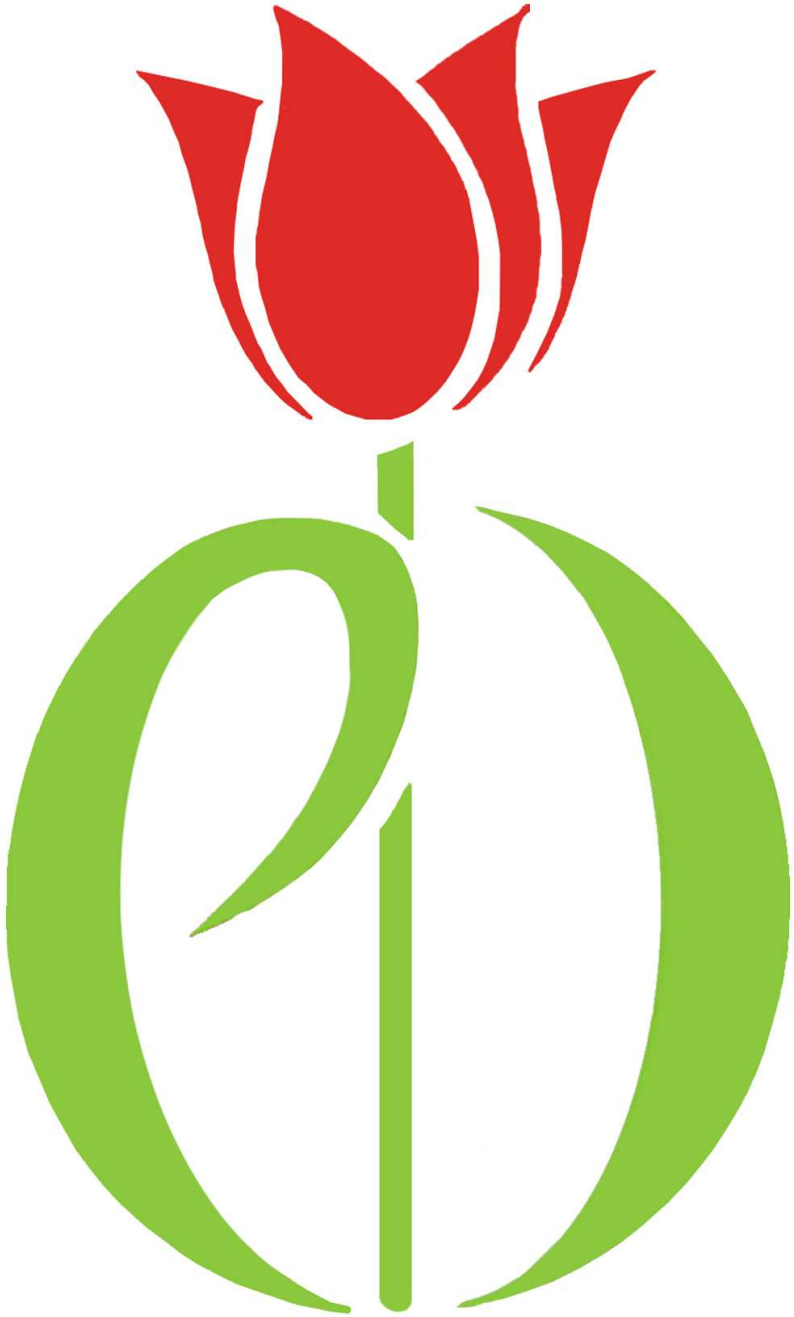
How T-cells influence microglia to form a feedback loop that accelerates disease progression in Parkinson's disease



Floris Christian Loeff BSc (0424625)
Master thesis Infection and immunity, Utrecht University
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Cover illustration
Copied from: http://www.parkinsonswny.com/

The illustration depicted on the cover is recently suggested to become the international Parkinson's disease logo.

Master thesis infection and immunity

Author: Floris Christian Loeff BSc (0424625), Infection and immunity, Utrecht University
Supervisor: Dr. Jeroen Hoozemans, VU University Medical Center
Reviewer Dr. Femke van Wijk, University medical Centre

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Abstract

Parkinson's disease (PD) is a movement disorder characterized by extensive degeneration of dopaminergic neurons in the nigrostriatal pathway. Although several gene mutations and toxins are correlated with inherited and induced forms of PD, causes of initiation of sporadic PD remains debated. In this thesis I will give a general introduction of PD and an overview of the literature on PD initiation. However most importantly, I will give an overview of the recent literature discussing the involvement of the adaptive immune system on the progression of PD. Here we will specifically focus on the involvement of T-cells in the pathogenesis of PD. Also, possibilities to influence PD by inducing differential T-cell subtypes, such as $T_{helper}1$ cells, $T_{helper}2$ cells, and $T_{regulatory}$ cells, will be discussed.

Introduction

Discovery

At the moment, Parkinson's disease (PD) is the most prevalent movement disorder and second to Alzheimer's disease the most predominant neurodegenerative disorder. PD is named after James Parkinson who described the clinical symptoms in 1817 in his monograph "essay on the shaking palsy". However, descriptions of a disease with Parkinson-like symptoms were made long before the common era (Manyam, 1990). In the beginning of past century it became clear that the cause of PD was loss of neurons in the Substantia Nigra pars compacta (SNc). And after the discovery of dopamine in 1958 it became clear that predominantly dopaminergic neurons were affected.

Clinical symptoms and diagnosis of Parkinson's disease

PD is characterized by four major symptoms which are: involuntary movement (dyskinesia), tremor at rest, rigidity, and slowness or absence of voluntary movement (bradykinesia). Due to the progressive nature of the disease not all of these symptoms are noticeable in the early stages; however, during the course of the disease, patients will generally develop all four. In early stages, PD does not severely impair the daily live of patient, aided by the fact that tremors decrease with voluntary movement. In later stages, on the other hand, patients may develop a stooped posture and, due to impaired postural reflexes, become prone to falling, which leads to wheelchair confinement in severe cases.

Besides the four symptoms mentioned above, other symptoms include impairment of cognitive processes (bradyphrenia), personality changes (active lively people become passive), and drooling (lack of active swallowing).

Currently, diagnosis of PD is based on neurological examination, which includes scoring of the four major symptoms, by a neurologist. Together with the patients medical history it is decided if treatment should be started. Interestingly, response to treatment, especially Levodopa administration, is a good predictor for PD. Although neurological examinations are increasingly perfected, it is estimated that accuracy in diagnosis is approximately 75-90%. Currently, the only definitive diagnosis for PD is presence of Lewy bodies, small inclusions of aggregated misfolded proteins, and neurodegeneration, extensive loss of nerve cells, in brain specimen after autopsy. However, great effort is put into imaging technologies and biomarkers to give an earlier and more accurate diagnosis (Brooks, 2010; Frasier *et al.*, 2010).

