestigd in experimenten waarbij deze beperkingen niet bestaan, zoals in diermodellen of in menselijk materiaal.
Een tweede beperking is het ontbreken van informatie over de functionaliteit van TLRs 2, 3 en 7. Nu we weten dat deze receptoren aanwezig zijn op IM-FEN cellen, is de volgende vraag wat de functie is van deze receptoren. In hoofdstuk 7 zijn preliminaire data gepresenteerd waar uit blijkt dat stimulatie van TLR1/2 op IM-FEN cellen leidt tot geprogrammeerde celdood (apoptose). Verder onderzoek is nodig om de functionaliteit van deze receptoren te bestuderen. Hiermee kan onder andere worden onderzocht wat de mogelijke rol van deze receptoren is bij het ontstaan en de ontwikkeling van de ziekte van Parkinson in de darmen.
Een technische beperking in de resultaten over de functionaliteit van TLR4 is het ontbreken van aanvullende informatie over de ontstekingsstimulerende stoffen. Met name het ontbreken van informatie over het effect van activatie of remming van genen van cytokinen op de productie van deze cytokinen beperkt de waarde van deze informatie. Het is namelijk mogelijk dat een verschil in genactiviteit niet tot uiting komt in de productie van het eiwit. Daarnaast moeten de gevonden resultaten worden geplaatst in een groter netwerk van immuunmodulerende stoffen, waarbij inzicht wordt verkregen in de interacties tussen al deze factoren, en de mogelijke overkoepelende effecten op andere celtypes, en het organisme als geheel.
Ondanks de bovengenoemde beperkingen, biedt dit proefschrift een bewijs voor het concept dat neuronen betrokken kunnen zijn bij de ontstekingsreactie in de darmen van patiënten met de ziekte van Parkinson, en voor de IM-FEN cellen als model om dit proces te bestuderen. De aanwezigheid van Toll-like receptoren, de veranderingen in expressie van ontstekingsgerelateerde signalen onder

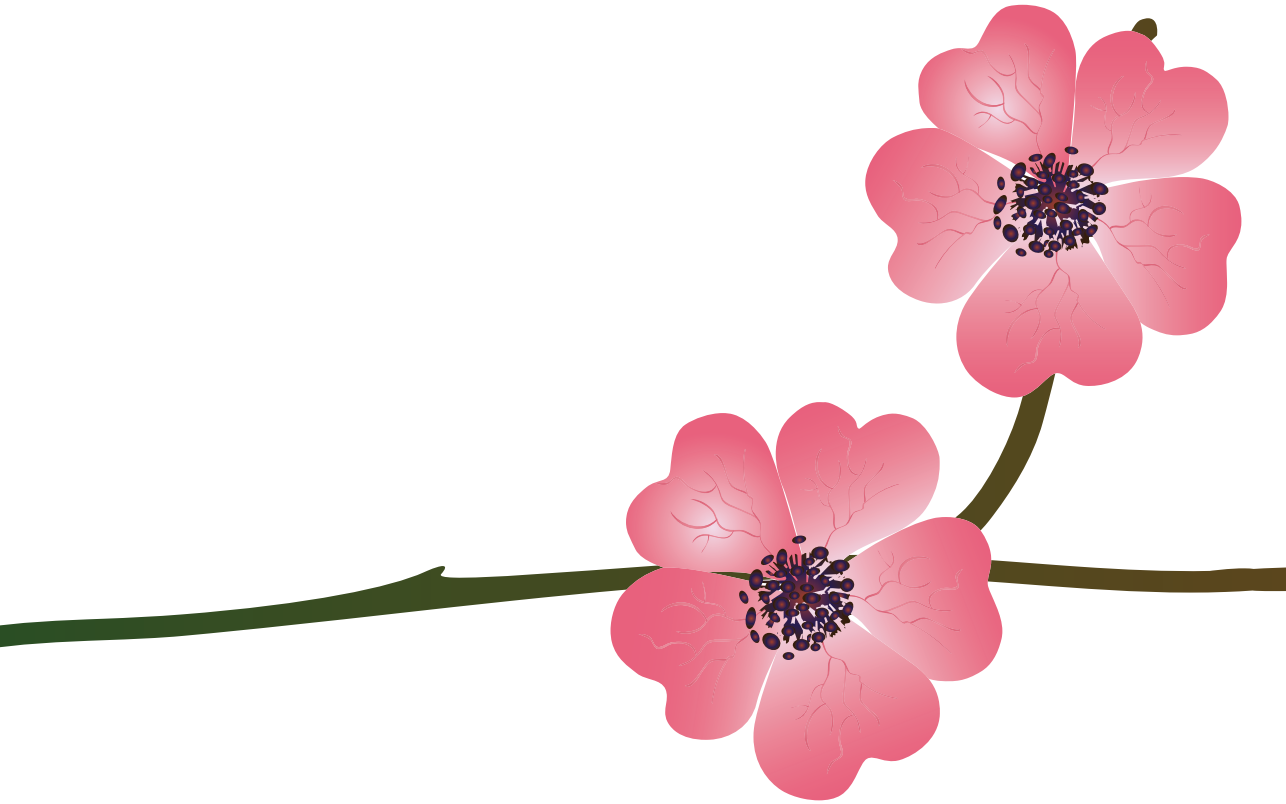
verschillende omstandigheden, en de verschillende reacties van de cellen op normaal of geklonterd α-Syn duiden allemaal op een mogelijke rol van neuronen bij de ontstekingen in de darmen in patiënten met de ziekte van Parkinson. Dit biedt aanknopingspunten voor toekomstig onderzoek, zowel in het laboratorium als in patiënten, wat uiteindelijk kan bijdragen aan de ontwikkeling van een snellere en/of betere diagnose en behandeling van (het vroege stadium van) de ziekte van Parkinson.

