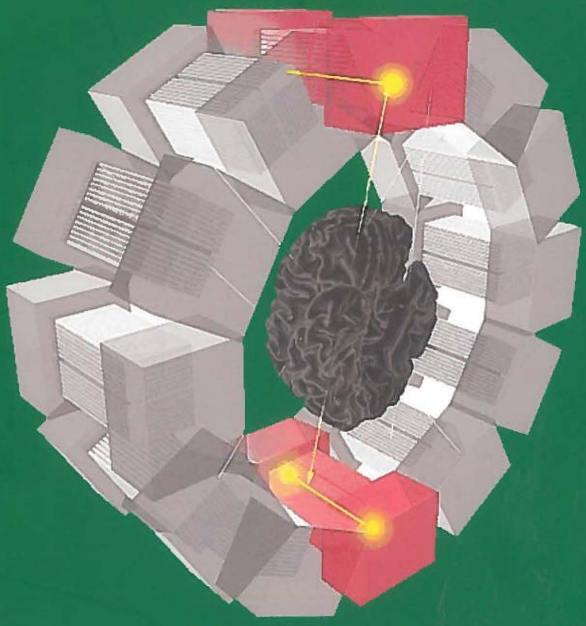
Pathogenetic mechanisms in Parkinson's disease

Bartels, Anna L.

Pathogenetic mechanisms in Parkinson's disease: Studies with Positron Emission Tomography



Anna L. Bartels

In this thesis, PET is used to study the role of blood-brain barrier (BBB) dysfunction and neuroinflammation as possible contributory factors in the pathogenesis of Parkinson's disease (PD). The first part of the thesis focuses on the involvement of BBB P-glycoprotein transporter (P-gp) function in PD pathogenesis using [^{11}C]-verapamil PET. Protective function of the BBB may be an important factor linking neurotoxic substances to the development and progression of neurodegenerative diseases. The second part of this thesis focuses on the role of neuroinflammation in the progression of PD. The use of [^{11}C]-PK11195 as a tracer for neuroinflammation was investigated in an animal PD model as well as in PD patients. Furthermore, the effect of anti-inflammatory treatment with a selective cyclo-oxygenase-2 inhibitor was studied *in vivo* with PET.

Pathogenetic mechanisms in Parkinson's disease:

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Anna Bartels

Stellingen
behorende bij het proefschrift

"Pathogenetic mechanisms in Parkinson's disease: studies with Positron Emission Tomography".

Anna L. Bartels

1. Dysfunctional blood-brain barrier P-glycoprotein is not a primary causative factor in the etiology of Parkinson's disease. (Chapter 3)

2. Impaired blood-brain barrier P-glycoprotein function is involved in progressive neurodegeneration. (Chapter 4)

3. Decreased blood-brain barrier P-glycoprotein function with aging is involved in vulnerability of the aging brain for accumulation of toxic substances and drugs. (Chapter 5)

4. The distribution volume of [^{11}C]-verapamil provides a measure for assessment of regional blood-brain barrier P-glycoprotein function. (Chapters 3-5)

5. The current analysis methods of [^{11}C]-PK11195 uptake in brain do not provide solid data to assess changes in specific tracer uptake by anti-inflammatory treatment. (Chapter 7)

6. COX-2 inhibition probably prevents up-regulation of P-glycoprotein-expressing vasculature, without an effect on microglia activation. (Chapter 8)

7. De kennis van een gevolg hangt af van de kennis van de oorzaak en sluit die in. (Spinoza, Ethica)

8. What is past is prologue. (Shakespeare, The tempest)

9. Van een theorie is het waarachtig niet de geringste charme dat zij weerlegbaar is. (Nietzsche)

10. Niets geloven we zo stellig, als wat we het minste weten. (M. de Montaigne)

11. Life is what happens to you, while you're busy making other plans. (John Lennon)

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Contents

Chapter 1

General Introduction

Parkinson's disease (PD) is a typical human neurodegenerative disease that usually starts in the 6th decade and progresses slowly but inexorably. The pathogenesis of PD is unknown, and the clinical syndrome that we recognise as PD has multiple causes and also displays multiple phenotypes. The key to development of any successful disease-modifying therapy depends upon a better understanding of the pathogenetic mechanisms of PD neurodegeneration. Genetic mutations are found in proteins involved in protein degradation, oxidative stress and mitochondrial function. Also the environmental toxins used in animal PD models inhibit mitochondrial function, increase free radical formation or can cause protein aggregation. Both genetic and environmental factors are considered important and have led to the notion of common final pathogenetic mechanisms in the aetiology of PD.

Questions of importance are: where does the pathological process start and what are the mechanisms that underly progression of neurodegeneration? In this thesis, two mechanisms that may be involved in PD development and progression of neurodegeneration will be investigated, namely blood-brain barrier (BBB) dysfunction and neuroinflammation. Loss of BBB function has been suggested as a possible (early) factor in the pathogenesis of PD, related to cerebral environmental toxin exposure. After initial damage, local substantia nigra brain stem neuroinflammation mediated by microglial activation possibly plays a pivotal role in the progression of PD.

Radiotracer imaging techniques allow *in vivo* assessment of biochemical parameters such as receptor density, neurotransmitter concentration or neuronal activation. Functional imaging studies using positron emission tomography (PET) can assess different biochemical mechanisms as disease unfolds, and could thereby offer insights into the pathogenesis of PD. Both PET and single-photon emission computed tomography (SPECT) have been used to identify dopaminergic defects in clinical and also pre-clinical

parkinsonism. PET and SPECT methods have been proposed as biomarkers to monitor disease progression. PET studies have demonstrated that early PD affects the dopaminergic projections to the posterior striatum, whereas in later disease stage also the anterior striatum becomes affected. Once disease has been established, the rate of progression is equal in striatal subregions [1;2], suggesting that the factors responsible for disease progression may be different from those that underlie the initial insult. The development of radiotracers for *in vivo* assessment of specific BBB function and of neuroinflammation now also allows investigating the involvement of these mechanisms in the pathogenesis of neurodegenerative disease.

Scope of the thesis

In this thesis, PET is used to study the role of BBB dysfunction and neuroinflammation as possible contributory factors in the pathogenesis of PD.

The first part of the thesis focuses on the involvement of BBB P-glycoprotein transporter (P-gp) function in PD pathogenesis using [^{11}C]-verapamil PET. Protective function of the BBB may be an important factor linking neurotoxic substances to the development and progression of neurodegenerative diseases. The P-gp transporter is present in high concentration in the BBB [3], where it functions as efflux pump to protect the brain from potentially hazardous substances. It has only recently become possible to measure P-gp function *in vivo* with PET and radiolabeled verapamil [4;5]. PD has been associated with a failure of the BBB to clear potentially toxic substances [6;7], which may have pathogenic implications. It remains an open question whether BBB P-gp functional impairment in PD may be a primary vulnerability factor or merely a consequence of reactive P-gp response to neurodegeneration.

The aim of this part of the thesis was to investigate to what extent the specific biochemical BBB functional impairment may play a role in the aetiology and the progression of PD and other parkinsonian syndromes. We used [^{11}C]-verapamil, a substrate for P-gp, using PET to study functional P-gp at the BBB in PD patients and in patients with progressive supranuclear paly (PSP) and multiple system atrophy (MSA) syndromes. Furthermore, we studied the effect of (normal) ageing on BBB P-gp function.

The second part of this thesis focuses on the role of neuroinflammation in the progression of PD. The factors responsible for disease progression in neurodegenerative diseases may be different from those that underly the initial insult. Upon neuronal injury, neuroinflammation with microglia activity is induced. This reaction can either be supportive for the recovery of neurons or result in neuronal death [8]. In PD, exacerbated microglia activation is suggested to enhance ongoing neurodegeneration through the production of neurotoxic substances. Epidemiological data indicate that anti-inflammatory agents such as non-

steroidal anti-inflammatory drugs (NSAIDs) may have a protective effect on PD [9;10]. Also, anti-inflammatory treatment reduced neuronal degeneration in experimental PD models [11] [12]. The role of neuroinflammation in PD pathogenesis however is still poorly understood.

Activated microglia show increased expression of the peripheral benzodiazepine receptor, which binds the PET ligand [^{11}C]-PK11195 to image activated microglia *in vivo* [13]. The aim of this part of the thesis was two-fold: first to investigate the use of [^{11}C]-PK11195 as a tracer for neuroinflammation in an animal PD model as well as in PD patients. Second to study *in vivo* the effect of anti-inflammatory treatment with a selective cyclo-oxygenase-2 inhibitor using [^{11}C]-PK11195 PET in the animal PD model as well as in a pilot study with PD patients.

A general introduction and the studies of these aims are presented in the following chapters:

In **chapter two**, a general introduction is given to describe PD. PD symptoms and associated basal ganglia pathophysiology is discussed. Special attention is paid to possible causes and pathogenesis of PD, with emphasis on the neurotoxin hypothesis in PD. Finally in this chapter PET imaging technology is introduced.

The goal of **chapter three** was to investigate whether dysfunctional P-gp at the BBB may be an early factor in the pathogenesis of PD. Dysfunctional BBB P-glycoprotein may cause increased accumulation of neurotoxic substances that are associated with the development of PD. A study with [^{11}C]-(*R*)-verapamil PET scans in early stage PD patients and healthy control subjects is presented. This study was performed in collaboration with the department of Nuclear Medicine and PET Imaging of the Free University Medical Centre in Amsterdam, the Netherlands.

For **chapter four**, [^{11}C]-verapamil PET scans were performed in patients with different stages of PD, and in patients with PSP or MSA, to study cerebrovascular P-gp function in the progression of neurodegenerative diseases with parkinsonism.

Chapter five investigates the effect of age, the main risk factor for neurodegenerative disease, on BBB P-gp function as measured with [^{11}C]-verapamil PET.

In **chapter six**, the role of neuroinflammation in the pathophysiology of PD is discussed. Evidence for a possible disease-modifying role of anti-inflammatory medication, especially inhibition of cyclo-oxygenase-2 (COX-2), is highlighted. The use of PET with the radioligand [^{11}C]-PK11195 is reviewed.

We have investigated the use of [^{11}C]-PK11195 and PET to measure neuroinflammation in PD patients. Results of this study are described in **chapter seven**, in which also preliminary results of the effect of COX-2 inhibition on [^{11}C]-PK11195 brain uptake in PD patients are presented.

Chapter eight describes the results of our study of BBB P-gp function, neuroinflammation and neurodegeneration in a PD animal model. Wistar rats were scanned with [^{11}C]-verapamil and [^{11}C]-PK11195 using a microPET scanner three weeks after a striatal lesion with 6-hydroxydopamine (6-OHDA), with or without receiving COX-2 inhibitory treatment. P-gp expression, microglial activation and dopaminergic neurodegeneration were assessed post mortem with immunohistochemistry. [^{11}C]-PK11195 uptake was compared to histological activated microglia count in the SN, to assess the use of [^{11}C]-PK11195 to measure neuroinflammation *in vivo*. The effect of COX-2 inhibition on P-gp function, neuroinflammation and dopaminergic neurodegeneration in this PD animal model was investigated.

Chapter nine provides an overview and general discussion of the studies presented in this thesis.

Chapter 2

Parkinson's disease: the syndrome, the pathogenesis and pathophysiology.

A shorter version of the introduction on Parkinson's disease is published in Cortex:

A.L. Bartels, K.L. Leenders: Parkinson's disease: the syndrome, the pathogenesis and pathophysiology. Cortex 2009, doi:10.1016/j.cortex.2008.11.010

Abstract

Parkinson's disease (PD) is characterised by a slowly expanding degeneration of neurons particularly in the mesencephalon. The causes are unknown although risk factors in the genetic and toxic domain are being discovered. An important pathophysiological feature in PD is the loss of part of the dopaminergic neurons in the substantia nigra resulting in a specific disorganisation of the complicated basal ganglia circuits. The relay functions at the level of the striatum e.g. are out of balance leading to disturbed subcortico-cortical interactions. At a functional level this is shown by problems in timing and scaling when performing movements and clinically this translates into initiation problems, bradykinesia and others. In this chapter an overview of the pathogenesis, basic pathophysiology and clinical problems of PD will be given.

In 1817, the neurologist James Parkinson described a disease that he called "Shaking Palsy", or "paralysis agitans": involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from walking to a running pace: the senses and intellects being uninjured [14]. His description led to the Parkinson's disease syndrome and the knowledge of the pathology and clinical spectrum of this disease has greatly evolved since then. In 1912, Friedrich Heinrich Lewy first described the inclusion bodies named after him that are now seen as pathologic hallmark of idiopathic PD. Tretiakoff then reported loss of pigmented cells in the substantia nigra pars compacta in patients with the encephalitic form of Parkinson's disease [15]. In the 1950's, Carlsson observed that 80 % of the dopamine in the brain is localised in the basal ganglia [16]. He made the link between loss of dopamine and Parkinson's disease. The dopamine-depletion theory was confirmed by postmortem biochemical studies showing decreased levels of dopamine and its metabolites in the nucleus caudatus, putamen, nucleus accumbens, substantia nigra and globus pallidus of PD patients [17]. More recently, positron emission tomography (PET) studies demonstrated impaired [^{18}F]-fluorodopa (FDOPA) uptake in the striatum of PD patients, reflecting the presynaptic dopaminergic defect in PD [18].

However, besides the dopaminergic deficit, PD is now believed to be a multicentric neurodegenerative disease. Differential effects on regions of the basal ganglia during the neurodegenerative process in PD have consequences for motor and cognitive capacity and the performance of several skills. It has been suggested that PD pathology follows a specific sequence, starting in the dorsal motor nucleus of the vagus nerve and the olfactory bulbs and nucleus, followed by the locus coeruleus, after which neuron cell loss appears in the substantia nigra pars compacta (SNc). Thereafter, less vulnerable nuclei and cortical areas gradually become affected [19]. Lewy bodies are found in the degenerating neurons and consist of neurofilaments with aggregated α-synuclein, and ubiquitin immunoreactivity. Whether Lewy bodies are causative or symptomatic in PD pathogenesis is not clear. Most likely however, the latter is the case [20].

Here, the epidemiology and pathogenesis of PD will be discussed, and an overview of clinical symptoms and pathophysiology in PD will be given.

Epidemiology of Parkinson's disease

Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's disease. The prevalence is 1-2/1000. However, 1 to 2 % of the elderly are affected because the incidence increases above the age of 50 [21]. Mean age of onset is 60 years. In about 5 %, age of onset is before 40 years; the disease is then classified as young-onset or early-onset PD. PD shows a difference in prevalence rates throughout the world [22]. On the other hand, incidental Lewy Body disease in post-mortem examinations, which is thought to reflect preclinical PD, is as frequently found in Nigerians as in western populations[23]. This may suggest a universal equal predisposition for PD, with a role for environmental factors influencing the actual prevalence of PD in different areas. The prevalence is highest in Europe and North America (between 100-300/100.000 compared to approximately 50/100.000 in Asia and Africa). It is slightly more common in men than in women. First-degree relatives of PD patients have a two- to threefold increased relative risk to develop PD [24]. A few genes have been identified that cause familial PD. However, disease concordance rates between monozygotic and dizygotic twin pairs revealed similar concordance rates when PD was diagnosed after the age of 50, suggesting that heredity is not a major etiologic component in most cases of PD [25]. A potential limitation of these studies, however, is the difficulty in estimating concordance rates based on clinical information alone. Indeed, FDOPA-PET scans revealed preclinical disease in apparently unaffected twins, and concordance rates as assessed by loss of dopaminergic function were higher in monozygotic than in dizygotic twins [26]. Together, epidemiological studies support the importance of both genetic and environmental factors as possible causes of PD, leading to the notion of final common mechanisms in PD pathogenesis.