Current therapies

Treatment of PD was severely aided by the detection of dopamine and its function as neurotransmitter. Subsequent research showed reduction of excretion of this neurotransmitter by SNc neurons of PD patients. Great effort was put into the development of drugs that were able to penetrate the brain and replenish dopamine levels. Levodopa, which unlike dopamine can penetrate the Blood Brain Barrier (BBB), was discovered as a treatment for PD in 1968 (Cotzias, 1968). Levodopa is converted inside the brain to dopamine, by the enzyme aromatic L-amino acid decarboxylase. Levodopa is in most cases administered orally, consequently it has to pass parts of the gastrointestinal tract and endure the first pass effect of the liver (Iwamoto *et al.*, 1987). Combinations of levodopa with other drugs such as carbidopa and benserazide counteract its conversion in the periphery by some extent, ensuring a substantial fraction of the drugs to reach the dopaminergic neurons.

Although treatment with Levodopa does ameliorate the symptoms of PD to great extent, it does not stop disease progression. Levodopa is even reported to boost disease progression via the formation of oxygen radicals when dopamine is metabolized, although this is still controversial (Agid, 1998).

Several side effects are reported when using Levodopa including, hypotension, nausea, and insomnia. Also, long-term use is correlated with motor fluctuations and alteration between "on" (good response to medicine) and "off" state (patient experiences symptoms) (Miyawaki *et al.*, 1997). This together with the fact that despite treatment considerable motor disability returns after 5-10 years and treatment does not positively influences life expectancy in PD patients (Morens *et al.*, 1996) provides the requirement for new and improved medicine.

Epidemiology and consequences to patient and society

Neurodegenerative diseases have increasingly become a problem in the last century as people live longer. Sporadic PD is typically a disease that is correlated with age, showing an average onset of

disease at age 55. A large study done under Europeans showed prevalence of the disease of 0.6 in 65-69 year olds and 3.6 in the age group 80-84 (De Rijk *et al.*, 1997b). Although age and environment seems to be a major factor in PD, gender, race, and ethnicity did not influence susceptibility (De Rijk *et al.*, 1997b; Morens *et al.*, 1996; Zhang and Roman, 1993). In the Netherlands approximately 50.000 and in the USA roughly one million patients are diagnosed with PD.

When treated with Levodopa, symptoms of PD take an average of 15 years to effect patients in such a way that they are fully dependent on help. And although, mortality rates are approximately 3 fold compared to healthy elderly (Louis *et al.*, 1997), life expectancy of PD patients is not severely effected.

Besides the consequences of PD on the health of patients, PD also forms a large financial burden on both patients and society. It is calculated that both in the USA and in the UK additional cost of healthcare is approximately $10.000 per patient per year (Findley, 2007; Huse *et al.*, 2005). The majority of this money was spent on inpatient care and nursing home costs whilst the smallest amount was spend on prescription drugs (~$1650). Although the cost to healthcare services seems small, indirect costs in the form of loss of productivity by both the patient and his non-professional caretakers (e.g. family members) are estimated to be $25.000-$40.000. However, these indirect costs are only present for several years as PD patients often are close to retirement.

Neurodegeneration in PD

Neurodegeneration is the common causative pathological process in Alzheimer, Parkinson, Huntington, amyotrophic lateral sclerosis, and other neurodegenerative diseases. However, each of these diseases is characterized by a specific location in the brain where neurodegeneration is most prominent. In PD, neurons of the nigrostriatal dopaminergic pathway are most affected (Fig. 1). This pathway connects the substantia nigra with the striatum and is predominantly involved in initiating movement. Cell bodies of the neurons in this pathway are located in the SNc. Degeneration of this pathway leads to a deficiency of dopamine in the striatum, causing the symptoms of PD. As a result of the presence of neuromelanin in the nigrostriatal neurons, degeneration is visible in brain specimen by depigmentation of the SNc.

Interestingly, neurodegeneration of the nigrostriatal pathway is not completely symmetrical but appears to be correlated to the expression of dopamine transporter mRNA (Uhl *et al.*, 1994). Also, dopaminergic neurons of the ventral tegmental area, which are located flanking the SNc, are less affected than the SNc (Uhl *et al.*, 1985). Together this results in more extensive depletion of dopamine in the putamen than in the caudate (Bernheimer *et al.*, 1973; Price *et al.*, 1978). Strikingly, neuronal terminals in the putamen seem to be more effected than the neuronal cell bodies in the SNc (Bernheimer *et al.*, 1973), suggesting a dying back mechanism of neuron loss.

Besides dopaminergic neurons from the nigrostriatal pathways other neuronal pathways are also affected to some extent in PD. For example neurodegeneration is also found in neurons in the hypothalamus, cerebral cortex, olfactory bulb, and sympathetic ganglia and also in neurons using other neurotransmitter such as, noradrenalin (locus coeruleus), serotonin (raphe), acetylcholine (nucleus basalis of Meynert) (Hornykiewicz and Kish, 1987). Depending on the type and extent of neurons lost in these other brain locations, other symptoms might develop which include depression and dementia.

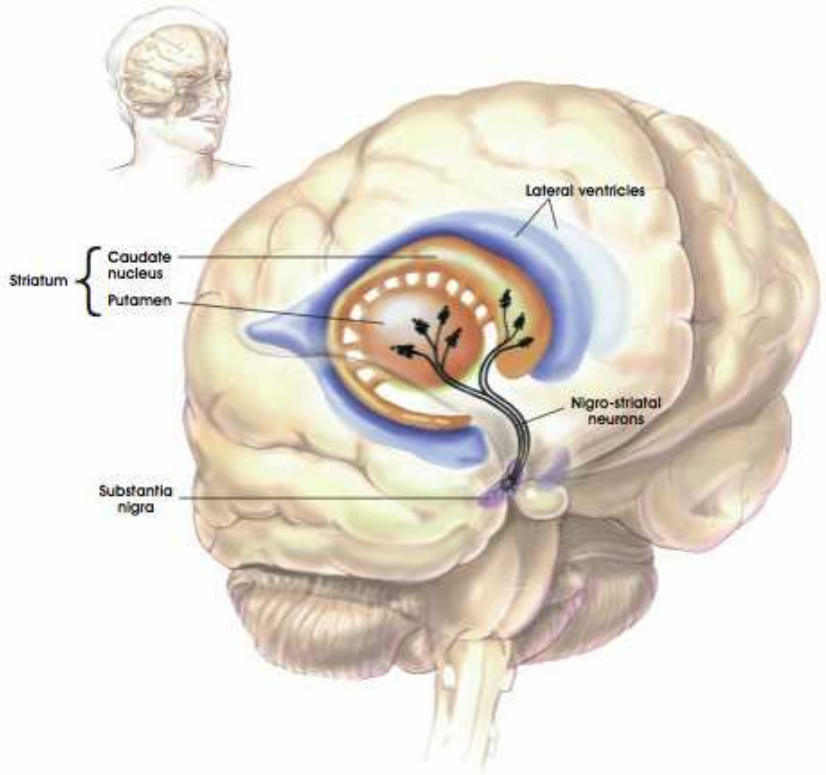

Figure 1; Neurons of the nigro-striatal pathway are most effected in PD. These neurons connect the substantia nigra with the striatum and are part of the pathway that initiates movement. Picture adapted from (Terese Winslow and Lydia Kibiuk, 2010).

Etiology of PD

When discussing the etiology of PD it is important to distinguish between Parkinsonism, also called Parkinson syndrome, which is a collection of diseases leading to PD-like symptoms and PD itself, which is characterized by neurodegeneration in the SNc and Lewy body formation. Although 80% of Parkinsonism cases are induced by PD, some causative factors of Parkinsonism do not apply for PD.