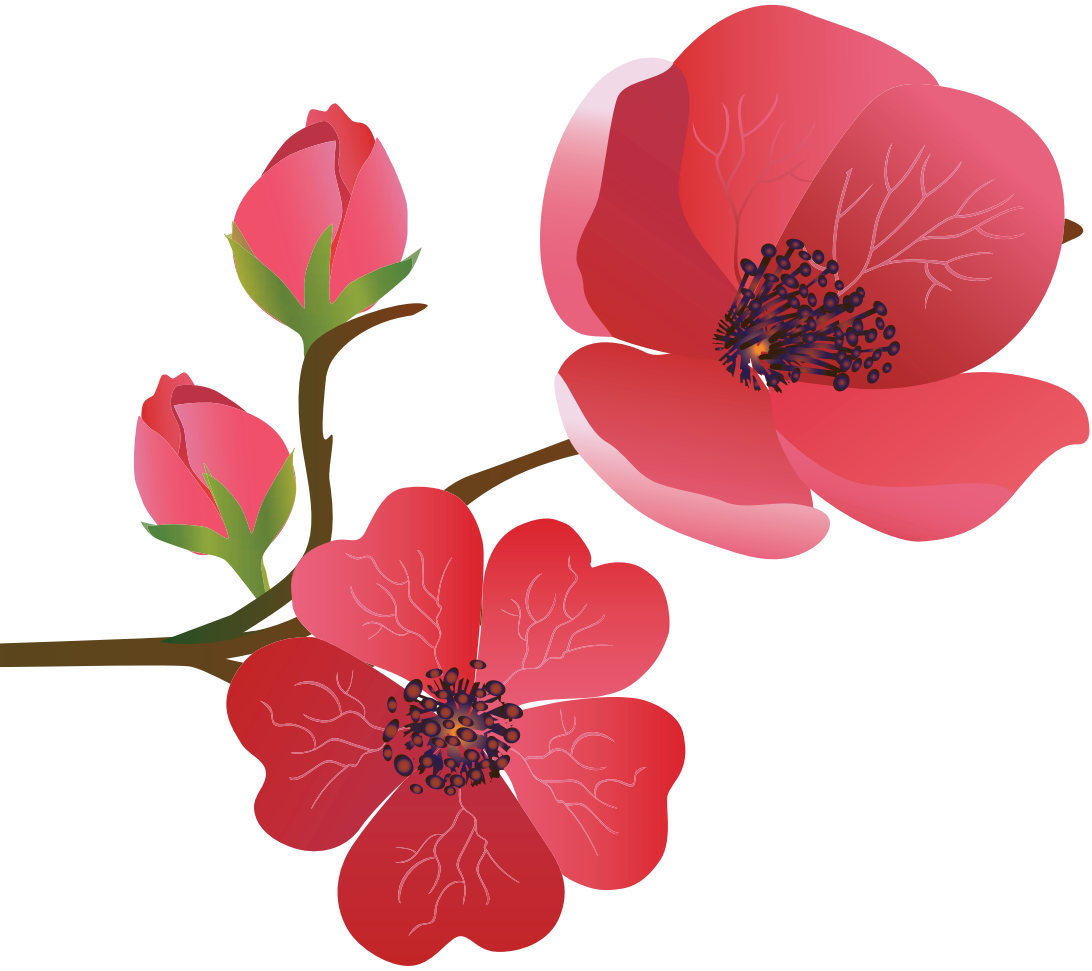
Er zijn veel vragen die in toekomstig onderzoek kunnen worden bestudeerd. De effecten van TLR stimulatie op neuronen uit de darmen moet als eerste worden onderzocht, inclusief effecten op de productie van ontstekingsstoffen, celdood, het ontstaan van α-Syn klontering, en veranderingen in electrische activiteit. Resultaten gepresenteerd in **hoofdstuk 7** laten zien dat IM-FEN cellen in staat zijn om α-Syn op te nemen, wat ze geschikt maakt voor toekomstig onderzoek naar het effect van TLRstimulatie op α-Syn opname door deze cellen. In diermodellen van de ziekte van Parkinson kan vervolgens ook het effect van TLRstimulatie op neuronen in de darmen worden onderzocht, inclusief ontstekingsreacties, celdood van neuronen, klontering van α-Syn, en het functioneren van het verteringssysteem. En daaropvolgend kan worden bestudeerd wat het effect is van het specifiek verwijderen van een bepaalde TLR op deze uitkomsten, om te bepalen hoe belangrijk een enkele TLR is voor deze processen. In patiënten kan worden onderzocht welke TLRs voorkomen op neuronen die geklonterd α-Syn vertonen, om te bepalen welke TLRs meer relevant zijn voor het ontstaan en ontwikkelen van de ziekte van Parkinson. Omdat het bekend is dat zowel TLR2 als TLR4 belangrijk zijn in de ziekte van Parkinson verdienen deze twee subtypes de hoogste prioriteit in toekomstig onderzoek. Echter is het ook belangrijk dat de betrokkenheid van de andere subtypes wordt bepaald, omdat het niet bekend is of deze receptoren een rol spelen bij de ziekte van Parkinson.