Clinical symptoms in Parkinson's disease

The progressive degeneration of the nigrostriatal system causes dopaminergic denervation of the striatum. When clinical motor symptoms appear, putamen dopa uptake is reduced by at least 35 % [27]. The relationship between the nigrostriatal dopaminergic system and endogenous striatal dopamine levels, which are more severely depleted in symptomatic PD, is not yet fully elucidated.

Parkinsonism has three cardinal motor symptoms: bradykinesia, rigidity and tremor. Classical for PD is an asymmetrical onset of motor symptoms. The presence of two of the three primary signs and a consistent response to an adequate dose of levodopa are considered by most experts to be essential for the clinical diagnosis of Parkinson's disease. If this is not the case, other parkinsonian syndromes, such as progressive supanuclear palsy (PSP), multiple system atrophy (MSA) or vascular parkinsonism should be suspected.

Tremor is the most visible signature of PD; it is for this reason that James Parkinson called the disease "shaking palsy". In some PD patients, tremor is the predominant symptom, while in others tremor is absent or mild, which also led to the distinction of "tremor-dominant PD". Because the clinical presentation of PD can be quite variable, a number of subtypes have been described, including tremor-predominant versus postural instability/gait disorder (PIGD)-predominant type, or benign versus malignant PD according to the progressive course of the disease, and the distinction of young onset PD; but those classifications are arbitrary.

Tremor results from oscillatory movements of agonist and antagonist muscles. PD tremor is characteristically present at rest, but also an action tremor can be seen. Tremor is most pronounced in the distal parts of the limbs; in later stages of the disease, it can spread to involve the lips, jaw and tongue. In a later stage of the disease, tremor often subsides and bradykinesia and rigidity are more progressive. Sometimes, the term akinesia is used for one of the three primary signs of PD. Akinesia is the inability to initiate movement. Bradykinesia - slowness of movements- and hypokinesia -decrease of amplitude of movements- are designated lesser gradations of akinesia. Bradykinesia and

hypokinesia cause multiple secondary symptoms of PD, such as masked facies, sialorrhea, hypophonia and micrographia.

Gait problems form another spectrum of PD symptoms, and together with loss of balance reflexes they can cause dramatic immobility and risk of falling in later disease stages. PD gait is characterised by shuffling, small steps, decreased armswing and a forward bended posture. Furthermore, freezing of gait (FOG) may occur in 30-60 % of the PD patients [28;29]. FOG is a sudden disturbance of gait, where patients feel stuck with their feet being "glued to the floor". FOG often happens in challenging situations with increased "mental stress", and can often be overcome by applying external tricks, for example visual cues [30]. The influence of attention and mental loading on FOG [31] are an example of the important interplay between motor and cognitive processes that is also involved in PD symptomatology.

Although dementia may eventually develop with PD in 20-40 % of cases [32], most often a characteristic course of cognitive changes, not primarily classified as dementia, accompanies the disease. The pattern of cognitive deficits in Parkinson's disease often includes executive impairment similar to that seen in patients with frontal lesions, as well as episodic memory impairment, visuospatial dysfunction and impaired verbal fluency [33]. Mostly problems are seen with tasks that depend on context-specific planning. This deficit has been called impairment in "mental flexibility". On neuropsychological tests, behaviours most closely associated with "frontal" or executive functions were impaired (see review paper by J. Koerts et al. Cortex 2008), although PD was not associated with a full "frontal lobe syndrome". PD patients particularly can have difficulties developing and maintaining a set of useful plans [34]. Impairment in task-set switching was found in early stage PD patients, but was only apparent when competing information was present, i.e. when the load on selection mechanisms was increased [35]. In more advanced PD stages, cognitive symptoms may worsen and PD is in some cases then classified as PD dementia (PDD). Hallucinations may occur and are often associated with cognitive decline [33].

Language problems in PD can be related to the motor problems, resulting in hypophonia or dysarthria. But also deficits in sentence

comprehending can be seen, which is influenced by cognitive deficits in PD [36]. Semantic processing deficits in Parkinson's disease (PD) have been related to striatal dopamine deficiency. Automatic semantic activation was compromised in PD patients when off medication [37] or with deep brain stimulation of the subthalamic nucleus (STN) in off mode [38].

Additional non-motor symptoms (see table 1) can appear already early in the course of the disease. Insidious onset of perceptive (olfactory) disturbance [39], mild vegetative symptoms or cognitive signs is often found to precede the motor symptoms in patients with PD. These clinical observations of early non-motor symptoms led to a novel neuropathological concept of neurodegeneration in PD, which starts in nondopaminergic areas, notably the enteric nervous system and then rises via spinal cord and brainstem to nigral and subsequent cortical neurons [40;41]. Basal ganglia-cortical networks may be differentially affected and result in impairments in motor, visuomotor and cognitive behaviour in PD.

Table 1: Additional PD symptoms

Cognitive:	bradyphrenia, impaired attention and executive function, visuospatial deficits, dementia, language problems.
Psychiatric:	depression, anxiety, sleep disturbances, sexual dysfunction.
Craniofacial:	hypomimia, impaired accommodation, sialorrhea and dysphagia, olfactory hypofunction, dysarthria.
Autonomic:	orthostatic hypotension, impaired gastro-intestinal motility, bladder dysfunction, sexual dysfunction, abnormal thermoregulation and increased sweating.
Skin:	seborrhoea.
Sensory:	cramps, paresthesia, pain, numbness, tingling.
Musculoskeletal:	scoliosis, peripheral oedema.
Other:	fatigue, weight loss

Pathophysiology: basal ganglia circuits in Parkinson's disease

The basal ganglia (BG) constitute mainly five subcortical nuclei: putamen, nucleus caudatus, globus pallidus, nucleus subthalamicus and substantia nigra. The BG are thought to play a role in the initiation of voluntary movements, facilitation of some motion suppressing others, and comparison of motor commands with feedback from evolving motion. In addition to their role in motor control, they are involved in various emotional and cognitive functions. Brown and Marsden proposed that the BG function to facilitate the synchronisation of cortical activity underlying the selection of an appropriate movement, or an appropriate sequence of thoughts [42].

In the classical model, the basal ganglia form a complex network of parallel loops that integrate cerebral cortical regions (associative, oculomotor, limbic and motor regions), basal ganglia nuclei and the thalamus [43] (For a representation of basal ganglia inputs and outputs see fig. 1).

The motor circuit within this complex consists of cortical motor areas that project to the striatum, especially the putamen. From the putamen, neurons in the "direct pathway" project to the Globus Pallidus pars interna (GPi) and the Substantia Nigra reticulata (SNr), the output nuclei of the basal ganglia. The neurons in this pathway bear Dopamine D1 receptors and co-express the peptides Substance P and Dynorphin. This pathway provides a direct inhibitory (GABA-ergic) effect on GPi/SNr neurons, hereby reducing the inhibitory effect of these nuclei on the thalamus and thus "facilitating" movement. The "indirect pathway" connects the putamen with the output nuclei via the Globus Pallidus pars externa (GPe) and the Subthalamic Nucleus (STN). Neurons contain D2 receptors and the peptide Enkephalin. Stimulation of striatal projection neurons in the indirect pathway leads to inhibition of the GPe, disinhibition of the STN and excitation of the GPi/SNr, enhancing the inhibitory effect on the thalamus and "suppressing" movements.

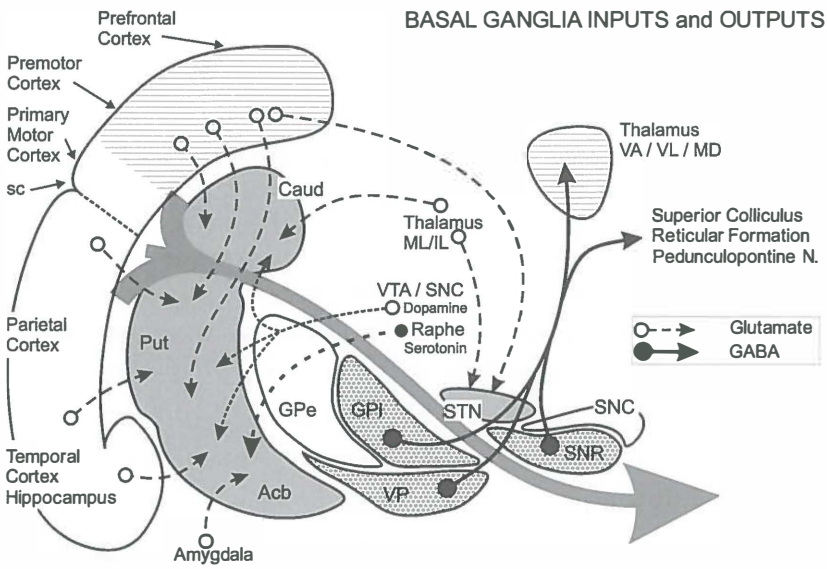

Figure 1: Basal ganglia inputs and outputs.

Schematic, semi-sagittal representation of the cerebral cortex, the different nuclei of the basal ganglia, and the thalamus, illustrating the most important afferent and efferent connections of the basal ganglia. The inputs of the basal ganglia are primarily directed at the striatum and the subthalamic nucleus (basal ganglia input structures). The ouputs of the basal ganglia stem from the internal segment of the globus pallidus, the substantis nigra pars reticulate, and the ventral pallidum (basal ganglia output structures).

Abbreviations: sc, central sulcus; Acb, nucleus accumbens; Caud, caudate nucleus; Put, putamen; VA/VL/MD, ventral anterior/ventral lateral/mediodorsal thalamic nucleus; ML, midline thalamic nuclei; IL, intralaminar thalamic nuclei; SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; STN, subthalamic nucleus; VP, ventral pallidum; VTA, ventral tegmental area. (Courtesy by H.J. Groenewegen, Parkinsonism and Related Disorders, Wolters E.Ch, van Laar T, Berendse H.W. Eds. VU University Press Amsterdam 2007)

Thus it was suggested that the brake-accelerator function of the basal ganglia is based on the net result of opposing effects of inhibitory inputs from the direct pathway and excitatory inputs from the indirect pathway. The model further proposes that dopamine exerts a dual effect on striatal neurons: exciting neurons of the direct pathway through stimulation of D1 receptors and inhibiting neurons of the indirect pathway via stimulation of D2 receptors, thus facilitating movement [44].

According to this "classical", direct-indirect pathway-model, dopamine deficiency leads to reduced inhibition of the indirect pathway and reduced excitation of the direct pathway, with the net result of an excessive activation of the basal ganglia output nuclei (GPi and SNr) and inhibition of thalamocortical and brainstem motor systems, obviously leading to parkinsonian motor features. This model serves as a good starting point, but provides no insight into the pathophysiology of specific motor abnormalities in PD. Different aspects of parkinsonian motor symptoms and non-motor symptoms cannot be explained simply as a result of augmentation in the inhibitory output from the basal ganglia.

Several suggestions to refine this model have been proposed. The basal ganglia are a highly organized network formed by discrete and finely-arranged cortico-BG-cortical parallel motor loops, which provide positive feedforward signaling for movement preparation and execution. Internal BG circuits would mainly serve a feedback-stabilizing function. Dopamine depletion destabilises this network and leads to large increases in neuronal synchronization and oscillary activity in several basal ganglia loops [45]. The basal ganglia are not only arranged in separate parallel circuits, but each cortical motor area (area 4, 6, SMA, DLPFC) is organized in a somatotopic manner. Functional specificity of these areas is selective: thus, normally a large proportion of pallidal neurons discharge only in relation to a specific task or a part of a motor sequence. Synchronisation of circuits in GPi, as is the case in PD, could lead to motor defects under special circumstances where critical components of the network are required [46;47].

In addition to the motor circuit, there are at least three other circuits connecting the basal ganglia to the thalamus and cortex: the oculomotor circuit, involved in control of saccadic eye movements, the dorsolateral prefrontal circuit, probably involved in aspects of memory and orientation in space, and the lateral

orbitofrontal circuit, which is thought to be involved in the ability to change behavioural set [43], signaling changes in reinforcement contingencies and behavioural control [48]. Those circuits project via the caudate nucleus. A distinction can be made between an "association neostriatum", including anterior regions (caudate and anterior putamen) and a posterior "motor striatum" [49]. Variation in PD symptomatology can thus result from deficits in selective regions in the striatum and its connections with specific cortical pathways.

Furthermore, Graybiel suggested a three-pathway model of the basal ganglia that takes into consideration the division of the striatum into striosomes and extrastriosomal matrix. The matrix compartment is innervated by the motor cortex and by the dopaminergic neurons of the ventral tegmental, the dorsal substantia nigra and the retrorubral area. The striosomes are innervated by e.g. the prelimbic cortex and dopamine neurones of the ventral part of the substantia nigra. According to Graybiel, the third pathway originates in this striosomal compartment of the striatum and projects to the SN. The striosomes receive input from regions as orbitofrontal and anterior cingulate/posterior medial prefrontal cortex, that mediate motivational, emotional and mnemonic processes in behavioural control, while the striatal matrix is more involved in sensorimotor and associative circuits [50].

Relationships between motor and cognitive performance in PD and striatofrontal dysfunction have been investigated using PET with (18F)-fluoro-2-deoxy-D-glucose (FDG) [51]. Resting state FDG uptake can be taken as a marker of local synaptic activity, which is both sensitive to direct neuronal damage and secondary functional disruption distant from the primary site of pathology. Furthermore, scanning during performance of a task can assess the response of a brain network, which has been applied in PET [52] as well as in fMRI studies. Using PET and network analysis, Lozza et al found that bradykinesia and global motor function in PD patients was associated with increased activity in the striatum and the mediodorsal thalamus, with decreased activity in prefrontal and cingulate regions. Executive dysfunction was associated with increased metabolic activity in left pallidum and mediodorsal thalamus, with decreased activity in ventromedial frontal regions, striatum and left hippocampal gyrus [51]. It should be noted that the putamen was also involved in this "cognitive circuit" [53], while it is generally

implicated in motor circuits. Muller et al confirmed this notion by finding that cognitive deficits correlated with dopamine transporter (DAT) binding in both the head of putamen and caudate in PD patients [54].

It is still unclear how the denervation in PD perverts normal functioning of the BG-cortical loops. Recent work has demonstrated abnormally synchronised oscillatory activity at low beta frequencies in BG circuits in PD [46]. Medium spiny neurons (MSN) in the striatum normally seldom fire and are thought to filter out uncorrelated synaptic cortical inputs, while the output neurons of other BG nuclei exhibit intrinsic tonic activity. Normal function of the BG is characterised by uncorrelated activity between their functional subcircuits. After nigrostriatal dopamine depletion, cross-connection between the "parallel" BG subcircuits become more active, resulting in abnormal synchronised activity within the BG. The cellular mechanisms underlying the pathological changes in oscillatory BG activity, involving dopaminergic, glutamatergic and GABAergic modulation, are largely unknown. Treatment with dopaminergic drugs or high-frequency stimulation of the GPi or STN may perhaps suppress the synchronised oscillatory activity, and remove the excitation of the BG output nuclei GPi and SNr. However, deep brain stimulation does not appear to be a useful treatment for cognitive and psychiatric dysfunction in patients with Parkinson's disease. STN more than GPi stimulation may exacerbate cognitive or emotional symptoms, which effect is comparable with overstimulation due to certain dopaminergic drugs [55]. Moreover, STN stimulation may not correct dysfunctional sensory association cortices in PD [56]. Furthermore, cognitive and emotional symptoms in PD may also relate to lesions in the mesocortical dopaminergic system, projecting from the ventral tegmental area (VTA) to frontal regions [57].