Only 5-10% of PD cases have a clear cause in the form of a genetic mutation such as Ala53Thr and Ala30Pro in α-synuclein (Polymeropoulos *et al.*, 1997). This type of PD is referred to as inherited PD and is often characterized by an early onset of the disease. The rest of cases is referred to as sporadic PD and is characterized by an average age of onset of disease of 55 years.

The etiological cause of sporadic PD is hard to pinpoint, however, there are certain factors that might increase the risk op developing PD. One such a factor is genetic variation. Although these variations do not directly cause PD, they might cause differences in expression pattern of proteins or produce mutated proteins increasing the risk of PD (Yang *et al.*, 2009). Recently is was suggested that half of the risk of developing PD can be identified genetically (Hardy, 2010).

A second risk factor for developing PD is of exogenous origin. It is suggested that both chemicals and infectious agents are able to initiate PD. Although some chemicals such as, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat have already proven to cause Parkinson-like symptoms it is hard to say if these chemicals could also be involved in sporadic Parkinson or result in the same symptoms via an other pathway (Dauer and Przedborski, 2003). Although development of sporadic PD is unlikely to result from one particular chemical or infectious agent, as they would already have been found after 200 years of research, they might increase the risk or accelerate disease progression. Interestingly, PD is one of few disease in which smoking and the consumption of coffee is inversely correlated with disease development (Morens *et al.*, 1995)

The last risk factor in sporadic PD described in this section is age. Although age itself can not cause PD, over time, accumulation of small mistakes made by biochemical processes inside the cell might eventually pass a threshold causing a positive feedback loop (Beckman and Ames, 1998). Neurons would be especially susceptible as they are postmitotic cells and therefore last a lifetime, as compared to other cell types which maximally last for days or several years. One example of accumulation of damage involves oxidative stress and is discussed below.

Taken together PD is probably caused by the accumulation of damage inside the cell due to aging, possibly ameliorated by exogenous chemicals or infectious agents. Some people would be more sensitive to this cell damage due to gene variations.

Molecular mechanisms in PD

The molecular mechanisms behind neurodegeneration in PD are highly complicated and interwoven with both pathological and normal pathways. In addition, research into these mechanisms is complicated by the number of cell types involved, including neurons, microglia, and astrocytes. Also, these cells are part of a difficult 3d structure inside the brain, which is hard to copy in cell culture studies. However, over the years more and more evidence is pointing towards two main mechanisms in PD, oxidative stress and misfolding of α-synuclein.

Oxidative stress

Over the years many studies have shown that oxidative stress is involved in the initiation of PD. Oxidative stress is characterized by an imbalance in the production, the functional requirement, and deletion of Reactive Oxygen Species (ROS) in a cell, resulting in ROS accumulation. Some examples of ROS are hydrogen peroxide, superoxide, peroxyl radicals, nitric oxide, and hydroxyl radicals. Tight regulation of these ROS is required, because of their reactive and destructive nature against nucleic acids, proteins, lipids, and other molecules. Production of ROS is part of the normal cell metabolism by mitochondria, via the electron transport chain. It is estimated that 1-2% of oxygen molecules is converted into superoxides by mitochondria in a normal brain (Cadenas and Davies, 2000). These low levels of ROS are part of important signaling pathways in cells. Next to PD, overproduction of ROS is associated with other diseases including, atherosclerosis, myocardial infarction, and Alzheimer's disease.

Brain tissue is one of the most susceptible types of tissue to accumulate ROS due to high oxygen consumption. Approximately 20% of the total oxygen consumption of the body is used by the brain. In addition dopaminergic cells are extra fertile to ROS accumulation because normal dopamine metabolism produces ROS (Dauer and Przedborski, 2003) and are therefore thought to be in a permanent state of oxidative stress (Greenamyre *et al.*, 2002).

The second inducer of oxidative stress in PD is failure of complex 1, causing mitochondria to produce excessive amounts of ROS. This was first shown in an experiment that coupled the effect of MPTP (chemically induces PD-like symptoms in animal models) to inhibition of complex 1 in the mitochondrial electron transport chain (Nicklas *et al.*, 1987) and was later confirmed in *post mortem* PD substantia nigra brain (Schapira *et al.*, 1989). Subsequent studies have shown that in PD complex 1 activity is reduced 30-40 percent (Mann *et al.*, 1992). Also, the effect was shown to be transmissible via mtDNA in cybrid cells (healthy cells without mitochondria fused with platelets, which contain motochondria but no nucleus, of PD patients), resulting in 20 percent decrease in complex 1 activity and increase of ROS production (Swerdlow *et al.*, 1996)

Taken together, both normal mitochondrial metabolism and dopamine metabolism induce a permanent state of oxidative stress in neurons. This is supported by the finding that in the SNc of PD cases biological markers for oxidative damage are elevated (Przedborski and Jackson-Lewis, 2000). The result of this oxidative stress would include damage to nucleic acids, proteins, lipids, and other molecules resulting in cell apoptosis, one of the cell death pathways that is highly activated in PD (fig. 2) (Perier *et al.*, 2005; Perier *et al.*, 2007; Vila and Przedborski, 2003). Protection of cells against oxidative stress via intake of antioxidants may protect against PD (De Rijk *et al.*, 1997a).