Aanvullende kennis van TLRs op neuronen in de darmen van patiënten met de ziekte van Parkinson kan leiden tot nieuwe biomarkers voor de diagnose van (een vroeg stadium van) de ziekte van Parkinson. Ook kan dit inzicht geven in welke soort ziekteverwekker(s) betrokken is/zijn bij het ontstaan van de ziekte van Parkinson, zoals voorgesteld in de Braak's hypothese.

Als bepaalde subtypes Van TLRs meer of minder voorkomen op neuronen in de darmen van patiënten met de ziekte van Parkinson, vergeleken met gezonde controle personen, kan dit een aanwijzing zijn voor het soort ziekteverwekker die mogelijk betrokken is bij het ontstaan van de ziekte van Parkinson, omdat TLRs ieder een specifieke groep ziekteverwekkers heeft waar ze op reageren, zoals bacteriën of virussen. Mocht de Braak's hypothese kloppen, dan zouden deze TLRs ook mogelijke doelwitten kunnen zijn voor toekomstige preventieve behandeling

van de ziekte van Parkinson. Ook is het mogelijk dat TLRs betrokken zijn bij de verspreiding van geklonterd α-Syn, wat ze een mogelijk doelwit maakt voor de behandeling van verder gevorderde stadia van de ziekte van Parkinson.
In dit proefschrift is een eerste bewijs geleverd dat zenuwcellen een belangrijke rol kunnen spelen bij ontstekingen in de darmen in patiënten met de ziekte van Parkinson. Zowel TLRs als de bestudeerde signaalstoffen kunnen in de toekomst mogelijk als doelwit dienen voor (vroege) diagnose en/of behandeling. Hiervoor is echter nog veel aanvullend onderzoek nodig in zowel zenuwcellen, als in diermodellen en patiënten.