Besides the dopaminergic neuronal BG system, PD symptoms may be the consequence of progressive degeneration of other ascending subcortical neuronal systems, i.e. cholinergic, noradrenergic and serotonergic systems [19]. Motor symptoms are secondary to an altered dopamine-acetylcholine balance due to reduced striatal dopaminergic tone and subsequent cholinergic overactivity, which can be treated with anticholinergics [58]. In a post-mortem study in PD patients, the activity of choline acetyltransferase (ChAT) was found to be unchanged in the striatum, indicating that intrinsic striatal cholinergic neurons

are not affected in PD. A marked decline in ChAT activity, however was seen in the hippocampus and cortical areas [59]. Cognitive dysfunction and hallucinations in PD are associated with deficits in cholinergic transmission due to degeneration of ascending projections from the nucleus basalis of Meynert. Current treatment of more severe cognitive deficits or dementia in PD is based on compensation of this profound cholinergic deficiency [32]. Also, the brainstem pedunculopontine nucleus (PPN) has been shown to be involved in PD pathology and has been related to gait disturbance and FOG in PD [60]. Another factor may be the impaired noradrenergic innervation in the forebrain and neocortex from the damaged locus coeruleus, which could contribute to cognitive slowing in PD [61]. Also serotonergic deficiency in the striatum and mediofrontal cortex, resulting from damage to the raphe nucleus, has been related to cognitive disorders and depression in PD [62].

In conclusion, damage to multiple neuronal systems causing complex biochemical changes may explain the variable clinical picture in PD patients, including various motor symptoms, cognitive dysfunction, depression, vegetative symptoms, pharmacotoxic hallucinations, etc. The pathophysiological basis of dysfunctional BG-cortical loops has changed the concept of the PD syndrome, primarily described as a motor disorder; "the senses and intellects being uninjured", to a syndrome involving changes in the organisation of mental as well as motor function.

Pathogenesis of Parkinson's disease

Most cases of Parkinson's disease (PD) are sporadic and are known as idiopathic PD. Several possible causes have been proposed, such as exogenous toxins, inflammation, genetic mutations and combinations of these factors. A generally accepted hypothesis is that Parkinson's disease is the result of an interaction between genetic and environmental factors. According to this theory, an interaction of genetic predisposition and environmental factors induces mitochondrial respiratory failure [63] and oxidative stress within nigral neurones, leading to cell death.

However, immigrant surveys that try to differentiate between a genetic and an environmental cause of PD favour environmental causes [64]. The recognition that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can produce a parkinsonian syndrome very similar to PD [65] led to the hypothesis of exogenous toxins as causative factors in PD. This was strengthened by the fact that people who lived in an environment with pesticides related in structure to MPTP had a relatively high risk for developing PD [66]. It was hypothesized that retrograde transport of neurotoxin is instrumental in the patterns of cell loss in the midbrain. The toxicity of MPTP is mediated through MPP+, which is believed to accumulate in dopaminergic neurons. Toxicity is achieved by mitochondrial respiratory failure and oxidative stress. There are some links between the neuromelanin content and the vulnerability of the dopaminergic neurons for degeneration [67]. These include the generation of free radicals in the synthesis of neuromelanin and the binding by neuromelanin of neurotoxic compounds as MPP+.

Finally, studies in which multiple agents were employed in rats at subtoxic concentrations were associated with nigrostriatal degeneration [68], which elucidates a possibly important role of toxins in the development of PD in humans. Association of pesticide exposure with increased risk of PD, and the use of MPTP, rotenone and paraquat to create models of selective nigrostriatal degeneration, point to pesticides as candidate neurotoxins [69].

Furthermore, the oxidation hypothesis suggests that PD patients have a deficient antioxidant system. Oxidative metabolism of dopamine by monoamine-oxidase (MAO) leads to the formation of peroxide, which is normally cleared by glutathion. As glutathion is decreased in PD SNc [70] more toxic free oxygen radicals may be formed (oxidative stress), which damage the dopaminergic neurons [71].

Neurodegeneration in PD may also result from a decreased activity of the proteasome. Several studies suggest that defects in the capacity of the ubiquitin-proteasome system (UPS), that degrades proteins to clear excess or misfolded proteins from the brain, may be a common feature in both genetic and sporadic forms of PD [72]. In some hereditary forms of PD, mutations in components of the UPS, namely parkin and ubiquitin C-terminal hydrolase, are responsible for development of more rare hereditary forms of PD [73] [74] [75].

Furthermore, environmental toxins can interact with α-synuclein [69]. Misfolded or excess levels of α-synuclein, associated with the development of PD, can resist or inhibit the capacity of the UPS to clear proteins from the brain [76].

Several genetic mutations can cause parkinsonian syndromes that clinically closely resemble idiopathic PD. The characterisation of single-gene mutations in some cases of familial PD has provided insights into the pathogenetic mechanisms that result in PD neurodegeneration [77]. The three main disease mechanisms caused by these gene mutations implicated in PD are dysfunction of the UPS, abnormalities of the oxidative stress response and mitochondrial defects.

Finally, activated glial cells (astrocytes and microglia) might also intervene in the mechanism of degeneration by perpetuating or even amplifying the primary neuronal insult [78]. In postmortem investigations of humans exposed to MPTP, activated microglia were detected 16 years after the drug exposure [65], supporting the role of neuroinflammation in the ongoing process of degeneration after the initial toxic insult. Activated microglia may form an abundant source of oxygen-free radicals in the respiratory burst system of activated microglia, from which large numbers of superoxide ions are released [78]. The resulting oxidative stress has been linked to nigral mitochondrial respiratory chain deficits. Also rotenone toxicity to dopaminergic neurons as a toxic PD model has been first attributed to its inhibition of mitochondrial complex 1 activity, but has recently been linked to the presence of activated microglia [79]. The possible role of neuroinflammation in the progession of PD pathology is further discussed in chapter six.

Several mechanisms that are involved in "detoxification" have been suggested to influence the pathogenesis of PD. For example, there might be an excess enzyme activity to activate neurotoxins [80]. Second, aberrant activity of cytochrome P450 mono-oxygenase, which is involved in detoxification of endogenous and exogenous neurotoxins, has been related to PD, although evidence is not conclusive [81;81]. Another mechanism that may be important for detoxification involves the blood-brain barrier. Proteins that are involved in cellular efflux prevent penetration of the brain by various potentially toxic compounds. They include P-glycoprotein (P-gp) and multidrug resistance

associated protein (MRP). P-gp is expressed at the BBB endothelium in very high concentrations [3]. In humans, two P-gp transporters exist, encoded by multidrug resistance-1 (MDR1) and MDR3 genes, adjacently located at chromosomal region 7q21. The MDR1 product is thought to participate in drug disposition while the MDR3 gene encodes for a phospholipid flippase protein expressed on hepatocytes. The MDR1 gene contains 28 exons encoding for a 1280 amino acid transporter. With the presence of the highly conserved ATP binding site, this protein is a member of the so-called ATP-Binding Cassette (ABC) transporter superfamily. Polymorphisms of the MDR1 gene, associated with altered P-gp expression and function, may affect the uptake of neurotoxic xenobiotics, thereby modulating interindividual susceptibility to develop neurodegenerative disorders such as Parkinson's disease. In a study involving 107 PD patients with and without history of exposure to pesticides, a significant association between parkinsonian patients exposed to pesticides and C3435T polymorphism of the MDR1 gene was found [6]. Another study found a higher frequency of the 3435 TT genotype, associated with decreased P-gp expression and function, in early-onset PD [82]. Thus it appears that mutation of the MDR1 gene may predispose to damaging effects of pesticides. It is assumed that pesticides in general are substrates of P-gp and that exposure of the brain to such agents may cause neurodegenerative diseases.

More studies are required to confirm the role of BBB P-gp function in the aetiology of Parkinson's disease. Its function and importance at the BBB in aging and in PD neurodegeneration will be described in the chapters three, four and five.

PET imaging and movement disorders

Positron emission tomography (PET) is a technique that allows us to measure the tissue concentration of injected radioactive tracers. The positron emitting isotopes of carbon, nitrogen, oxygen and fluorine allow the labeling of a wide variety of substrates, metabolites and drugs, or analogs of these compounds.

Quantitative tracer kinetic techniques can then be implemented for the measurement of functional processes of the human brain, to obtain insight in biological processes responsible for diseases. The basis of PET imaging is the emission of positrons from a radioactively labelled compound that collide with electrons upon uptake in tissue and are annihilated (See Fig. 2). In the process, two 511 keV gamma rays originate simultaneously and leave the annihilation site in opposite directions. Positron imaging was introduced by Brownell in 1951. Current ring PET cameras take advantage of the annihilation characteristics. A ring of scintillation detectors surrounds the patient. If two events are detected simultaneously in two opposed detectors, one assumes that an annihilation occurred somewhere on an imaginary line connecting the two detectors. By acquiring a large number of lines, e.g. 10^6, tomographic reconstruction methods can be used to reconstruct images of the tracer distribution. Studies of the whole brain are performed by serially taking tomographic sections, with data correction for radioactive decay and photon attenuation. Using advanced computer software, this information is translated into a three-dimensional image, showing the location of the radiolabeled molecule in the body (brain) as a function of time.

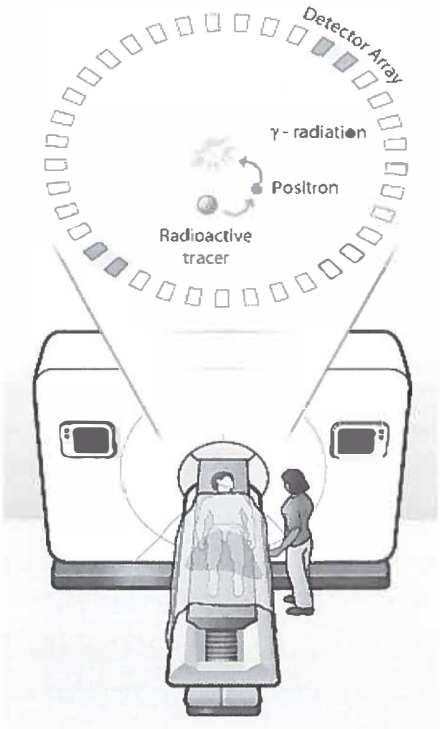

Figure 2: Schematic representation of positron annihilation and a PET camera. Adapted from http://www.csulb.edu

Up to date, the PET studies performed on patients with a movement disorder have fallen into two main categories. Firstly, changes in regional blood flow and cerebral metabolism were measured. Increases and decreases of synaptic activity in the brain are accompanied by proportional changes in local glucose consumption and capillary perfusion, which can be measured using various radiotracers. Secondly, the integrity of a number of neurotransmitter systems could be assessed. In movement disorders such as Parkinson's disease, progressive supranuclear palsy, multiple system atrophy, dystonia, Huntington's disease and Wilson's disease, especially the pre- and postsynaptic components of the dopaminergic system are investigated. These diseases disrupt the nigrostriatal and efferent striatal pathways in different manners, and so produce

varying patterns of dysfunction of the dopaminergic system. Later, also PET studies of other neurotransmitter systems and also of other biochemical mechanisms became possible. Below I will discuss the use of PET tracers to study *in vivo* BBB P-gp function and neuroinflammation.

P-gp function measured with verapamil-PET

Until recently, only techniques were only available to study the presence of P-gp in tissues at the mRNA and protein level. The presence of P-gp, however, does not yield information about the functionality of these efflux pumps. Positron emission tomography (PET) using radiolabeled substrates of P-gp can be used to study P-gp function *in vivo.*

Verapamil is actively transported as a substrate by P-gp. Direct binding of verapamil to P-gp was shown using a verapamil photoanalogue. Furthermore, verapamil was found to be actively transported outward [83]. So verapamil functions as a P-gp substrate and competitively inhibits P-gp transport function. PET in combination with [^{11}C]-verapamil has been shown to be a suitable methodology for the in vivo assessment of the P-gp status in human BBB [5]. It measures pharmacokinetics of verapamil in brain tissue and therefore allows the quantification of BBB P-gp expression. Hendrikse et al. used homozygous mdr1a gene disrupted mice (mdr1a -/- mice) and wild type mice (mdr1a +/+) to develop a method for in vivo measurement of P-gp function and to study the effect of a P-gp reversal agent on its function in vivo. [^{11}C]-verapamil levels were lower in mdr1a +/+ mice compared to mdr1a -/- mice. After co-administration of cyclosporin A (CsA), [^{11}C]-verapamil accumulation in the brain was increased in mdr1a +/+ mice to levels comparable with those in mdr1a -/- mice. This indicated that [^{11}C]-verapamil PET is able to measure reversal of P-gp mediated efflux [84]. Moreover, it is of clinical interest that P-gp function can be completely blocked in the brain with a reversal agent.

For quantification of P-gp function *in vivo*, a mathematical model describing the pharmacokinetics of uptake and clearance of verapamil is

necessary. Quantification of racemic [^{11}C]-verapamil may be difficult, as the two enatiomers have different pharmacokinetic properties. Because the (R)-enantiomer is less metabolized in man and has lower affinity for calcium channels, this enantiomer reaches higher plasma concentration. Luurtsema et al. argumented that the (R)-enantiomer would be the best candidate as PET tracer for measuring P-gp function *in vivo* [85]. However, the elimination half-life from plasma of both isomers is not significantly different, indicating that the concentration ratio of both isomers in plasma is constant [86]. Uptake of verapamil is identical for both stereoisomers, as it is diffusion limited. Using biodistribution of (R)- and (S)- [^{11}C]-verapamil in mdr1a knockout and wild type mice, no stereospecific P-gp mediated transport for [^{11}C]-verapamil was found [85] . In conclusion, the reported difference in systemic clearance in plasma of both stereoisomers results in a constant concentration ratio of both isomers in plasma. Thus, the measured distribution volume will be an average of the two distribution volumes of both stereoisomers and will exhibit a constant bias only, which does not invalidate or change the results of studies that used the racemic verapamil mixture [87].

[^{99m}Tc]-sestamibi has been used to study P-gp function with single photon emission computer tomography (SPECT) (Piwnica-Worms D et al 1993), however it was shown that [^{99m}Tc]-sestamibi is not only a substrate for P-gp but also for multidrug resistance associated protein (MRP) [88], while [^{11}C]-verapamil has proven to be a more specific P-gp substrate [5].

The beta-receptor antagonist carvedilol also is a P-gp substrate. Compared to [^{11}C]-verapamil less CsA was needed to reach maximal brain distribution volume, suggesting that [^{11}C]-carvedilol kinetics may be a more sensitive tool to in vivo measure P-gp function [89]. Furthermore, P-gp-mediated effects were observed for two 5-HT(1a) receptor ligands, with more effect for [^{18}F]MPPF than for [carbonyl-^{11}C]-WAY100635. Brain uptake of the beta-adrenergic receptor ligands [^{11}C]-carazolol and [^{18}F]-fluorocarazolol was increased in P-gp knockout mice and CsA-treated rats. However both the specific and nonspecific binding of [^{18}F]-fluorocarazolol were doubled by CsA. The uptake of [^{11}C]-carazolol increased five- to sixfold, but this uptake was not receptor-mediated [90]. Furthermore, while searching for a PET method to

determine the density and occupancy of the dopamine D(3) receptor, evidence was found that suggested that the dopamine D(3) antagonist GR218231 could be a substrate of the P-glycoprotein efflux pump. Modulation of P-glycoprotein with CsA caused a 12-fold higher [^{11}C]-GR218231 uptake in the brain, indicating that the low cerebral tracer uptake was caused by the P-glycoprotein efflux pump in the blood-brain barrier [89].

Up to date, [^{11}C]-verapamil or its stereo-isomer [^{11}C]-*R*-verapamil are the most suitable tracers for *in vivo* measurement of P-gp activity.