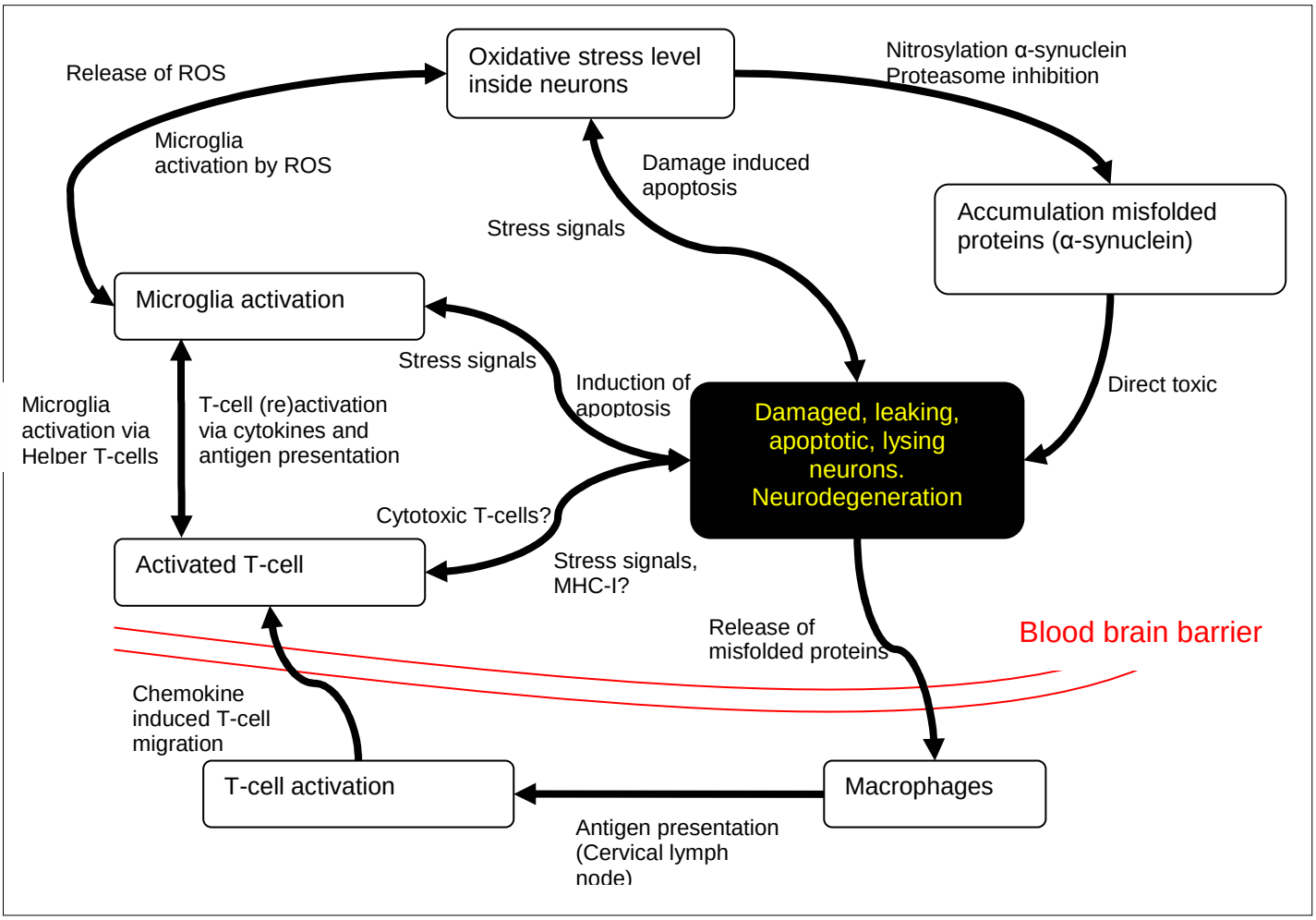

Figure 2; Schematic representation of the feedback loops in PD (Double arrows; effects clockwise). Depending on initiation of PD some loops may exert more effect than others. For example, mutations in α-synuclein in inherited PD will greatly induce protein accumulation, neurodegeneration, and T-cell activation, whilst on the other hand MPTP-induced PD will greatly induce oxidative stress, neurodegeneration, and microglia activation.

Abnormal protein aggregation

As mentioned above, continuous exposure to ROS leads to damage in proteins. One of these proteins is α-synuclein. α-Synuclein is a natively unfolded 140 amino acid long soluble protein that is involved in transport of synaptic vesicles. It belongs to a larger family of highly conserved proteins, which include β-synuclein and γ-synuclein. α-Synuclein can both be phosphorylated and nitrated.

Much of the knowledge on the involvement of α-synuclein in PD came from the research of Lewy bodies. Besides neurodegeneration the second condition that characterizes PD is the formation of Lewy bodies. Lewy bodies are spherical eosinophilic cytoplasmic protein aggregates containing oxidized and nitrosylated α-synuclein, parkin, ubiquitin, and neurofilaments (Braak and Del Tredici, 2009; Giasson *et al.*, 2000). Formation of these Lewy bodies is the result of accumulation of misfolded α-synuclein in the cytoplasm of neurons. Lewy bodies are initially formed inside cells, however upon apoptosis or lysis of dying cell, both Lewy bodies and soluble misfolded α-synuclein leak into the extra cellular space where they are thought to induce an immune response (see below).

Strikingly, accumulation of proteins seems to be a feature of several neurodegenerative diseases, including Alzheimer's, Huntington, Frontotemporal dementia and PD. Accumulation can be toxic to cells by formation of fibrils, interfering with cellular transport, and by depletion of resources. In PD, dopamine is shown to be a stabilizing factor for α-synuclein fibril formation, by formation of dopamine-α-synuclein adducts, rendering dopaminergic neurons increasingly susceptible to α-synuclein accumulation (Conway *et al.*, 2001).

Accumulation of α-synuclein and formation of Lewy bodies is greatly enhanced in several inherited forms of PD. For example, the first mutation identified to cause inherited PD was in α-synuclein, Ala53Thr, and is correlated with early-onset PD (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997). However, this mutations was not found in sporadic PD patients (Higuchi *et al.*, 1998). Other mutations such as in, Parkin, Park1, and DJ-1 are all known to enhance α-synuclein accumulation by inhibiting the proteasome, inducing mitochondria dysfunction, or disrupting synaptic vesicle formation (Hardy, 2010).

Taken together, genetic, oxidative, or spontaneous factors can induce misfolded and nitrosylated α-synuclein to accumulate and aggregate. These aggregations either have a direct toxic effect or induce an immune response thereby initiating the neurodegenerative process in PD (fig. 2).

Role of inflammation in Parkinson's disease

Much of the early research into PD did not recognize inflammation as being involved in PD. Most of this early misgiving came from the concept that the CNS was immune privileged. This concept was first described in the beginning of the twentieth century when it became apparent that the immune response to transplanted tissue into the CNS did not resemble that of the immune response in the periphery (Murphy J.B. and Sturm E., 1923). Consequently it was thought that immune cells were not present in the CNS and inflammation could not take place. Fortunately, in the following decades it became clear that nuances had to be made and nowadays thousands of studies show that indeed both the innate and the adaptive immune system play a major role in safeguarding the CNS. However, they also play a major role in the CNS pathology, including in PD.

Innate immunity

The first line of defense against pathogens and pathological processes is the innate immune system. The innate immune system comprises a number of mechanisms and cell types that prevent the host from being infected. Cells of the innate system act via non-specific reactions to either stress or danger signals spread by human cells or pathogens.

Blood brain barrier

The first lines of defense against CNS infection are the barriers that prevent pathogens from entering the CNS. As is true for any place in the body, before a pathogen or toxin is able to penetrate into the systemic blood flow it has to pass either the skin or the lining of the respiratory or gastrointestinal tract. The anatomical properties of these barriers prevent large compounds from passing. Also these barriers are lined with specialized immune cells that scrutinize everything that tries to pass.

However due to the important and delicate nature of the brain, it is protected via an extra barrier. This barrier, between the systemic blood flow and the brain tissue, is called the Blood Brain Barrier (BBB). The BBB consists of tightly packed capillary epithelial cells that prevent large molecules from passively entering to the brain (Rubin and Staddon, 1999). Small molecules are allowed to pass, however larger molecules have to be actively transported over this barrier (Persidsky *et al.*, 2006).

Although the function of the BBB is critical in the protection against external pathogens, it remains to be discussed whether or not it is protective in PD. Although the involvement of toxins and pathogens in the etiology of PD cannot be ruled out, it might be the case that PD is entirely resulting from internal processes in the brain (see "etiology of PD"). If the latter was the case, the BBB would not be involved in the prevention of PD initiation. However, the BBB would be highly involved in the

propagation of the diseased by disallowing active immune cells from entering the brain, which is discussed later.