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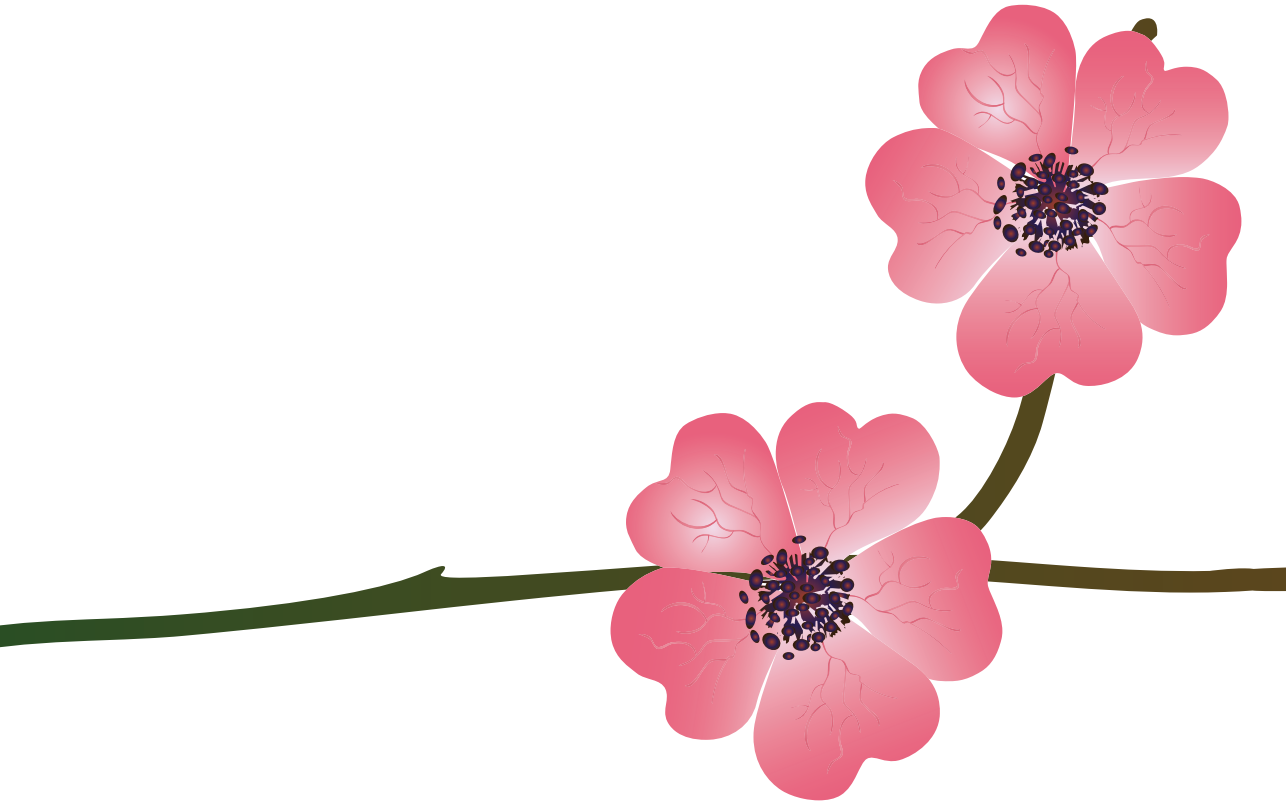