Neuroinflammation measured with PK11195 PET

Microglia are an interesting target for imaging of neuroinflammation, because their distribution occurs at the primary site of pathology, as well as secondarily in the remote projection areas of the damaged neurons [13]. Inflammation through activated microglia can be measured directly in vivo using PET and the radiolabeled isoquinolone 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) [91-93], a ligand that binds the peripheral benzodiazepine receptor (PBR). PBR is expressed at relatively low levels on resting microglia and astrocytes in the normal brain [92]. Activated microglia are distinguished by the exceptional abundance of PBRs. The availability of specific ligands to these PBRs, such as [11C]-PK11195, paved the road to in vivo animal and human PET studies measuring neuroinflammation in neurological injury. Various ligands have been synthesized that bind specifically to PBR [94-96]. N-2,5-dimethoxybenzyl)-N-(5-fluoro-2-phenoxyphenyl) acetamide (DAA1106) has been reported to bind to PBR with higher affinity compared with PK11195 [97], and may prove a better tracer for *in vivo* imaging of neuroinflammation. However, these newer tracers had not been characterized in humans at the time of the research project of this thesis.

PET modelling

Mathematical modelling methods are applied to relate the measured radioactivity to the biochemical parameters that are investigated. The calculations are based on models that describe the radiotracer distribution using compartments that correspond to physiologically separate pools of ligand concentration [98]. In the standard three-compartment model (two tissue model), the first compartment (Cp) defines the radioligand concentration in arterial plasma, the second compartment (Cf) the free and non-specifically bound ligand concentration in (brain) tissue and the third compartment (Cb) the specifically bound ligand (receptor compartment). The exchange of the radioligand between the different compartments can be calculated using rate constants. K1 is the delivery rate constant of the tracer from Cp to Cf and K2 describes the rate constant of return of Cf to Cp. K3 is the rate constant of transfer from Cf to Cb and K4 is the rate constant of the return from Cb to Cf (see fig.3). There will be different kinetics of ligand transfer from free to non-specifically bound or to specifically bound ligand, which would assume a third tissue compartment with K5 and K6 exchange constants with Cf, however the model equations then become too complicated to obtain reliable equation fits. So, it is usually assumed, that the kinetics of non-specific components are fast, leading to a rapid equilibrium between free and non-specific compartments. The availability of free ligand for specific binding would then be the same as for a system without non-specific binding and the model can be simplified to the two-tissue-compartment model. If the two-tissue model does not provide good model fits, sometimes a single-tissue compartment model is used (see fig.3).

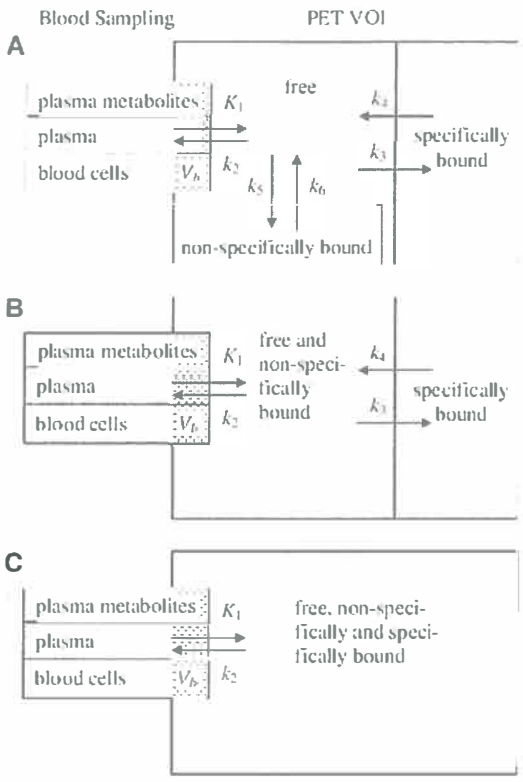

Figure 3: Compartment models for quantitative analysis of PET studies.
Figure from Cunningham and Lammertsma 1995.
*(**A**) The standard compartmental model used to describe the kinetic behaviour of PET radioligands in brain tissue is also referred to as three-tissue compartment, six rate constants model. Two simplifications of this model were introduced by Koeppe et al (1991). (**B**) The two-tissue compartment, four rate constants model with rapid equilibration between the free and nonspecifically bound compartments. (**C**) The one-tissue, two rate constants model with rapid equilibration between all tissue compartments with respect to blood–brain barrier transport rates. PET VOI =volume of interest (brain).*

The outcome parameters can be calculated from the rate constants K1 to K4. Outcome parameters of analysis models can be the distribution volume (DV) of a tracer or the tracer binding potential (BP), which is mostly used in receptor binding studies. The DV is the ratio of (brain) tissue to free plasma tracer

concentration at equilibrium. The BP is the product of the receptor density with the affinity of the tracer, and corresponds to the ratio of B_{max} (the total receptor concentration) over K_D (the dissociation constant at equilibrium). When equilibrium of ligand binding is achieved during the scanning time, BP can be derived from the PET measurements.

Methods for *in vivo* quantification of PET experiments are divided into kinetic, equilibrium and graphical methods. Kinetic models estimate the rate constants that characterize the tracer transfer between plasma, brain and receptors. Equilibrium methods derive this information from analysis of the activity distribution at equilibrium, as described above. In the graphical approach, model equations are transformed into linear plots, the slopes of which are the measures of tracer binding.

An example of the graphical analysis is the Logan analysis [99], which is also used in this thesis for calculation of the DV of [^{11}C]-verapamil. The Logan-plot has been developed for analysis of studies with reversible radioligands, and can in practice be applied before steady state in the whole system is reached. A single-tissue compartment model as well as Logan plots provided good model fits and test-retest results for [^{11}C]-verapamil [100].

To analyze [^{11}C]-PK11195 PET data, different quantification models have been applied before, requiring either accurate arterial blood measurements as the input function or assuming the existence of a reference region. A reversible two-tissue compartment model showed an optimal model when a metabolite corrected plasma curve was available [101]. Also Logan graphical analysis provides a fast method for generating parametric DV images of [^{11}C]-PK11195, however it is a measure of total (specific and non-specific) binding [102]. The simplified reference tissue model (SRTM) [103] proved to be the best model for quantification of specific binding (binding potential, BP) when no arterial plasma input was available [104]. The cerebellum is mostly used as reference region, however it may be inadequate as activated microglia could be present[105]. Therefore, cluster analysis [106]has been developed as an alternative method to define a group of voxels with a "normal" [^{11}C]-PK11195 binding.

Chapter 3

Blood-brain barrier P-glycoprotein function is not impaired in early Parkinson's disease

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Abstract

Introduction: The cause of Parkinson's disease (PD) is unknown. Genetic susceptibility and exposure to environmental toxins contribute to specific neuronal loss in PD. Decreased blood-brain barrier (BBB) P-glycoprotein (P-gp) efflux function has been proposed as a possible causative link between toxin exposure and PD neurodegeneration.

Methods: BBB P-gp function was investigated *in vivo* in 10 early stage PD patients and 8 healthy control subjects using *(R)*-[^{11}C]verapamil and PET. Cerebral volume of distribution (V_d) of verapamil was used as measure of P-gp function. Both region of interest (ROI) analysis and voxel analysis using Statistical Parametric Mapping (SPM) were performed to assess regional brain P-gp function. In addition, MDR1 genetic polymorphism was assessed.

Conclusion: In the present study, a larger variation in V_d of *(R)*-[^{11}C]verapamil was seen in the PD group as compared to the control group, without significant differences.

Decreased BBB P-gp function in early stage PD patients could not be confirmed.

Introduction

Parkinson's disease (PD) is a typical human neurodegenerative disease that usually starts in the 6th decade and progresses slowly but inexorably. Most cases of PD are sporadic and are known as idiopathic PD. Several possible causes have been proposed, such as exogenous toxins, inflammation, genetic mutations and combinations of these factors [107].

The classical hallmark of PD is the loss of dopaminergic cells in the substantia nigra. However, other catecholaminergic neurons in the midbrain are also damaged, It has been proposed that in particular the olfactory system is affected first, followed at a later stage by deeper structures, such as the substantia nigra[19]. This sequence would point to environmental factors penetrating the nervous system e.g. via the nose.

Dysfunction of the blood-brain barrier (BBB) P-glycoprotein (P-gp) efflux system could allow more, potentially neurotoxic, endogenous or exogenous substances to accumulate in the brain, over time damaging sensitive neuronal cells e.g. dopamine neurons in the mesencephalon. The P-gp transporter is highly concentrated at the BBB [3], where its function is important as an inhibiting factor of brain penetration of exogenic substances. Recently, a PET pilot study was performed to measure for the first time brain penetration of the P-gp substrate [^{11}C]-verapamil (racemic mixture) *in vivo* in PD patients [7]. Midbrain [^{11}C]-verapamil penetration was relatively high in 5 PD patients compared to 5 healthy controls, suggesting that diminished P-gp function may play a role in PD.

P-gp is of particular interest because of its involvement in the transport of MPP+ [108], the neurotoxin causing a parkinsonian syndrome strongly resembling PD. Furthermore, it appears that mutation of the MDR1 gene, encoding for P-gp in the brain, may predispose to damaging effects of pesticides leading to PD. Some of the known MDR1 single-nucleotide polymorphisms, notably a CT change at position 3435 at exon 26, are associated with altered P-gp expression and activity in human tissue [6]. Another possibility is that P-gp dysfunction is a later event, playing a secondary role in the cascade of pathological events and progression of PD neurodegeneration. A

compensatory mechanism with up-regulation of P-gp expression has also been suggested in the early pathogenesis of Alzheimer's disease (AD)[109], with loss of P-gp expression in capillaries only occurring at later disease stages.

[^{11}C]-verapamil appears to be a suitable PET tracer for the *in vivo* assessment and quantification of BBB P-gp functionality in humans [110]. The distribution volume of the P-gp substrate verapamil can be used as an inverse measure of P-gp efflux function.

Using *(R)*-[^{11}C]-verapamil and PET [85], the purpose of the present study was to assess whether BBB P-gp function is impaired in de novo and early stage PD patients as compared to healthy controls. In addition, the effect of the MDR1 gene polymorphism on *(R)*-[^{11}C]-verapamil accumulation was investigated.

Methods

Subjects

Ten male patients with idiopathic PD according to the criteria of Gelb et al were included in the study. Patients had early stage PD with Hoehn and Yahr disease stages 1 or 2 (table 1). Ten age-matched male healthy control subjects participated in the study. All subjects underwent physical and neurological examinations to exclude other neurological disorders or significant physical disease. Subjects using known P-gp modulating drugs (see for detailed list Loscher and Potschka 2005) were excluded from the study.

PET scan procedure

PET scans were performed in 3D-acquisition mode using an ECAT Exact HR+ scanner (CTI/Siemens, Knoxville, TN, USA). Prior to tracer administration, a 10 minute transmission scan in 2D-acquisition mode was performed to correct the emission scans for attenuation. The *(R)*-[^{11}C]-verapamil emission scan was performed with 21 time frames (time frames used were 1 x 30, 1 x 15, 1 x 5, 1 x 10, 2 x 15, 2 x 30, 3 x 60, 4 x 150, 5 x 300 and 2 x 600 s with total scan duration of 60.5 minutes). *(R)*-[^{11}C]-verapamil was

administered as a bolus injection. Emission scans were reconstructed using filtered back projection with a Hanning filter that resulted in a reconstructed spatial resolution of 7 mm full width at half maximum at the center of the field of view. Zoom factor 2 and matrix size 256x256x63 resulted in a voxel size of 1.2x1.2x2.4 mm. During the scan, arterial blood was withdrawn continuously using an online blood-sampling device (Veenstra Instruments, Joure, the Netherlands), which was calibrated against the PET scanner. At 2.5, 5, 10, 20, 30, 40 and 60 min after tracer injection, additional blood samples were drawn manually for determination of plasma and whole blood concentrations using a well counter cross-calibrated against the PET scanner. In addition, for these samples, plasma parent tracer and metabolite concentrations were determined using solid-phase extraction (SPE) combined with radio-high performance liquid chromatography (HPLC) and off-line radioactivity detection.

MRI scans

All subjects underwent MRI scanning on the same day of the PET scan. T1 weighted images with voxel size 0.98x0.98x1.49 were acquired using a 1T MRI scanner (IMPACT, Siemens Medical Solutions, Erlangen, Germany).

Determination of MDR1 genotype

From each subject 5 ml blood was collected in an EDTA coated vial. DNA was isolated and PCR amplification was performed for the determination of polymorphism in exon 26 (C3435T). PCR products were sequenced on an ABI PRISM 3100-Avant Genetic Analyser according to the protocol supplied by the manufacturer (Applied Biosystems, Foster City, CA, USA).

Image analysis

MRI scans were used for definition of regions of interest (ROI). First, the skull was removed from the MRI scan using a brain extraction tool which is part of the FMRIB software library (www.fmrib.ox.ac.uk/fsl, Image analysis group, Oxford, UK). Then, de-skulled brain MRI and corresponding PET scans were aligned using a mutual information algorithm included in the image registration package MIRIT. Correct alignment was checked visually in three directions.

ROIs were defined on the PET scan using the co-registered MRI scan and the PVE-lab programme. PVE-lab provides a fully automated procedure yielding a set of ROI that subdivides the brain in 57 regions (left and right), including whole brain.

Time-activity curves (TACs) were generated by projecting all ROI's onto the dynamic *(R)*-[^{11}C]-verapamil frames. A validated single tissue compartment model (for detailed description of the methodology see ref. [100], assuming similar kinetics of verapamil and HPLC metabolites and no brain uptake of polar metabolites, provided the best fit for the TACs. The volume of distribution (V_d) of *(R)*-[^{11}C]-verapamil was used as measure of P-gp function. V_d is a robust parameter, independent of inter-individual differences in tracer bioavailability and cerebral blood flow. V_d values for the various ROIs were compared between the two groups using a t-test with level of significance $p<0.05$. Ratios of midbrain/total grey matter V_d were calculated to assess whether there was a relatively increased midbrain V_d in PD patients.

In addition, parametric V_d images were generated using Logan analysis. Logan analysis is a graphical method of analysis, applicable to ligands that bind reversibly to receptors or enzymes, calculating V_d on a voxel-by-voxel basis. For comparison of regional differences between the groups, these parametric V_d images were analysed using a two-sample t-test and Statistical Parametric Mapping (SPM; version SPM2, www.fil.ion.ucl.ac.uk/spm, Institute of Neurology, London, UK). The individual MRI scans were spatially normalised to the SPM MRI template, and these parameters were subsequently used for spatial normalisation of the *(R)*-[^{11}C]-verapamil scans to the SPM MRI template. For image smoothing, a 10 mm Gaussian filter was used. Proportional scaling was preferably omitted as the Logan analysis provides quantitative *(R)*-[^{11}C]-verapamil V_d values. However, because relative regional differences can be missed with higher interindividual differences, analysis was repeated with proportional scaling to 100. Clusters were considered significant at a $p<0.05$, corrected for multiple comparisons (p-FDR = false discovery rate). As it has been reported that P-gp function decreases with age[111] and the present subjects were aged 40 to 72, the correlation between age and whole brain V_d was also investigated by adding age as a nuisance variable in SPM. Finally, to

compare the data of this study with the previous pilot study [7], the same analytical procedures as used in the pilot study were applied, including Logan analysis without correction for metabolites. Statistical analysis was repeated in SPM99, using the verapamil template that was constructed in the pilot study for spatial normalisation of the Logan images.

Results

Data were available from 10 patients with PD and 8 healthy controls. Data of two healthy subjects were excluded because of technical problems with blood sampling.

ROI analysis showed higher variability in the PD group, without reaching significant differences in V_d of *(R)*-[^{11}C]-verapamil between PD patients and control subjects for any of the ROIs. Ratios of midbrain/total brain V_d were not significantly different between the groups. Some example ROI V_d values are given in table 2 (not published).