Microglia

The second line of defense against infection of the CNS is the innate immune cells. Innate immune cells react upon non-specific danger and stress signals which they filter from their environment. A large number of different types of these cells are known in the periphery, however, only one major type is known in the brain, the microglial cells. Traditionally, microglial cells where simply thought to be a type of housekeeper cells and that their main role was to provide a constant homeostatic extracellular environment for the neurons and to clear cellular debris (Barron, 1995). However, currently microglial cells are known to be full-fledged immune cells capable of recognition, phagocytosis, processing and presentation of antigens, as well as recruiting other immune cells via the release of cytokines and chemokines. Microglial cells account for 5-20% of the total glial population depending on the region of the brain (Dobrenis, 1998).

In resting condition microglia are constantly surveying the CNS for signs of damage. Due to their high sensitivity they can react upon the slightest change in neuronal microenvironment (Kreutzberg, 1996). Activation of microglia is done either via direct interaction with neurons, neurons change their gene expression pattern upon injury, via the interaction with activated astrocytes, or via cytokines such as interferon(IFN)-γ and Tumor Necrosis Factor (TNF)(Mosser, 2003). Also microglia express a low amount of the Fcγ-receptor which allows them to react to the small amount of immunoglobulin that is present in the normal brain (Aihara *et al.*, 1994) in a highly specific manner.

When presented with one of the signals as described above, microglia get activated rapidly, but in a graded and highly controlled fashion (Kreutzberg, 1996). The first state of activation is characterized by an amplified state of alert. This state features increased expression of complement receptors, increased expression of adhesion receptors such as LFA-1, ICAM-1, VCAM-1, and CD1 and major changes to the internal cytoskeleton structure, causing the cell body to swell (hypertrophy) (Raivich *et al.*, 1999). Also the microglia may re-enter the cell cycle to increase the number of glial cells (hyperplasia) (Raivich *et al.*, 1999).

When the negative environmental stimulus continues, microglial cells transform into an active microglia. The active microglia cell is capable of secreting proinflammatory cytokines TNF-α, IL-1B, and IL-12, chemokines, proteases, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Banati *et al.*, 1993; Mosser, 2003). Also the phagocytic properties of the microglia are increased, to clear the pathogen and to remove damaged cells, together with the ability to process and present antigens, via MHC class II proteins (Dobrenis, 1998).

Normally after this last phase, when the pathogen is cleared, microglia enter an anti-inflammatory phase in which they facilitate neural repair and reconstruction (Mosser, 2003). However when the pathogen is failed to be cleared, microglia enter a chronic activated state in which they continuously secrete proinflammatory signals, ROS, and RNS and phagocytize foreign material and damaged cells.

Most of these general principles also apply for microglia activation in PD. The SNc, the area most effected by PD, contains the highest concentration of microglial cells in the CNS making it a region of increased surveying and increased susceptibility to toxin induced neurological damage (Kim *et al.*, 2000; Lawson *et al.*, 1990). Also the activation of microglia seem to depend on the same signals as elevated levels of the proinflammatory mediators, TNF-α, IL-1β, IL-6, ROS, and RNS are found in the SNc of mouse models of PD (McGeer *et al.*, 1988b; Mogi *et al.*, 1994; Nagatsu *et al.*, 2000). These activated microglial cells are present in high numbers in *post mortem* brains of PD patients (Ghosh *et al.*, 2007). However the actual prove came from ex vivo studies which showed dying dopaminergic cells being actively phagocytized by microglia (Giasson *et al.*, 2000). These findings were not only true for PD but also for other neurodegenerative disease such as Alzheimer's disease, Huntington, Multiple Sclerosis, and Amyotrophic Lateral Sclerosis.

However, there are also some important differences. For example the initial activation of microglia in PD is largely done in the absence of reactive astrocytes (Hirsch, 2000; Vila *et al.*, 2001). This could be explained by a direct mechanism of activation of microglia by neurons, rendering a messenger type of cell non-essential. The ability of neurons to actively express activating signals is backed-up by evidence that large numbers of reactive microglia are located near the cell bodies of neurons in the SN and not to their degenerating termini, where passive activating signal are released (Mirza *et al.*, 1999).

Finally, it is thought that microglia in PD do not reach the final level of assisting in repair. Microglial cells are continuously activated by pro-inflammatory signals including, T-cell factors, signals

from apoptotic neurons, and the direct stimulation of aggregated α-synuclein. This causes the microglia to go into a chronically activated state in which they continue to excrete ROS, RNS, and pro-inflammatory cytokines (Kosloski *et al.*, 2010). In conclusion, microglia are thought to ameliorate disease progression by secretion of ROS and RNS thereby inducing a feedback loop in which oxidative stress causes cell damage and protein accumulation, protein accumulation causes microglia activation, and microglia activation causes oxidative stress (fig. 2).

Complement system

The complement system is a highly complicated system with the ability to stimulate inflammation, assist in phagocytosis of antigens, and destroy invaders. The system is based on a cascade of proteases which is either activated by immunoglobulins or via direct hydrolytic activation of one of its proteases (C3). Whilst Individual fragments of the complement system are stimulatory to inflammation the full effect of the cascade is the formation of a macromolecule, called membrane attack complex (MAC or C5b-9). This complex normally causes lysis of bacteria by inserting itself into the bacterial membrane, however when assembly of such complexes is taking place in the vicinity of neurons, these could also be attacked in a process called bystander lysis. Increased quantities of subunits (C1q, C3b, C3d, C5b-9) of the complement system were found in the immediate vicinity of neurons in patients who suffered from traumatic head injury (Bellander *et al.*, 2001). A number of these proteases was also found in SN of PD and not in control SN when stained with antibodies (Yamada *et al.*, 1991; Yamada *et al.*, 1992). Also, increased levels of complement system mRNA was found in PD patients (McGeer and McGeer, 2004). Together this indicates that this pathway is also activated in PD. However it is extremely difficult to state whether or not this process is involved in the initiation of the disease or merely a weak bystander.

Adaptive immune system

Besides activation of the innate immune system, currently there is also a large body of evidence towards activation of the adaptive immune system in PD. The adaptive immune system is a highly specialized system of immune cells that react in an antigen specific manner and is able to generate "memory". The cells of the adaptive immune system are called lymphocytes and can be sub-divided into T-cells and B-cells. Both types of lymphocytes originate from multipotent hematopoietic stem cells in the bone marrow. Traditionally, the brain was considered to be shielded from an adaptive immune response, due to both the peripheral origin of the cells, which were thought to be unable to pass the BBB, and the lack of lymphatic drainage. However, recent studies show that naïve as well as activated T-cells and B-cells are able to penetrate into the CNS of healthy subjects, albeit in low levels (Anthony *et al.*, 2003; Hickey *et al.*, 1991).

The question remains whether or not these cells play a role in PD. Although recent studies have shown that B-cells are involved in CNS injury (Ankeny and Popovich, 2010), and that antibodies collected from PD patients are able to induce dopaminergic neurodegeneration when synergistically introduced with complement protein C5a into neuron-glia cell cultures (Wang *et al.*, 2007), there is little evidence of their involvement in PD. Also, B-cells are not found in *post mortem* human PD brain or in the MPTP mouse model of PD (Brochard *et al.*, 2009). Therefore B-cells are beyond the scope of this thesis. T-cells on the other hand are studied in much more detail and are discussed below.