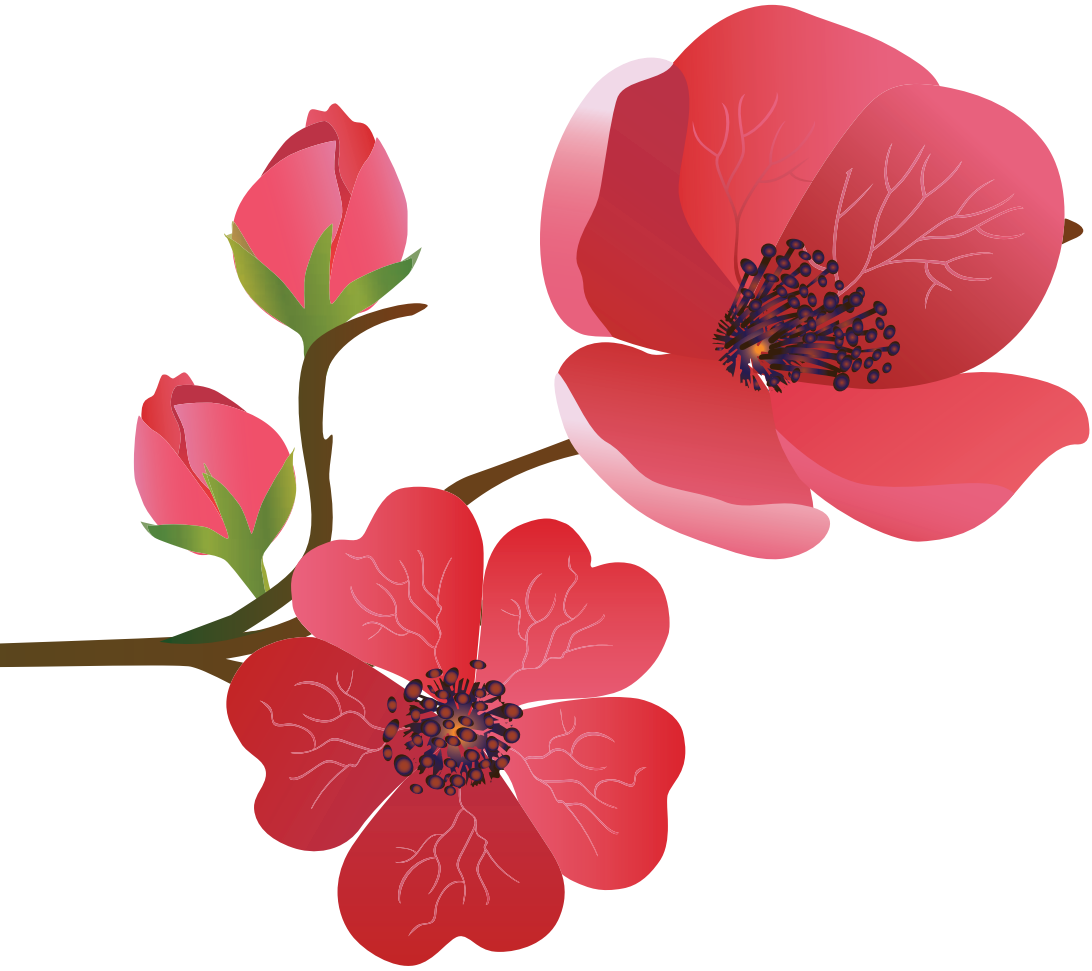

About the author

Carmen Rietdijk was born in Leidschendam, the Netherlands, on December 30th 1985. She grew up with her parents and brother in the small village of Benthuizen, and later the somewhat bigger village of Duiven. After graduating from secondary school (gymnasium) she initially chose to study Pharmacy at Utrecht University. However, after attending the first year of the bachelor program, she was drawn to the possibility of becoming a researcher. She chose to specialize in this direction during the second and third year of the bachelor program. After receiving her Bachelor of Science degree in Pharmacy at Utrecht University, she continued with the prestige master Neuroscience and Cognition at the same university. During this master program she performed a nine month internship at Yale University entitled 'Initial screening of the pharmacological profiles of the calcium permeable ion channels Transient Receptor Potential Ankyrin 1 and Sodium Leak Channel NALCN'. At Yale university she met her French husband, and together they moved to Bordeaux. After receiving her Master of Science degree in Experimental and Clinical Neuroscience at Utrecht University she worked at Université Bordeaux Segalen. A year later she accepted a PhD position at the division of Pharmacology at Utrecht University, on the topic of Toll-like receptors on enteric neurons. The results of this research project are presented in this thesis. During her PhD training she discovered a passion for teaching, and she obtained a BKO qualification. She currently works at Leiden University as a teacher for the bachelor program Bio-Farmaceutische Wetenschappen and the master program Pharmacy, on the topics of pharmacotherapy, research skills, statistics, and neuroscience.