SPM analysis showed no significant differences between the PD patients and healthy controls. Age as a nuisance variable in SPM did not reveal significant effect on the results. With a lower threshold for significance in SPM ($p<0.05$ uncorrected), two small clusters of decreased V_d were seen in PD patients relative to controls in bilateral thalamus region aligning the choroid plexus. This is possibly due to spatial volume effect after normalisation. Repeated analysis according to the methods as used in the pilot study did not reveal significant differences between the groups.

No correlation was seen between the different MDR1 C3435T polymorphisms and whole brain V_d . The healthy control group comprised of five homozygous TT alleles, compared to 3 homozygous cases in the PD group. Those cases were not associated with higher V_d of *(R)*-[^{11}C]-verapamil.

Discussion

This study did not confirm decreased BBB P-gp function in early stage PD as a primary causative factor in the pathogenesis of PD. In addition, analysis of the MDR1 gene did not reveal a primary, genetically determined lower level of P-gp function in the PD group.

The pilot study that was published recently [7] showed decreased midbrain P-gp function in 5 PD patients compared to 5 healthy control subjects. Results of this pilot study could not be confirmed, even when the same analytical procedures were used.

The PD patients in the present study had early stage PD with shorter disease duration as compared to the pilot study group. Differences of P-gp function according to disease stage can not be ruled out, but this cannot be assessed by the present study. The type of tracer was different in the two studies: the *(R)*-enantiomer of verapamil was used in the present study compared with the racemic verapamil mixture in the earlier pilot study. Both *(R)*- and *(S)*-enantiomers have equal transport for P-gp, but the peripheral metabolism of the (R)-enantiomer is slower than that of the racemic mixture [85]. This means that in studies with a racemic mixture, higher plasma concentrations of N-dealkylation metabolites, which enter the brain with comparable kinetics as *(R)*-verapamil, can be found. Performing no metabolite correction described the cerebral uptake equally well in a study that compared several tracer kinetic models for quantification of P-gp function [100]. Also in the present study, repeated data analysis without metabolite correction gave comparable results. Thus, as the difference between the stereoisomers is confined to the systemic clearance in plasma, the measured V_d will be some average of the V_d of both isomers, and it is unlikely that this difference accounts for the local (midbrain) difference in V_d of verapamil in the pilot study. Furthermore, the metabolite analysis performed in the present study did not show differences in peripheral tracer metabolism between the PD patients and controls.

In the present study, a larger variation in V_d of *(R)*-[^{11}C]-verapamil was seen in the PD group as compared to the control group. P-glycoprotein may

show regionally enhanced or diminished activity in different stages of neurodegenerative disease activity [109]. Under pathological conditions, such as the neurodegenerative or neuroinflammatory process in PD, "second line defense" mechanisms in perivascular glia can be up-regulated, including expression of P-gp[112] and other drug efflux transporters. P-gp could thus form a compensatory mechanism to increase clearance of harmful proteins that play a role in neurodegenerative diseases; its function being lost only at a later stage in progressive disease processes. More elaborate studies, with PD patients at different disease stages, will be necessary to investigate a possible role of P-gp function in the progression of neurodegenerative disease.

In conclusion, the present findings do not support the hypothesis of impaired BBB P-gp function as a primary causative factor in the development of neurotoxic events leading to PD.

Acknowledgement

We gratefully acknowledge support by the Michael J Fox Foundation, Biomarkers 2005.

Table 1: Patient characteristics.

Hoehn and Yahr	Age	UPDRS III	Years PD	Medication
2	51	18	2	ropinirole
1	60	16	1	--
2	46	27	1	pramipexole
2	40	16	4	pergolide
2	43	22	2	pramipexole
2	60	25	8	pergolide, levodopa
2	72	25	8	pramipexole, propranolol
1	56	10	2	--
2	65	24	1	pramipexole
2	49	18	2	Pergolide, levodopa
Mean, (SD)	54,2 (10,2)	20,1 (5,4)	3,1 (2,7)	

UPDRS = Unified Parkinson's Disease Rating Scale, III = part three, motor score. SD = standard deviation.

Table 2: example of some mean ROI V_d values.

	PD mean (SD)	CON mean (SD)	t-test p-value
Midbrain	0,57 (0,09)	0,55 (0,06)	0,57
Total gray	0,73 (0,12)	0,72 (0,04)	0,82
Total white	0,67 (0,11)	0,66 (0,04)	0,81
Total ROIs	0,70 (0,11)	0,68 (0,04)	0,70
Putamen	0,77 (0,14)	0,77 (0,06)	0,90
Caudate	0,66 (0,13)	0,63 (0,08)	0,67
Thalamus	1,03 (0,29)	1,00 (0,11)	0,82
Enthorinal	0,71 (0,13)	0,73 (0,10)	0,75
Cerebellum	0,72 (0,12)	0,71 (0,05)	0,84
CSF	0,70 (0,10)	0,65 (0,07)	0,23

Chapter 4

Decreased blood-brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA

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Abstract

Decreased blood-brain barrier (BBB) efflux function of the P-glycoprotein (P-gp) transport system could facilitate the accumulation of toxic compounds in the brain, increasing the risk of neurodegenerative pathology such as Parkinson's disease (PD).

This study investigated *in vivo* BBB P-gp function in patients with parkinsonian neurodegenerative syndromes, using [^{11}C]-verapamil PET in PD, PSP and MSA patients. Regional differences in distribution volume were studied using SPM with higher uptake interpreted as reduced P-gp function.

Advanced PD patients and PSP patients had increased [^{11}C]-verapamil uptake in frontal white matter regions compared to controls, while *de novo* PD patients showed lower uptake in midbrain and frontal regions. PSP and MSA patients had increased uptake in the basal ganglia.

Decreased BBB P-gp function seems a late event in neurodegenerative disorders, and could enhance continuous neurodegeneration. Lower [^{11}C]-verapamil uptake in midbrain and frontal regions of *de novo* PD patients could indicate a regional up-regulation of P-gp function.

Introduction

The causes of neurodegenerative brain diseases are unknown. Also the factors that result in continuous neurodegeneration in these diseases are not understood. Genetic susceptibility and exposure to neurotoxins are contributors to brain tissue malfunction and neuronal cell loss. Environmental toxins influence PD onset [113] and are also suggested to be involved in atypical parkinsonian syndromes [114] and progressive supranuclear palsy (PSP) [115]. Furthermore, the age-related neurodegenerative disorders are commonly characterised by the accumulation of insoluble neurotoxic ubiquinated proteins [116]. The neurotoxin hypothesis states that detoxification enzymes can influence susceptibility to PD, but recently another mechanism has also been recognised as important for detoxification of the brain. Proteins that are involved in cellular efflux prevent penetration into the brain by various potentially toxic compounds or their accumulation. They include P-glycoprotein (P-gp) [117] and multidrug resistance associated protein (MRP). At the blood-brain barrier (BBB), P-gp is expressed in a high concentration at the luminal side of the brain vascular endothelium [3] and functions as an efflux pump by translocating a substrate to the extracellular compartment. P-gp in the endothelial cells of the choroid plexus has a transport direction towards the CSF [118]. P-gp could thus play a role in brain detoxification via different routes.

The P-gp transporter is associated with the multidrug resistance gene (MDR1). Interestingly, a significant association was found between parkinsonian patients exposed to pesticides and C3435T polymorphism of the MDR1 gene, related to lower P-gp function [6;82]. Thus mutation of the MDR1 gene with diminished P-gp function increases vulnerability to damaging effects of pesticides. It is assumed that neurotoxic substances such as certain pesticides are substrates of P-gp [119] and that CNS exposure to such agents may continue to cause neurodegenerative diseases.

In a pilot study [7], impaired BBB P-gp transport was found in the midbrain of five PD patients compared to five healthy control subjects, measured with [^{11}C]-verapamil PET, pointing to a role of P-gp in the brain to prevent accumulation of toxic substances causing PD neurodegeneration. This

finding, however, could not be replicated in a study that measured P-gp function with [^{11}C]-(*R*)-verapamil PET in ten early stage PD patients compared to healthy controls[120].

Decreased expression of P-gp in BBB vasculature has been found in other neurodegenerative diseases, like Alzheimer's disease (AD) [109] and Creutzfeldt-Jakob disease (CJD) [121]. In AD, P-gp up-regulation in early pathogenesis was suggested as a compensatory mechanism to increase clearance of harmful proteins, while at later disease stages P-gp expression was lost [109].

We hypothesize that P-gp-mediated transport out of the brain is involved in clearance of harmful substances and that dysfunction of this system also occurs in late stage parkinsonian neurodegeneration. In the present study, we performed [^{11}C]-verapamil-PET brain imaging of new groups of *de novo* PD patients, PD patients in more advanced disease stages, progressive supranuclear palsy (PSP) - and multisystem atrophy (MSA) patients and a healthy control group, to study regional BBB P-gp function in patients with different parkinsonian pathology. PET with [^{11}C]-verapamil has been shown to be an appropriate methodology for the *in vivo* assessment of the functional P-gp status in human BBB [110]. The distribution volume (DV) of the P-gp substrate verapamil can be taken as a measure of P-gp efflux function, higher [^{11}C]-verapamil uptake meaning less P-gp efflux function. The effect of the MDR1 gene polymorphism was additionally investigated.

Materials and Methods

Patients were selected from the Movement Disorders Clinic of the University Medical Centre Groningen; healthy volunteers were asked to participate by advertisement. The study was approved by the Medical Ethics Committee of the University Medical Centre Groningen and all subjects gave their informed consent. PD was diagnosed according to the criteria of Gelb et al [122]. Ten de novo PD patients (diagnosed in the past year and without PD medication), and ten PD patients in more advanced disease stage (Hoehn and Yahr stages 2-3) participated in the study (table 1). Furthermore, five patients with PSP and four

patients with MSA compliant with the criteria for PSP or for MSA according to the NINDS-SPSP international workshop [123] and the consensus statement for the diagnosis of MSA [124] were included. Nine healthy age-matched subjects, who did not use medication, participated as controls. Patients did not use known P-gp modulating agents (cardiovascular drugs, antimalarial drugs, cyclosporine A, phenothiazines, hormones (eg. tamoxifen), certain antibiotics such as cefoperazone, ceftriaxone, erythromycine (see for a detailed list [125]. T1 weighted MRI scans were made in all subjects and did not show abnormalities or significant atrophy of cortical regions.

[^{11}C]-verapamil was synthesised as described before [126]. PET scans were made with an ECAT EXACT HR+ positron camera (Siemens/CTI, Knoxville, TN). After cannulation of the radial artery, all subjects underwent one hour dynamic PET scanning consisting of 21 frames after injection of [^{11}C]-verapamil (200-400 MBq). Serial arterial blood sampling to define the input function for ^{11}[C]-verapamil was done using a computerised sampling programme; six remaining samples were drawn manually. These samples (collected at intervals of ten minutes) were processed by high liquid chromatography to measure the fraction of unmetabolised parent compound. In this way the contribution to the total signal can be calculated. PET data were reconstructed to a 128x128x63 matrix with a plane separation of 2.425 and a bin size of 2.250 mm.

For anatomical demarcation, the frames 12 to 21 of the dynamic [^{11}C]-verapamil scans were summated using Clinical Application Programming Package (CAPP; Siemens, Erlangen, Germany). On this summated image, in CAPP regions of interest (ROIs) were manually drawn over the midbrain and whole brain to calculate distribution volume (DV) in the ROIs. Using Matlab (The Mathworks Inc., Natick, U.S.A.) and a linearization according to Logan [99], starting at t=5 minutes, DV images were constructed from the ^{11}[C]-verapamil scans, which was done for the whole brain ROI as well as pixelwise to obtain parametric DV images. Logan analysis is a graphical method of analysis, applicable to ligands that bind reversibly to receptors or enzymes, calculating the DV for dynamic PET data before steady state is actually reached. For comparison of regional differences between the groups, parametric DV images

were analysed using statistical Parametric Mapping [127] (SPM99, Wellcome Department of Cognitive Neurology, London, UK). SPM99 instead of the latest SPM version was used to be able to compare results with those of the pilot study [7]. Individual DV images were spatially normalised to the [^{11}C]-verapamil DV template that was constructed in the pilot study. After spatial smoothing by convolution with a Gaussian kernel of 20 mm, a two-sample t-test was done. To visualize the statistical parametric maps, the threshold was set to $P < 0.001$ uncorrected. Because higher variation in DV between individuals may render smaller differences between groups statistically insignificant in SPM, t-tests were repeated after global calculation of the DV images to 100 in SPM. We also used this SPM analysis method to be able to compare our results with the previous pilot study [7].

From each subject 10 ml blood was collected in an EDTA coated vial for determination of MDR1 C3435T polymorphism on exon 26.

A polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay was performed with the primers MDR1F 5'-TGCTGG-TCCTGAAGTTGATCTGTAAC-3' and MDR1R 5'-ACATTAGGCAGTG-ACTCGATGAAGGCA-3'. PCR was carried out with an initial denaturation of 12 min at 95° C followed by 30 cycles of denaturation at 94° C for 1 min, primer annealing for 1 min at 60° C, and 1 min extension at 70° C. When the mutation was present (3435T), digesting of the 248bp PCR-product produced the 172-, 60bp and 16-bp fragments. The digested PCR products were analysed on a 2% agarose gel with ethidium bromide staining. Whole brain verapamil DV of all subjects was plotted against the C3435T polymorphism (CC, CT and TT genotypes), and correlation coefficient of genotype with DV was calculated in all subjects and in the separate groups.

Results

[^{11}C]-verapamil uptake was high in the pituitary, ventricles and skin, moderate in gray matter and low in white matter and bone. Visual inspection of the scans revealed no obvious differences between the groups. The Logan analysis provides the DV of a tracer, and is defined by a ratio between the influx of the tracer K_1 and the efflux out of the brain K_2, of which the latter reflects the efflux function of P-gp. The influx K_1 of [^{11}C]-verapamil was of the same magnitude in all groups, which confirms that differences in DV reflect differences in P-gp efflux function. Mean DV in the more advanced PD group was 0,56 (SD 0,12), de novo PD showed 0,50 (SD 0,08), PSP 0,66 (SD 0,05), MSA 0,66 (SD 0,07) and controls 0,59 (SD 0,13). K1 in the PD group gave 0,037 (SD 0,013), PSP 0,038 (SD 0,005), MSA 0,036 (SD 0,006) and controls 0,036 (SD 0,006). Differences in DV between the groups were not statistically significant.

Pixel by pixel t-test ($p<0.001$ uncorrected) in SPM did not show increased midbrain [^{11}C]-verapamil uptake in either of the PD groups as compared to the control group. The advanced PD group showed significantly increased ^{11}[C]-verapamil uptake in frontal white matter regions compared to controls (SPM coordinates 34, 30, 26 and -16, 48, 12, near gray matter Brodmann areas 9 and 10, p corrected at cluster level 0.000, Fig 1); a cluster with decreased uptake was seen in the cerebellar tonsils (maximum effect at coordinates 40, -54, -46). The de novo PD group had lower uptake in brainstem and cerebellar tonsils (brainstem coordinates 2, -26, -26 with p corrected at cluster level 0.000, Fig 2) compared to the control group and did not show clusters with higher uptake. Plots of the local SPM responses showed a considerable separation of the groups, although there are some overlapping cases, where the PD groups show larger variation in local response values. Comparison of de novo and advanced PD groups revealed a cluster of pixels reflecting higher uptake in the advanced PD group in the anterior cingulate cortex (coordinates 4, 28, -6) and in the caudate head (coordinates 14, 14, 2 with p corrected at cluster level 0.01).