T-cells in PD

Introduction

T-cells are an important link in the process of inflammation, due to their ability to orchestrate both the adaptive and the innate immune system. In addition, T-cells are able to both induce and suppress inflammation by means different T-cells subsets. The major subsets of T-cells include, $CD4^+$ T-helper (T_h)-cells, $CD8^+$ T-cytotoxic (T_c)-cells, and T-regulatory (T_{reg})-cells, all of which express the antigen specific T-cell receptor (TCR). Both recent and older studies show infiltration of all of these subsets in both *post mortem* human PD brain specimens and in ex-vivo MPTP-intoxicated mouse brain specimen (Brochard *et al.*, 2009; Hickey *et al.*, 1991; McGeer *et al.*, 1988a). Also, dopaminergic neurodegeneration was attenuated in immunodeficient mice, lacking mature T-cells (Brochard *et al.*, 2009).

Activation

One general prerequisite for activation of T-cells is the presentation of an antigen by either normal cells via MHC-I or by antigen presenting cells via MHC-II, in conjunction with a co-stimulatory signal. Recently, a computer based study (using predictive tools for proteasome cleavage, Transporter associated with Antigen Processing (TAP) Binding, processing and MHC binding) has shown that both α-synuclein and nitrated-α-synuclein can be processed and presented via both MHC-I and MHC-II (Benner *et al.*, 2008). Thus α-synuclein, the major component of Lewy bodies, can be presented by both normal cells and antigen presenting cells. However previous studies have already shown that antigen presentation via brain epithelial cells, expressing MHC-I, is very poor (Pryce *et al.*, 1989), regardless of their ideal position on the boundary between the CNS and the peripheral blood flow. Also, although expression of MHC-II by microglia is increased upon activation by ROS and their ability to potently induce T-cell activation in a cell culture experiment (Tezel *et al.*, 2007), it is unlikely that initial activation of T-cells takes place in the brain itself. This is supported by the fact that activation of T-cells is inhibited and the lifespan of T-cells is decreased by oxidative stress (Fonseca *et al.*, 2001; Klemke *et al.*, 2008; Malmberg *et al.*, 2001), one of the major factors in PD and present in large extent in the area of PD inflammation.

Fortunately this does not exclude T-cells from being activated against α-synuclein. Recent evidence showed drainage of nitrosylated-α-synuclein to the cervical lymph nodes in an MPTP-treated mice (Benner *et al.*, 2008) (fig. 2) as was shown before for amyloid-beta in Alzheimer (Weller, 1998). Also, antigen-presenting cells within the cervical lymph nodes were reported to increase their surface expression of MHC-II, and peripheral leukocytes were activated with high efficiency (Benner *et al.*, 2008). Although these active lymphocytes are peripheral, microglial induced chemokines gradient attract them to pass the BBB (Babcock *et al.*, 2003), which on itself is reported to be dysfunctional in PD (Kortekaas *et al.*, 2005) via the action of IL-17 and IL-22 (Kebir *et al.*, 2007). This was further confirmed by a study that showed the ability of splenocytes harvested from nitrated-α-synuclein immunized mice to enhance neuronal loss when adoptively transferred into MPTP-treated mice (Benner *et al.*, 2008). Taken together this evidence strongly indicates T-cell activation occurs in the periphery rather than in the CNS (Aloisi *et al.*, 2000). This would also explain the neuroprotective role for non-steroidal anti-inflammatory drugs (NSAIDs) in Parkinson's Disease and other neurodegenerative diseases (Gagne and Power, 2010). Even though most NSAIDs are unable to pass the BBB efficiently (Parepally *et al.*, 2006), they are able to sufficiently suppress T-cell activation in the periphery (Paccani *et al.*, 2002).

Pro- and anti-inflammatory effect of T-cells subsets

Upon activation of T-cells and their subsequent infiltration into the SNc, distinction has to be made between the different subsets of T-cells to clarify what kind of effect they have. Next we discuss the effect of T_c-cells, T_h-cells, and T_{reg}-cells.

In a typical immune response, cytotoxic T-cells, also known as killer T-cells, are capable of inducing cell death when their TCR is activated by an antigen-MHC-I complex of the target cells. In PD the antigen would be α-synuclein, and the target cells would be neurons. However, it is questionable whether or not T_c-cells play a major role in PD neuronal death. Although, increased numbers T_c-cells are found in close proximity to activated microglia and degenerating neurons within the SN of PD patients (McGeer *et al.*, 1988b), removal of T_c-cells did not reduce neurodegeneration in MPTP-treated mice (Brochard *et al.*, 2009). Also, one of the requirements for antigen presentation by neurons is an efficient proteasome, however the proteasome is intensively and irreversible inhibited by the oxidative metabolites of dopamine (Zhou and Lim, 2009). Therefore neurons would not be efficient in presenting the antigen to T_c-cells and would therefore not be recognized as a target cell.

Helper T-cells on the other hand are much more likely to be involved in disease progression in PD. Although T_h-cells can be considered useless when on their own, together with other immune cells some of the T_h-cells subsets are strong inducers of cell death. T_h-cells can be subdivided into several subtypes, of which T_h1 and T_h2 cells are two examples (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). The function of these subtypes is very important as T_h1 and T_h2 cells drive the immune response toward cell–mediated (macrophages/microglia) or humeral (B-cell/antibody) respectively.

Most T_h-cells do not have phagocytic or cytotoxic properties; however, they force the environment towards a pro-inflammatory state. For instance, T_h1- and T_h17-cells express IL-2, interferon-γ, and TNF-α which induce release of ROS and nitric oxide by microglia (Kosloski *et al.*, 2010). Besides its pro-inflammatory effect T_h17-cells are also capable of inducing direct damage to the neurons via the release of granzyme B (Kebir *et al.*, 2007). Unlike T_c-cells, T_h-cells are not dependent on re-activation via neurons, but react via the antigen-MHC-II complex of the antigen presenting

microglia (Tezel *et al.*, 2007). Therefore re-activation of T_h-cells is not effected by a faulty proteasome as this is not reported in microglia. A good indication of the involvement of T_h-cells in PD comes from the fact that removal of CD4+ T-cells reduced neurodegeneration significantly in MPTP-treated mice (Brochard *et al.*, 2009). Also an increase is reported in the number of T_h-cells in the periphery of PD patients compared to healthy subjects (Hisanaga *et al.*, 2001).

Fortunately, not all T_h-cell subsets are pro-inflammatory. T_h2-cells are thought to have anti-inflammatory properties in PD. In a normal immune reaction, T_h2-cells would induce the humeral response via activation of B-cells, and therefore induce a pro-inflammatory response. However, no large humeral response is reported in PD and therefore the pro-inflammatory effect of T_h2-cells might not be present. The anti-inflammatory effect on the other hand, can be explained by the inhibition of T_h1-cells via the excretion of IL-4 and IL-10. Inhibition of T_h1-cells would indirectly inhibit microglia.