In the PSP group, significantly increased uptake was seen in basal ganglia regions (maximum effect in lentiform nucleus, coordinates 26, -10, 6

with p corrected at cluster level 0.06, fig.3). When plotting the effect, one PSP patient did not show higher uptake compared to the healthy controls (Fig 3). Notably, this was also the patient with shortest disease duration (< 1 year) and only mild parkinsonian symptoms (UPDRS motor part score 12). The PSP group had also increased uptake in frontal regions compared both to controls and to the advanced PD group, but this did not reach significance and plotting the effect showed high variance between the PSP patients. MSA patients did not show significant differences compared to controls, however after lowering the threshold to $p<0.01$, also increased uptake in basal ganglia regions was seen, with p corrected at cluster level 0.98 and uncorrected p 0.056 in putamen (28, -6, -2). Plot of the SPM effect showed more variance between the MSA patients with one patient in the control range. This patient had lower disease severity compared to the other MSA patients, with 2 years disease duration and UPDRS motor score 24.

All results did not remain statistically significant after correction for multiple comparisons in SPM (FDR correction with level of significance set at $p<0, 05$); possibly due to the higher inter-subject variation. T-tests in SPM were repeated after global calculation of the DV images to 100, which showed comparable results and did not reveal additional foci with relative DV differences between the groups.

The frequencies of the determined MDR1 polymorphism in our study group did not differ from the frequencies seen in the general population [128]. Furthermore, C3435T genotypes did not significantly correlate with differences in [^{11}C]-verapamil DV in the brain in all groups.

We compared [^{11}C]-verapamil PET data of the present study with data of the previously published pilot study that was performed by our group. All subjects showed comparable K_1 and DV values. Mean injected dose was lower in the pilot study with 111 MBq given as lowest dose; for a better signal-to-noise ratio the cut-off dose for the current study was set at 200 MBq. The previous pilot control group showed relatively lower midbrain uptake compared to the nine control subjects of this study, which, however, only was shown in SPM after global calculation of the mean DV to 100. Neither group used medication nor was there a significant age difference between both

control groups, although the pilot group had one outlier of 86 years. We could not demonstrate a definite methodological explanation for the midbrain effect in the pilot control subjects. However in our opinion, global calculation of the mean may have affected the results in the midbrain area, which is a small region with a low uptake signal and is thereby prone to artefacts. Finally, because of the lower injected tracer dose, data of the previous pilot study were not further used for the present study.

Discussion

The results of this study do not support the idea of a primary dysfunctional BBB P-glycoprotein efflux system in the pathogenesis of Parkinson's disease, but finds decreased P-gp function at later disease stages. This is in line with a most recent study that measured BBB P-gp function in mostly early stage PD patients using [^{11}C]-(R)-verapamil, where higher variance in tracer uptake was seen in several regions in the PD group, but no significant decreased P-gp function was found[120].

More advanced PD patients showed frontal regions with diminished P-gp function relative to normal controls and de novo PD patients. Regional frontal decreases in both advanced PD and advanced PSP possibly reflects the affected frontostriatal neuronal projections with frontal dysfunction in these disease stages [129]. The increased uptake lies in white matter regions rather than cortex. In general, white matter shows similar [^{11}C]-verapamil uptake as gray matter. Involvement of white matter regions and vasculature in neurodegenerative disorders that can be seen as "proteinopathies", involving accumulation of misfolded proteins, is a topic of important discussion. It was shown in vitro that ß-amyloid is transported by P-gp [130-132]. Interestingly, the deposition of $A\beta_{1\text{-}40}$ in cerebral amyloid angiopathy (CAA), which is found in around 90 % of AD patients, is predominantly found in the penetrating arterioles that supply the periventricular white matter [133]. Another study associated failure of interstitial fluid drainage from the white matter in AD with the deposition of Aβ in the perivascular fluid drainage pathways [134]. Thus,

decreased P-gp function in white matter regions may influence protein removal and accumulation at sites more distant from the white matter. Furthermore, it is suggested that Lewy Body-related pathology initially involves the neuronal perikarya, dendrites, and axons, with abundantly present "Lewy axons" in the limbic subcortical white matter [135]. These authors also find that impairment of axonal transport and synaptic transmission leads to the formation of Lewy Bodies, a hallmark of functional disturbance long before neuronal cell death. White matter P-gp dysfunction might also influence removal of toxic substances such as pesticides, which could cause harm to distant sites of the white matter after alterations of axonal transport, leading to "dying-back" patterns of neurodegeneration [136].

In PD, greater disease severity and a more rapid neurodegenerative process have been associated with white matter hyperintensities [137]. However, although specific methodology or rating scales were not used to assess white matter pathology on the MRI's in this study, we could not detect any visually apparent white matter hyperintensities in any subject.

Both *de novo* and more advanced PD patients showed lower [^{11}C]-verapamil uptake in the cerebellum. The cerebellum is closely linked to midbrain nuclei, but is by itself not affected with progression of PD pathology [19]. Possibly, P-gp function in these regions is increased in PD, but does not deteriorate in later stages as these regions are not vulnerable to PD pathology. On the other hand, in MSA cerebellar degeneration can be part of the disease spectrum. We did not find changed P-gp function in the cerebellum in the MSA patients. However the MSA patients in this study did not show symptoms of cerebellar dysfunction, and MRI scans did not show cerebellar atrophy.

PSP and MSA patients showed basal ganglia regions with increased [^{11}C]-verapamil uptake, pointing at decreased P-gp function in regions with neuropathological features in these disorders. Brain atrophic changes are not likely to play a role in these results, because MRI scans did not show overt atrophy in these regions. Moreover, tissue loss would lead to decreased [^{11}C]-verapamil uptake in these regions. PSP patients also showed decreased P-gp function in frontal regions. Although this result was not significant, plotting this effect showed higher variation between the PSP patients with one PSP

patient in early disease stage who showed values overlapping with the controls (Fig 3). These results may possibly point at regionally decreased P-gp function with advancing disease stage.

Regionally decreased P-gp function could thus occur not only in more advanced PD, but may also play a role in other neurodegenerative diseases with parkinsonism, and likely plays a role in advancing neurodegenerative processes in general. It has been argued that decreased cerebrovascular P-gp impairs the ability of the brain to expel excess proteins, and that the resulting accumulation of protein in cells could overwhelm cellular ubiquitin-proteasomal degradation [121]. P-gp was even found to interact actively with the proteasome complex, and a role for P-gp in the transport of proteasome-derived peptides was suggested [138]. Loss of P-gp function may possibly also lead to increased accumulation of insoluble α-synuclein with ongoing neurodegeneration in PD. P-gp dysfunction in late disease stage may also contribute to neuronal damage due to increased accumulation of toxins. It is known that environmental factors causing damage, such as pesticides, are closely linked to mechanisms underlying the formation of α-synuclein pathology and PD-like neurodegeneration [139]. Furthermore, it is recognised that the prevalence of dementia is higher in PD patients than in age-matched controls, which often is found to be defined by AD pathology in conjunction to the Lewy bodies [140]. Decreased P-gp function to expel proteins in advanced PD could also play a role in the co-existence of α-synuclein and β-amyloid in PD with dementia (PDD), a patient group that was not investigated in the present study.

In the study presented here, *de novo* PD patients showed lower [^{11}C]-verapamil uptake compared to normal controls in the brainstem, which may indicate a regional up-regulation of P-gp function in early stage PD. The brainstem regions lies posterior to the pons, comprising the locus coeruleus and lying slightly superior to the dorsal motor vagal nucleus, a region that is affected early in PD pathology [141], containing projection neurons of the intermediate reticular zone, coeruleus-subcoeruleus complex and caudal raphe nuclei.

A limitation of the methods, using [^{11}C]-verapamil uptake as a measure of P-gp efflux function, is the low uptake signals of this tracer in the normal condition, as it is removed by P-gp. Intersubject variation in tracer uptake can

be substantial, whereby small regional effects may be overlooked in the statistical comparison. On the other hand, global calculation can be applied to investigate regional patterns within disease groups rather than absolute P-gp measures. This should be applied with care, as small regions with low uptake signals may be prone to artefactual changes. In the case of the present study, the brainstem effect was also seen without proportional scaling in SPM, and is therefore more likely a significant result.

Initial up-regulation of P-gp expression or increase of P-gp function in reaction to neuronal damage may be associated with a neuroinflammatory process in Parkinson's disease. In PD as well as in other neurodegenerative diseases, extensive microglia activation, the key event in neuroinflammation, is seen [78;142;143]. Microglia themselves express P-gp, which increases upon microglia activation [144]. Several factors that are involved in inflammatory processes, like TNF-α, IL-6 and nitric oxide (NO) may increase P-gp function [145;146]. Furthermore, Tan et al showed that exposure of BBB endothelial cells to activated T-lymphocytes induced endothelial cell death, whereas P-gp expression and function was increased in surviving cells [147]. The authors conclude that P-gp could play a regulatory role in promoting survival of cerebral endothelial cells and maintenance of BBB function. In conclusion, neuronal damage with a subsequent neuroinflammatory reaction could, by activating the P-gp system promote clearance of the brain of potentially toxic compounds.

An initially compensatory mechanism with up-regulation of P-gp expression has also been suggested in the early pathogenesis of Alzheimer's disease (AD). At later disease stages, P-gp expression in the capillaries was lost. P-gp in the brain could thus form a compensatory mechanism to increase clearance of neurotoxic substances that play a role in neurodegenerative diseases. A similar role for P-gp was also suggested in Creutzfeldt- Jakob Disease (CJD), where decreased P-gp expression was suggested to facilitate the accumulation of PrP^{Sc} prions [121]. Whether P-gp also transports α-synuclein, a major constituent of Lewy bodies that causes neurodegeneration in PD when aggregated, has so far not been investigated. Furthermore, it is known that P-gp contributes to the cellular efflux of some pesticides [119] and that environmental

toxins influence PD onset [113]. However, decreased P-gp function was not seen in de novo PD patients, indicating that impaired BBB P-gp efflux function does not play a primary role in the initiation of pathology in idiopathic PD.

The findings of our study could have implications in the therapeutic options in PD and other proteopathies. P-gp activity can be modulated by a variety of substances, but most studies focus on P-gp inhibition, e.g. in the prevention of multidrug resistance in oncological treatments [148]. However, P-gp activity can also be up regulated, for example by St. Johns worth or rifampicin [149;150]. Selectively increasing P-gp mediated BBB efflux function would provide a possible novel therapeutic means to prevent further accumulation of insoluble toxic compounds or proteins leading to ongoing neurodegeneration.

We conclude that cerebrovascular P-gp function is regionally decreased with advanced stages in several parkinsonian neurodegenerative diseases, implying decreased regulatory and protective function of the BBB with ongoing neurodegeneration.

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Staff of the central laboratory of the University Medical Centre Groningen is thanked for determination of the MDR1 C3435T genotype.

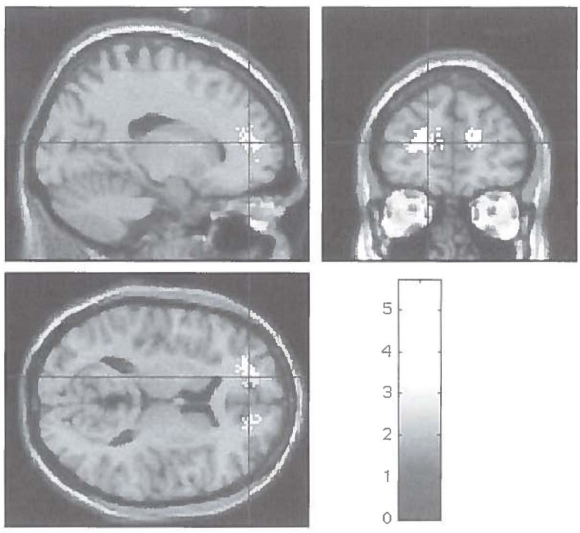

Fig. 1a

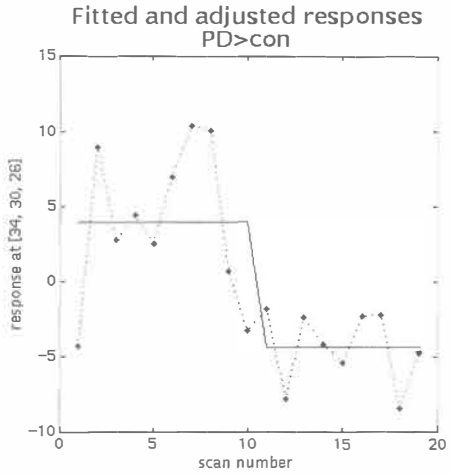

Fig. 1b

Fig 1.a: SPM t-test on MRI overlay showing p<0.001. Increased [^{11}C]-verapamil uptake in the frontal lobe in advanced PD patients compared to healthy controls; SPM coordinates 34, 30, 26 and -16, 48, 12 (Brodmann areas 9 and 10) with p corrected at cluster level 0.000.

1.b: Plot of the frontal effect (PD patients scan number 1-10 and controls 11-20) shows little overlap.

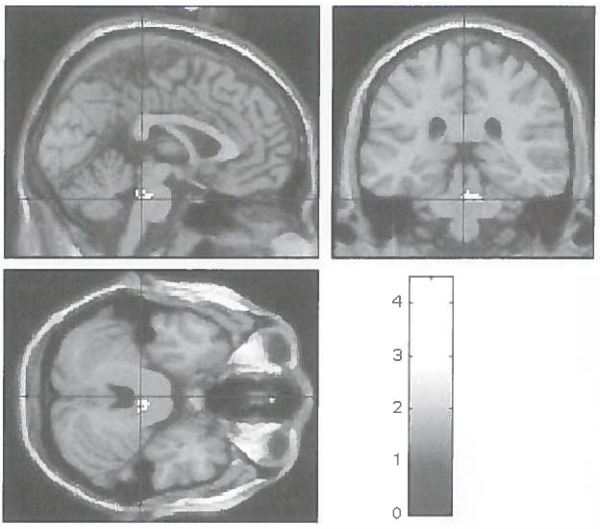

Fig. 2a

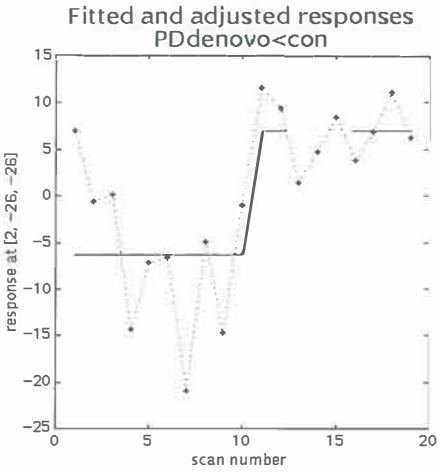

Fig. 2b

Fig 2a: de novo PD patients showed lower brainstem [^{11}C]-verapamil uptake in the brainstem compared to controls, $p<0.001$; SPM coordinates 2, -26, -26 with p corrected at cluster level 0.000.

2b: Plot of the brainstem effect shows more variation in PD de novo values (scan number 1-10) with some overlap with the control group (scan number 11-20).

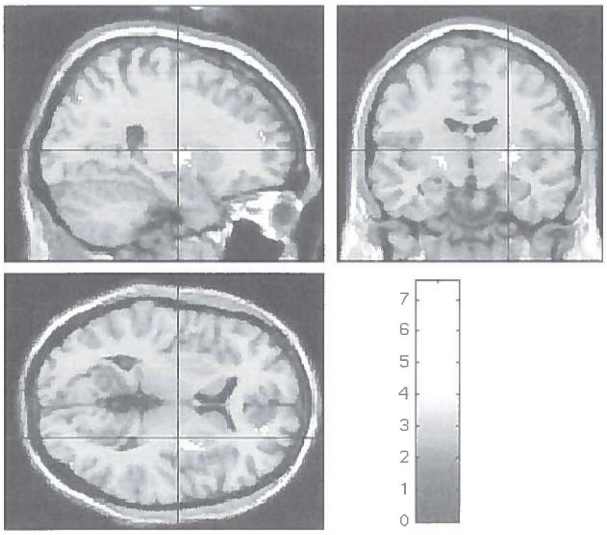

Fig. 3a

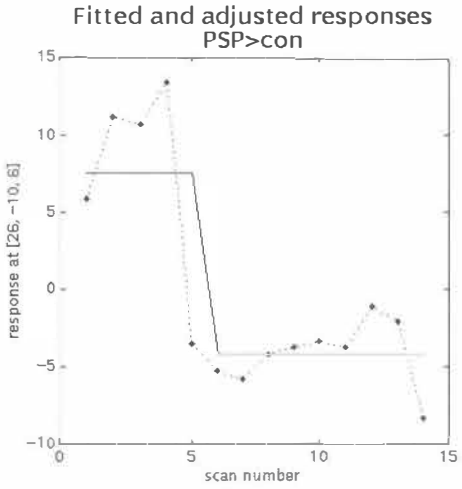

Fig. 3b

Fig 3a: PSP patients showed higher [11C]verapamil uptake in lentiform nucleus and putamen compared to controls, $p<0.001$; SPM coordinates 26, -10, 6 with p corrected at cluster level 0.06.

3b: Plot of the basal ganglia effect shows higher values in four of the five PSP patients (scan number 1-5). PSP patient number 5 had shortest disease duration (<1year).

Table 1: Patient characteristics.

Diagnosis (N)	Mean age	Disease duration	UPDRS III	medication
PD (N=10)	63 (8)	5 years SD 2	21 SD 7 ("on"state)	Levodopa + dopamine-agonist (ropinirole or pramipexole or pergolide)
PD de novo (N=10)	66 (9)	< 1 year	19 SD 8	x
PSP (N=5)	63 (8)	3 years SD 2	20 SD 8	Amantadine (2 patients)
MSA (N=4)	59 (11)	3 years SD 2	27 SD 3	Levodopa (2 patients)
Controls (N=10)	59 (10)	x	x	x

Mean values for the groups are given with standard deviations (SD).

Chapter 5

Blood-brain barrier p-glycoprotein function decreases in specific brain regions with aging: a possible role in progressive neurodegeneration.

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Abstract

Cerebrovascular P-glycoprotein (P-gp) acts at the blood-brain barrier as an active cell membrane efflux pump for several endogenous and exogenous compounds. Age-associated decline in P-gp function could facilitate the accumulation of toxic substances in the brain, thus increasing the risk of neurodegenerative pathology with aging. We hypothesized a regionally reduced blood-brain barrier P-gp function in older healthy subjects.

We studied cerebrovascular P-gp function using [^{11}C]-verapamil PET in seventeen healthy volunteers with age 18-86. Logan analysis was used to calculate the distribution volume of [^{11}C]-verapamil in the brain. Statistical Parametric Mapping was used to study specific regional differences between the older compared with the younger adults.

Older subjects showed significantly decreased P-gp function in internal capsule and corona radiata white matter and in orbitofrontal regions.

Decreased blood-brain barrier P-gp function in those regions could thus explain part of the vulnerability of the aging brain to white matter degeneration. Moreover, decreased blood-brain barrier P-gp function with aging could be a mechanism by which age acts as the main risk factor for the development of neurodegenerative disease.

Introduction

Advancing age regulates the onset of many neurodegenerative diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD). Immigrant surveys attempting to differentiate between genetic and environmental causes favour an environmental cause in the aetiology of PD [64], and epidemiological studies suggest that chronic exposure to pesticides may be a causative factor [69]. The contention put forward in this study is that the link between environment, genetic factors and age affecting the brain can possibly be found at the level of the blood-brain barrier and its protective function.

The blood-brain barrier (BBB) limits entrance of proteins and polar compounds into brain parenchyma. Passive transport across the BBB depends highly on the physico-chemical features of the compound. Importantly, the concept of the BBB has expanded to accommodate newly recognised functions: proteins that are involved in cellular efflux prevent accumulation in the brain of various potentially toxic compounds. They include P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) [151]. P-gp is expressed in high concentrations at the luminal side of the BBB endothelium [3], and functions as active efflux pump by extruding a substrate from the brain to the blood, which is important for maintaining loco-regional homeostasis in the brain.

P-gp was shown to play an important role in protecting the brain from accumulation of potentially toxic substances. In P-gp knockout mice, higher brain uptake of the P-gp substrate ivermectine was seen, causing severe neurotoxicity and death [152]. Environmental toxins have important effects on central nervous system (CNS) degenerative changes. Exposure to environmental toxic substances combined with ageing could increase the risk for developing PD. It has indeed been demonstrated that the ageing nigrostriatal dopamine pathway has increased sensitivity to pesticides [68].

Dysfunctional P-gp in the BBB has already been suggested to play a role in the development of neurodegenerative diseases, such as PD [6;7] and AD [109]. Genetic variations in the MDR1 gene, associated with decreased P-gp function at the BBB [153], has been associated with higher risk of PD in

interaction with exposure to neurotoxic substances [6;82]. Decreased P-gp expression was also found in patients with Creutzfeldt-Jakob Disease (CJD), which was suggested to facilitate the accumulation of the pathogenic prion PrP^{Sc} [121]. In AD, a compensatory mechanism with up-regulation of P-gp expression in the early pathogenesis was suggested, while at later disease stages P-gp expression in the capillaries was lost [109]. It was shown that ß-amyloid (Aß) is transported by P-gp in vitro[131], and in elderly non-demented humans, BBB P-gp expression was found to be inversely correlated with the number of Aß plaques [154]. Furthermore, deficiency of the BBB in white matter areas was suggested to play a role in white matter lesions [155], the incidence of which also increases with ageing.

It can thus be hypothesized that P-gp forms a compensatory mechanism to increase clearance of harmful proteins that play a role in neurodegenerative diseases. Decreased P-gp function in the BBB, possibly with a genetic predisposition, may increase the risk of neurological diseases as a result of increased exposure to exogenous and/or endogenous toxic substances. If this hypothesis is true, it can give insight in a mechanism by which age is the main risk factor for neurodegenerative diseases.

Relatively few papers have been published so far on the impact of advancing age on P-gp activity and expression in different tissues, and more studies are warranted to characterize the impact of age on P-gp activity at crucial P-gp locations such as the BBB[156]. Effect of age on BBB P-gp expression has first been studied in rodents. In rats and mice, decreased BBB P-gp expression was found with senescence [157-159]. It is now also possible to measure functional P-gp *in vivo* in humans using [^{11}C]-verapamil and positron emission tomography (PET) [5;125]. Verapamil is a substrate for P-gp and is hence actively transported out of the brain by P-gp. The volume of distribution (DV) of [^{11}C]-verapamil in the brain inversely reflects P-gp function in the BBB. Recently, a pilot study of P-gp function *in vivo* in the human blood-brain barrier (BBB) with [^{11}C]-(R)-verapamil PET found decreased P-gp activity as measured in overall brain of five older healthy subjects compared to a younger group[111].

The present study is the first to assess regionally accentuated loss of BBB P-gp function in relation to age. We hypothesized decreased BBB P-gp

function with ageing with possible accentuated decline in white matter regions. We again studied *in vivo* P-gp function using [^{11}C]-verapamil PET in seventeen healthy volunteers with age 18-86. The C3435T polymorphism of the MDR1 gene, associated with diminished P-gp function, was also assessed.

Methods

The study was approved by the Medical Ethics Committee of the University Medical Centre Groningen and all subjects gave written informed consent. Volunteers were recruited by open advertisement. Seventeen Caucasian volunteers, fourteen male and three female, with a broad age range (18-86) participated in the study. Since the subjects presented as two age clusters (see fig. 1) they were divided in two groups for comparison in SPM as follows: an older (N=10, age 47-86 with mean age 60 ± 11) and a younger group (N=7, age 18-27 with mean age 24 ± 2). Subjects did not suffer any disease and did not use medication. Subjects with risk factors for small vessel disease (diabetes, hypertension, high cholesterol, cardiac vessel disease) were excluded. MRI scans (1.5 T) were made to assess possible pathology by visual inspection and were judged by a radiologist and a neurologist.

[^{11}C]-verapamil was synthesised as described before [126]. PET scans were made with an ECAT HR+ positron camera (Siemens/CTI, Knoxville, TN, USA).

After cannulation of the radial artery for blood sampling, [^{11}C]-verapamil (200-400 MBq) was injected into the antecubital vene. All subjects underwent one hour dynamic PET scans consisting of 21 frames. Serial arterial blood sampling was done to define the input function for [^{11}C]-verapamil using a computerised sampling programme; six remaining samples were manually drawn. Six samples (collected at intervals of ten minutes) were further processed by high liquid chromatography to quantify the fraction of unmetabolized parent compound.

Using Matlab (The Mathworks Inc., Natick, TN, U.S.A.) and a linearization according to Logan [99], starting at t=5 minutes, parametric images for distribution volume (DV) were constructed from the [^{11}C]-verapamil scans.

Logan analysis is applicable to quantify P-gp function in the BBB using [^{11}C]-verapamil and PET [87]. A larger DV reflects decreased P-gp function. Logan also provides a rate for tracer influx K1, which is a measure of tracer availability and in the case of verapamil independent of blood flow. K1 was comparable between the groups. DV was calculated in a ROI, containing whole brain. Whole brain DV was compared between the younger and older groups using t-test and was correlated to age using Spearman correlation. For comparison of regional differences between the younger and older groups, parametric DV images were analysed using SPM99 [127] instead of the latest SPM version, in order to compare results with the pilot study [7]. Individual DV images were spatially normalised to the [^{11}C]-verapamil DV template that was constructed before [7]. After spatial smoothing by convolution with a Gaussian kernel of 20 mm, a two-sample t-test was done. To visualize the statistical parametric maps, the threshold was set to $P < 0.001$ uncorrected. Clusters with a false discovery rate (FDR) corrected value of $p < 0.05$ were considered significant.

From each subject 10 ml blood was collected in an EDTA coated vial for determination of MDR1 C3435T polymorphism on exon 26. A polymerase chain reaction (PCR)-based restriction fragment length polymorphism assay was performed with the primers MDR1F 5'-TGCTGGTCCTGAA-GTTGATCTGTAAC-3' and MDR1R 5'-ACATTAGGCAGTGACTCGA-TGAAGGCA-3'. PCR was carried out with an initial denaturation of 12 min at 95° C followed by 30 cycles of denaturation at 94° C for one min, primer annealing for 1 min at 60° C, and 1 min extension at 70° C. When the polymorphism was present (3435T), digesting of the 248bp PCR-product produced 172-, 60bp and 16-bp fragments. The digested PCR products were analysed on a 2% agarose gel with ethidium bromide staining.

Results

All younger subjects were male; mean age 24 ± 2. The older group consisted of seven male and three female subjects; mean age 60 ± 11. No gross atrophy was present at visual inspection. No white matter hyperintensities were seen.

The DV of [^{11}C]-verapamil in a whole brain ROI showed a significant difference ($t(15) = -4.2$; $p<0.001$) between the younger and older volunteers: 0.38 ± 0.09 in younger subjects and 0.61 ± 0.13 in older subjects. Correlation of brain DV to age gave Spearman correlation $r = 0.66$ with $p = 0.0038$ (Fig 1). Global tracer influx rate K_1 was not significantly different between both groups (mean 0.04 ± 0.01 in the older and 0.05 ± 0.03 in the younger group).

Comparison of the two groups on a voxel level in SPM showed large clusters with higher uptake in the elderly group in contrast with the younger subjects, in bilateral orbitofrontal regions, white matter regions of mostly the posterior internal capsule and corona radiata and in cerebellum (Fig 2). Clusters were significant after FDR correction at $p<0.05$. A region outside the brain, around the optic chiasm, was also seen. No clusters of higher uptake in the younger group compared to the older group were seen.

All volunteers were screened for the C3435T polymorphism in the MDR1 gene. One homozygous TT - and one homozygous CC genotype was found in the young volunteer group as well as in the elderly group, the rest had heterozygous CT alleles. DV of [^{11}C]-verapamil in the large brain ROI did not differ between the homozygous and heterozygous MDR1 genotypes.

Discussion

This *in vivo* study strongly indicates that the function of P-gp in the BBB decreases with increasing age, particularly in white matter regions and in orbitofrontal regions.

Neuroimaging is a valuable tool to study active efflux function *in vivo* at the human BBB. Most of the *in vitro* studies reported so far have failed to show a significant age-related alteration of BBB permeability in the absence of neurological disease [160], however these studies did not study active BBB efflux function.

Increased total brain uptake of [^{11}C]-verapamil in elderly volunteers was also shown in the pilot study by Toornvliet et al [111], however regional alterations of active efflux functions in the brain had not been studied *in vivo* so

far. Interestingly, the present study showed a specific regional decrease of P-gp function in older subjects when compared at voxel level that may play a role in regional vulnerability to neurodegenerative changes.

Decreased P-gp function was seen in the older group in orbitofrontal and posterior internal capsule regions. It has been found that fibre populations within prefrontal regions and posterior internal capsule are most vulnerable to age-related degeneration [161]. Ageing mostly affects frontal lobe circuitry, underlying decline of frontally-based processes in normal adult ageing [162]. Deficiency of the BBB in white matter areas has been suggested to play a role in white matter lesions [155], but quantitative analysis of BBB ultrastructure in the ageing human did not reveal changes in passive BBB transport while the capillary walls in the white matter became thinner [163]. Decreased BBB P-gp function in white matter regions may contribute to the development of white matter pathology with consequently functional denervation of cortical areas.

In AD pathology, marked damage and dysfunction not only of the gray matter but also in the white matter is reported [164]. Cerebral amyloid angiopathy (CAA) is a very common finding in AD [165]. Also white matter changes that are independent of ischemic infarcts occur early in the course of AD. In a transgenic model of amyloid deposition, Aß accumulation and deposition was linked with white matter abnormalities in the absence of ischemic infarcts, as detected with diffuse tensor imaging [166]. Interestingly the deposition of $Aß_{1-40}$ in CAA is predominantly found in the penetrating arterioles that supply the periventricular white matter [133]. Therefore, decreased P-gp mediated Aß efflux could cause myelin damage and lead to white matter injury. Another study associated failure of interstitial fluid drainage from the white matter in AD with the deposition of Aß in the perivascular fluid drainage pathways [134]. In an AD mouse model, increased deposition of Aß in the perivascular fluid drainage pathways was also seen after administration of a P-gp inhibitor [167]. Finally, brain trauma involving white matter transport has also been shown to be a risk factor for developing AD. It was shown that Aß deposition is related to traumatically impaired white matter transport, leading to pathological accumulation of amyloid precursor protein and Aß formation in axonal membrane compartments [168]. Interestingly, in a study using PET and PIB as a marker for

amyloid deposition [169], white matter PIB binding was observed in both healthy controls and AD subjects. The authors did not have an explanation for this finding, although aspecific uptake could not be excluded. However, PIB is also reported to be a non-specific marker of Aß-related cerebral amyloidosis, rather than a specific Aß-plaque marker [170]; and binding in white matter might thus also be related to white matter vascular changes with amyloid disposition. We suggest that decreased P-gp function with aging in posterior white matter regions connecting to temporal and parietal lobes (and the proximity of our lesion close to hippocampus) may be related to white matter changes with aging, possibly due to increasing Aß burden. Increased PIB binding was also seen in the orbitofrontal regions in healthy controls, with larger increase in AD subjects [169]. Furthermore, possibly factors favouring Aß deposition also promote α-synuclein pathology, as Aß deposits have been associated with increased α-synuclein pathology [171]. Notably, accumulation of phosphorylated α-synuclein in the aging brain was shown to initially involve impairment of axonal transport in subcortical white matter, later leading to the formation of Lewy bodies [135]. Decreased P-gp efflux function may thus play a role in the vulnerability of the brain for the accumulation of insoluble proteins. More research is necessary to decipher the mechanisms of formation of insoluble protein deposition and neurodegeneration.