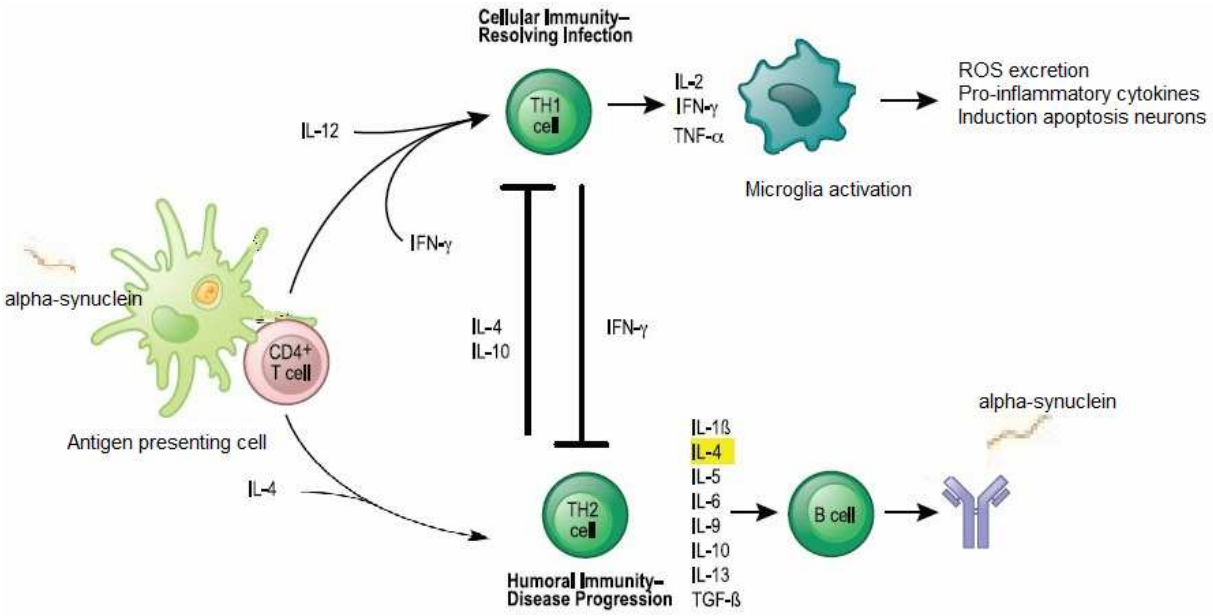

Figure 3; T_h-cells are activated by antigen presenting cells. Upon activation and depending on environmental stimulatory factors a decision is made toward a predominantly T_h1 or T_h2 response. It is suggested that a balance has to exist between the T_h1 and the T_h2 response, to prevent chronic inflammation. In PD, little research is done on the involvement of humeral immunity, however, the chronic nature of microglia activation in PD suggest that primarily T_h1 cells are activated. Illustration adapted from (Ezra *et al.*, 2010).

T_h1- T_h2 balance

It is often thought that a proper immune reaction is characterized by a balance between the T_h1 and T_h2 reaction. This does not mean that they have to be equal, but there has to be an equilibrium at which both reactions are able to control the other. Although, some find the existence and necessity of this balance controversial (Kidd, 2003), shifting of this balance is related to multiple diseases. A shift towards T_h1 is associated with delayed type hypersensitivity DTH due to excessive macrophage or microglia activation, causing autoimmune diseases such as Multiple Sclerosis (MS) and insulin dependent diabetes mellitus. A shift toward T_h2 on the other hand is associated with allergies (Singh *et al.*, 1999). In PD the T-cell reaction seems to shift towards a T_h1 response, causing the extended chronicle activation of microglia.

Normally, the faith of the immune reaction towards either a T_h1 or T_h2 is decided by the antigen presenting cell and its environment. In the case of PD this would be the antigen presenting cells in the cervical lymph nodes or antigen presenting microglia during reactivation in the CNS. One of the effects that could influence the faith towards is T_h1 or T_h2 is the oxidative state of the antigen presenting cell. In antigen presenting cells glutathione is the one of the most important regulatory antioxidants (Kidd, 1997) and is known to modulate the immune response to either T_h1 or T_h2 (Peterson *et al.*, 1998). In the oxidized state, a cell contains a reduced concentration of glutathione and is associated with a high T_h2 response, on the other hand a non-oxidized state favors a T_h1 response (King *et al.*, 2006; Murata *et al.*, 2002). However, this contrasts the situation of microglia which are in a highly oxidized environment. This supports the idea that the initial activation of T-cells and therefore the decision towards a T_h1 or T_h2 response happens in the periphery rather than in the

CNS. Upon reactivation in of T-cells in the CNS this initial decision towards T_h1 could be enhanced by dopamine, which is known to induce a T_h2 to T_h1 shift in activated T_h-cells (Levite, 2008), independent of microglial oxidation state.

In general, a better understanding of this T_h1/T_h2 balance together with knowledge about the factors that influence it might result in new therapeutical possibilities. For instance, in experimental allergic encephalomyelitis (EAE), a mouse model for multiple sclerosis and characterized by an extensive T_h1 response, induction of a shift towards T_h2, via orally induced tolerance, IL-4 treatment, or interferon-beta therapy (Chen *et al.*, 1994; Matsui, 2008; Racke *et al.*, 1994) diminishes demyelination and enhances remyelination (Gaupp *et al.*, 2008). In conclusion, a therapy aimed towards inducing a T_h2 response might be beneficial in PD. However, it might also accelerate disease progression by stimulation of B-cells and subsequent induction of a strong antibody reaction against α-synuclein, therefore caution has to be taken when introducing such a therapy.

Regulatory T-cells as a therapy for PD

T_{reg}-cells are a special subset of T-cells that have the sole purpose of inhibiting inflammation. Once an immune response has been successful in removing the antigen, both immune T_c-cells and T_h-cells along with their activating signal molecules have to be suppressed to limit unwanted side effects such as damage to self-tissue. T_{reg}-cells achieve this by excretion of the anti-inflammatory cytokines IL-10 and Tumor Growth Factor (TGF)-β. Also, T_{reg}-cells are able to consume pro-inflammatory cytokines. Consumption of pro-inflammatory cytokines causes a disruption in the positive feedback loop between helper and effector cells, resulting in cytokine deprivation induced T_h-cell apoptosis (Pandiyan *et al.*, 2007).

In PD, adoptively transferred T_{reg}-cells have shown to be neuroprotective in MPTP-intoxicated mice (Reynolds *et al.*, 2007), despite the ability of dopamine to reduce their suppressive activity (Kipnis *et al.*, 2004). The protection of T_{reg}-cells is thought to be caused by inhibition of microglial cytokine excretion and induction of microglia apoptosis (Reynolds *et al.*, 2009). However, in human no increase was found in the relative number of T_{reg}-cells in PD and Alzheimer patients compared with controls (Rosenkranz *et al.*, 2007). This indicates that the body does not actively induce T_{reg}-cells, and leaves a possibility to induce them via medicine or to therapeutically introduce cultured T_{reg}-cells.

Specific T-cells as a biomarker for PD

At the moment there is no diagnostic tool that is fully capable of detecting PD before clinical symptoms are visible. This is unfortunate as it is estimated that 60%-80% of SNc dopaminergic neurons is already lost at onset of clinical symptoms (Dauer and Przedborski, 2003). Therefore enormous effort is put into the development of biomarkers (Frasier *et al.*, 2010), which would predict sporadic PD before clinical symptoms appear. On the basis of such a biomarker screening could take place in groups of people at risk for having PD in a pre-clinical symptom stage, and an early diagnosis could be achieved. This would result in therapy to begin earlier and thereby possibly slow down or prevent disease progression. Also, an early onset biomarker would be a great tool in research. It would give insight into the early stages of the disease and would shed a light on the involvement of oxidative stress and α-synuclein accumulation.

One type of biomarker that could be used is based on the specific reaction of T-cells against nitrated-α-synuclein epitopes (Benner *et al.*, 2008). A great advantage for such a biomarker would be the high specificity, as many other biomarkers tent to have difficulty to differentiate between the neurodegenerative diseases. Also, the specific T-cells would be easily harvested from the peripheral blood flow. Presence of specific T-cells in the blood can be tested via their ability to lyse cells presenting nitrated-α-synuclein (T_c-cells) or their ability to bind to such cells (T_c-cells, T_h-cells, T_{reg}-cells). However, the question remains in which stage of the disease specific T-cells can be visualized.

New therapies

Currently, therapies for the treatment of Parkinson's disease are palliative. They neutralize some of the symptoms for several years, but are unable to slow disease progression. Also, side effects are common and severely effect patients on a daily basis. Therefore, great effort is put into the development of new and better delivery of the current medicine. Besides the ordinary, also several out of the box approaches are being explored including, cell and tissue transplantation, immunization, and neuroprotective agents and immune modulatory approaches.

Tissue transplantation

Cell and tissue transplantation have proven to be indispensable in a wide variety of diseases such as corneal blindness (corneal graft), leukaemia (haematopoietic stem cell transplantations), and mitral stenosis (heart valve transplantation). However, although research into transplantation in PD has been done for several decades now, cell and tissue transplantation is not yet a widely applied technique in PD. This can partly be explained by large variation in success between studies (Winkler *et al.*, 2005) which on itself can be attributed to the ethical difficulty to perform a large scale sham-controlled trial, consequently the number of patients in trials were small and results were hardly significant.

Also the theory behind tissue transplantation poses some difficult questions. For instance, although transplantation would restore the number of the dopaminergic neurons lost due to PD neurodegeneration, it remain questionable whether or not these newly introduced neurons form the complex connections with the original neuronal tissue. Secondly, tissue transplantation does not halt disease progression, recent research even indicates that Lewy bodies are formed in the cells of the tissue graft (Kordower *et al.*, 2008; Olanow *et al.*, 2009). Thirdly, the tissue would be introduced locally into the brain despite the less than localized nature of PD (see neurodegeneration in PD). Lastly, tissue transplantation is a very expensive treatment, due to the chirurgical procedure.

Immunization

A second option for a new therapy would be immunization. Although, a clinical trial against Alzheimer's disease immunization has failed, due to initiation of encephalitis (Senior, 2002), immunization with α-synuclein in animal models for PD showed good results (Agbo *et al.*, 2009; Benner *et al.*, 2004; Masliah *et al.*, 2005). Also, immunization is traditionally directed toward stimulation of B-cells, however, recently more attention is emerged for immunization strategies directed towards T-cell subtypes such as T_c-cells and T_h2-cells (Chen *et al.*, 1994; Zaiss *et al.*, 2010). When such a strategy would be developed for PD, inducing either T_h2-cells or T_{reg}-cells, it might slow disease progression to a point where life expectancy would be equal to healthy subjects.

Neuroprotective and immune modulatory approaches

The third option currently investigated is the use of neuroprotective and immune modulatory medicine. For instance, activation of antioxidant genes such as ECSOD have proven to positively influence EAE (Qi *et al.*, 2007). In PD, activation of an antioxidant gene would be a great tool to compete with oxidative stress.

Immune modulatory approaches would include NSAIDs which have proven to be beneficial in PD (Ardestani, 2010; He *et al.*, 2001; Teismann and Ferger, 2001) due to their anti-inflammatory nature. However, when new types of neuroprotective and immune modulatory molecules and genes were to be found, this would greatly influence the therapy of PD.

Conclusion

When discussing PD it is hard to definitively indicate what factors initiate the disease. The most likely candidates are oxidative stress or accumulation of misfolded proteins which result in damage to neurons. In addition to the normal presence of these factors and the accumulation of damage with age, exogenous and genetic factors might play a role in amplifying them. Also, genetic factors might influence the cells sensitivity to this damage. Upon damage, neurons either expire via a direct toxic effect or by inducing apoptosis.

Disease progression on the other hand is characterized by a fast degeneration of neurons. It is unlikely that this is caused by only one of the factors mentioned above. Therefore, in this thesis, we propose that one factor is able to initiate a cascade of effects resulting in a series of feedback loops (fig. 2) explaining the progression of PD. Depending on the initial initiation factor, some of these loops will be more prominent than others. For example, MPTP induced PD will induce the feedback loop which includes, oxidative stress, neuronal cell death, and microglia activation, whereas mutations in α-synuclein will induce the feedback loop including, accumulation of misfolded proteins, neuronal cell death, T-cell activation, and microglial activation.

In the past decades there has been increasing interest towards the involvement of the immune system in Parkinson's disease. As a result, the role of microglia in the progression of PD is well established. However, it remains debated if microglia are also able to initiate neurodegeneration. Recently, Cx3cr1 knock-out mice, in which communication between microglial cells and neurons is impaired, showed to be highly protected against neuron loss in a model for Alzheimer's disease (Fuhrmann *et al.*, 2010).

Comparable studies knocking-out microglial functions in PD might be helpful in future research on the role of microglia in PD. Moreover, because this study showed a combined *in vivo* measurement of neuronal cell loss and microglial infiltration it could shed light on the question if microglia activation is just a result of neuronal cell death or if microglia are activated beforehand, via active excretion of signals by neurons, and induce apoptosis pathways in neurons.

Other parts of the immune system that show increasing interest of the field are the T-cells and its wide variety of subsets. Many studies over the last years have shown involvement of T-cells in PD pathogenesis (Brochard *et al.*, 2009; Fiszer *et al.*, 1994; Hisanaga *et al.*, 2001; Reynolds *et al.*, 2007; Reynolds *et al.*, 2009). However, T-cells could also pose new ways in the treatment of PD. For instance, the T-cell subset T_{reg} cells are in theory able to induce anti-inflammatory signals, therefore expansion of these cells in PD patients might be beneficial (Brusko *et al.*, 2008). Also, the balance between the $T_{helper}1$ and $T_{helper}2$ cells has received much attention and is thought to be an important factor in the excessive activation of microglia in PD. Unfortunately much of this research is done in animal models for PD. It is therefore needed to design new studies measuring the presence and state of activation of these cells in blood samples of PD patients, starting with PD patients carrying mutations of the α-synuclein gene as we expect them to induce T-cells more significantly.

Future research is likely to focus more and more on the influence of immune cells on PD. When starting this research it has to be kept in mind that much can be learned from research done in other diseases such as, Alzheimer's (immunization studies) and rheumatoid arthritis (T_{reg} studies), and from abnormal T-cell activation in for instance delayed type hypersensitivity ($T_{helper}1$ and $T_{helper}2$ balance studies).

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