Another link between P-gp function and neurodegeneration may be found in the accumulation of toxins. It has been known for some time that toxins and proteins (viruses) can distribute from the nasal cavity into the olfactory neuron, and could then trans-synaptically spread into other brain regions. Also manganese, leading to a parkinsonian syndrome, can enter the brain directly by this route [172]. It was shown that P-gp also functions in the barrier system of the nose epithelium [173;174]. Interestingly, the orbitofrontal regions where the present study showed decreased P-gp function with aging also comprises the olfactory bulbs. Decreased efflux function in this region with aging may thus account for the increasing vulnerability of the aging brain for the entrance of toxic compounds.

Since similar influx rates K_1 between both groups were calculated, it is not probable that differences in DV of [^{11}C]-verapamil can be attributed to a

higher passive permeability of the BBB. Atrophy may increase partial volume effects. However, this would primarily lead to an underestimation of DV in elderly persons; therefore it can not account for the differences that were seen between younger and older subjects in the present study.

Conflicting evidence exists about the functional involvement of MDR1 polymorphisms [175]. Differences in [^{11}C]-verapamil uptake in this study were not explained by differences in MDR1 genotype frequencies between the groups. A recent study did not support the assumption that BBB P-gp function is different between the haplotypes of the MDR1 gene (3 single nucleotide polymorphisms: C1236T, G2677T and C3435T)[176].

Decreased BBB P-gp function with age can also account for increased exposure to toxins and to several drugs in the elderly. As a consequence of reduced brain elimination of P-gp substrates with CNS effects, such as certain anti-convulsants and psychopharmaca, these drugs should be used in older patients with the above findings in mind.

Importantly, the P-gp transporter could serve as a target for novel therapeutic options in age-related neurodegenerative diseases. P-gp function can be modulated by a variety of substances [177], either inhibiting (such as cyclosporine A, verapamil, atorvastatine) or stimulating (St John's wort, rifampicin) P-gp function. These drugs can modulate P-gp function in several tissues. A challenge in the further development of neuroprotective options will be to identify an agent that selectively modifies cerebral P-gp function and enhances the loco regional clearance from the brain of potentially toxic substances by P-gp. It should be noted that besides the P-gp pump, which is highly concentrated at the BBB [3], other efflux pumps of the ATP-binding cassette (ABC) family have overlapping functions in tissue defence [178]. It was shown, for example that P-gp and the multidrug resistance related protein 1 (MRP1) together serve a protective role by preventing the accumulation of their overlapping substrates in the brain [118]. Possibly, deficiency of more active BBB transport systems contribute to the accumulation of neurotoxic substances and the development of neurodegeneration, and P-gp can be seen as the prototype of these transport systems.

In conclusion, this study shows decreased P-gp efflux function at the BBB with aging. Most profound functional P-gp decrease was seen in internal capsule and corona radiata white matter regions and in orbitofrontal regions. Age-associated decline in P-gp function could facilitate the accumulation of toxic proteins or cause a more pronounced influx of neurotoxic substances, thus increasing the risk of neurodegenerative pathology.

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Fig.1

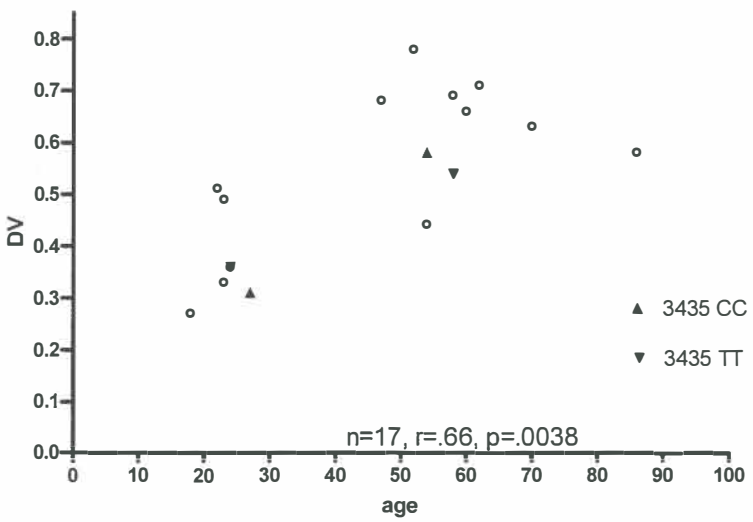

Fig.1. Age and distribution volume (DV) of [^{11}C]-verapamil in a total brain ROI in 17 subjects. DV is positively correlated to age with Spearman r = 0.66 (p = 0.0038), indicating decreased BBB P-gp function in older subjects. Subjects with MDR1 3435CC and 3435TT polymorphisms are also depicted.

Fig.2

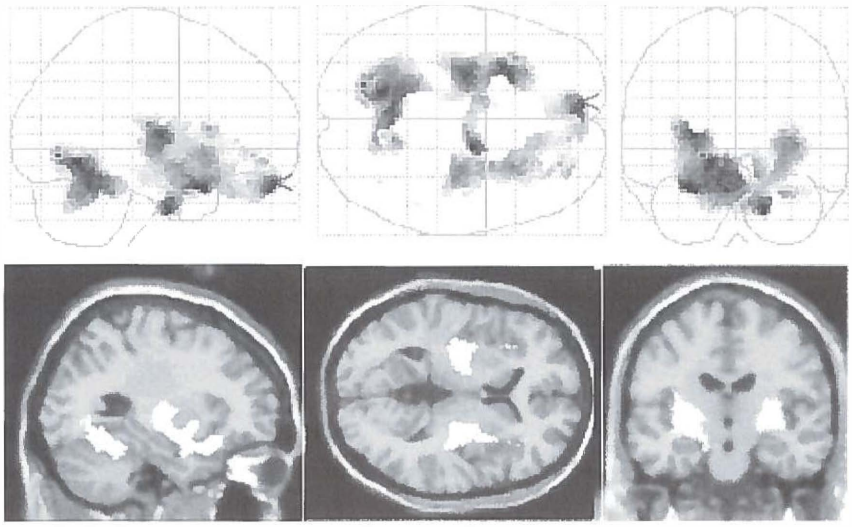

Fig.2. Orthogonal SPM projections of increased [^{11}C]-verapamil uptake in older healthy subjects as compared to the younger group. The clusters projected on the SPM glassbrain show increases inwhite matter regions and orbitofrontal regions. In addition, the foci of increase in capsula interna white matter regions are shown merged with standard MRI sections. Significance was set at $p<0,05$ with FDR correction for multiple comparisons.

Chapter 6

Neuroinflammation in the pathophysiology of Parkinson's disease: evidence from animal models to human *in vivo* studies with [^{11}C]-PK11195 PET

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Abstract

Increasing evidence suggests that neuroinflammation is an active process in Parkinson's disease (PD) that contributes to ongoing neurodegeneration. PD brains and experimental PD models show elevated cytokine levels and up-regulation of inflammatory-associated factors as cyclo-oxygenase-2 and inducible nitric oxide oxidase. Anti-inflammatory treatment reduced neuronal degeneration in experimental models. In this review, we summarise the place of neuroinflammation in the pathophysiology of PD. *In vivo* PET studies are discussed. These methods provide a means to monitor *in vivo* potential clinical relevance of anti-inflammatory treatment strategies in PD.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease whose incidence increases with age. Prevalence varies between 105 to 258 patients per 100.000 persons [21]. Although dopaminergic treatment continues to be useful to improve motor function, physical and mental disabilities increase as the disease continues are usually refractory to therapy and come to dominate the later stages. To date the disease cannot be cured, but an extensive search for neuroprotective therapy is ongoing to halt the pathological cascade that results in neurodegeneration.

Most cases of PD are sporadic and are considered idiopathic. Genetic susceptibility and exposure to neurotoxic substances may contribute to brain dysfunction and neuronal cell loss. Several pathogenic mechanisms have been discovered to contribute to neuronal cell loss in PD. In recent years, the involvement of neuroinflammatory processes in neurodegeneration has gained increasing attention. A glial reaction has been described in affected regions in other neurodegenerative diseases, e.g. Alzheimer's disease, suggesting that it likely represents a phenomenon common to several neurodegenerative disorders [143]. Studies using PD animal models have supported the relevance of microglia activation in the context of nigral cell death.

Here, we will discuss the role of neuroinflammation in the pathogenetic mechanisms in PD. An overview is given of microglial involvement in neurodegeneration, with special focus on the role of cyclo-oxygenase-2. Thereafter *in vivo* studies of neuroinflammation in patients with neurodegenerative diseases and the importance of *in vivo* PET studies for monitoring the clinical relevance of anti-inflammatory treatment are reviewed.

Brain microglia and neuroinflammation

Microglia cells are the resident immune cells in the brain. In response to pathological stimuli microglia express surface molecules like major histocompatibiliy complex (MHC) and release proinflammatory and potentially

cytotoxic substances like cytokines, cyclo-oxygenases, caspases and others [179]. These microglia are called activated microglia. Local activation of microglia can occur without the lymphocytic infiltrations characteristic of classical inflammatory brain disease, which has led to the concept of "neuroinflammation" in a variety of primarily non-inflammatory neurological conditions.

In PD, activated microglia were first reported post-mortem in the substantia nigra [180]and subsequently in the putamen, hippocampus, cingulate and temporal cortex of PD patients [181].

It is unknown whether the glial reaction is mostly a consequence of neuronal death in neurodegenerative diseases, or occurs early in the cascade leading to nerve cell death. Microglia can be activated directly by products of microorganisms, environmental toxins and protein aggregates like amyloid-β, or indirectly after neurodegeneration is induced. In animal models of parkinsonism, activation of microglia by a range of triggers, e.g. MPP+, 6-OHDA, Mn-EBDC (a fungicide) or rotenone, may lead to neuronal damage[182]. Microglial activation occurs prior to the appearance of a dopaminergic lesion in the rotenone PD model [183], supporting the role of inflammation early in the degenerative cascade. Alternatively, it has also been shown that dopamine neuron loss can in itself result in activated microglia, thus initiating a self-propagating sequence of steady cell damage [184].

Microglial activation can play a homeostatic role in the brain by scavenging excess neurotoxins, removing dying cells and cellular debris. Moreover, they release trophic factors like brain derived neurotrophic factor (BDNF) to promote collateral sprouting in injured neurons [185]. Furthermore, alpha-synuclein aggregates may lead to activated microglia via a mechanism that involves phagocytosis of aggregated alpha-synuclein by microglia [186]. When activation is excessive and ongoing, however, the release of cytokines has a toxic effect on neurons.

In postmortem investigations of humans exposed to MPTP, activated microglia were detected 16 years after the drug exposure [65], supporting the role of neuroinflammation in the ongoing process of degeneration after the initial toxic insult.

Oxidative stress in PD pathology has been linked to nigral mitochondrial respiratory chain deficits. An abundant source of oxygen-free radicals is the respiratory burst system of activated microglia, in which large numbers of superoxide ions are released [78]. Also rotenone toxicity to dopaminergic neurons as a toxic PD model has been first attributed to its inhibition of mitochondrial complex 1 activity, but has recently been linked to the presence of activated microglia [79].

Mediators released from activated microglia

The question which mediators released from activated microglia contribute to the dopaminergic toxicity has been studied by different groups, with varying results. Besides being a source of free radical release, activated microglia also release glutamate, which is potentially neurotoxic in PD. An important feature of inflammatory-related processes in PD is the marked increase in cytokine levels, including TNF-alpha, in the striatum and cerebrospinal fluid of PD patients [187]. The density of glial cells expressing proinflammatory cytokines was elevated in the SNpc of PD patients, suggesting that cytokine production occurs in the vicinity of dopaminergic neurons[188].

The pro-inflammatory cytokines TNF-alpha and IL-1beta, other reactive oxygen species (ROS) and reactive nitrogen species (RNS) have received much attention [189]. Glial cells in the SNpc of PD patients [184] show a marked expression of enzyme inducible NOS (iNOS), which is virtually absent in the brain under normal conditions

Other reactive species (ROS) have been implicated in the pathogenesis of PD [190]. Increased production of ROS in the SNpc generated by activated microglia after MPTP administration was found to be mediated by NADPH-oxidase. NADPH-oxidase is upregulated in the SNpc of MPTP-treated mice as well as PD patients [191]. Another enzyme capable of generating ROS is cyclo-oxygenase (COX)-2. Upon donation of electrons to COX, co-substrates such as dopamine become oxidized to dopamine quinone, which is highly reactive with glutathione and protein amino acids such as cysteinyl, tyrosine and lysine and can ultimately

lead to the demise of the cell. Inhibition of COX-2 leads to decreased levels of cysteinyl-dopamine, suggesting that cell demise caused by COX-2 might be due to ROS generation [192].

Neuroinflammation and cyclo-oxygenase-2 in PD

Epidemiological data indicates that anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) may have a protective effect on PD [193]. Regular intake of nonaspirin NSAIDs but also high doses of aspirin have been associated with a 45 % lower risk of PD in two large cohorts [10]. The epidemiological data regarding NSAID's and the risk of neurodegenerative diseases are conflicting, however. Chen et al reported decreased risk of PD only in ibuprofen users but not in aspirin users[9]. Another study found lower risk of PD with aspirin but not other NSAID's[194]. NSAID's are also suggested to have a protective effect in experimental studies related to the development of Alzheimer's disease, but clinical studies with COX-2 inhibitors had no positive results[195]. For Parkinson's disease, however, stronger evidence exists for a role of COX-2.Increased expression of COX-2 and elevated levels of prostaglandin E2 (PGE2) have been implicated in PD pathogenesis [196]. Teismann et al found that COX-2 expression is induced specifically within SNpc dopaminergic neurons in postmortem PD specimens and in the MPTP mouse model of PD during the destruction of the nigrostriatal pathway. A lack of COX-2 but not of COX-1 decreased MPTP neurotoxicity. The selective COX-2 inhibitor rofecoxib blocked ventral midbrain PGE2 production in MPTP injected mice and attenuated neuron and fiber loss, demonstrating the crucial enzymatic function of COX-2 to its neurotoxic effects on dopaminergic neurons. This report, however, suggested that the neuroprotective effect of COX-2 inhibition was related to the blockade of COX-2-mediated dopamine oxidation and not to decreased microglial activation. Carrasco et al report COX-2 associated toxicity in 6-OHDA lesioned rats, whereas MPTP toxicity seemed COX independent[197]. Wang et al [198] showed that MPP increased PGE2 in mixed neuron-microglia cultures, but not in neuron -, microglia -, or astroglia alone

cultures. PGE2 increase was abolished by treatment with DuP697, a COX-2 selective inhibitor, which also reduced dopaminergic neurotoxicity. Sanchez-Pernaute et al examined the effect of the COX-2 inhibitor celecoxib on dopamine toxicity and microglial activation in a 6-OHDA rat model of PD using micro PET. They showed that COX-2 inhibition resulted in protection of damaged dopamine neurons. [11].

The observed benefit of celecoxib could also stem directly from neuronal inhibition of COX-2, which is one of the several enzymes capable of oxidizing dopamine to reactive dopamine quinone, as described above. Inhibition of COX-2 appears to be able, directly and through inhibition of microglia activation to result in a reduction of dopamine cell degeneration. Microgliosis may also be responsible for the COX-2 activation in neurons, leading to enhanced dopaminergic neurotoxicity, which in turn reinforces microgliosis. Inhibition of COX-2 activity may thus via different routes stop the vicious circle of neuroinflammation and -degeneration and be a valuable strategy in PD therapy. The precise role of COX-2 in PD is not yet clear, but importantly, it is now possible to monitor the effect of anti-inflammatory treatment directly *in vivo* with PET.

Neuroinflammation measured *in vivo* with [^{11}C]-PK11195 PET

Inflammation through activated microglia can be measured directly *in vivo* in PD patients using PET and the radiolabeled isoquinolone PK11195 [91-93], which is a ligand for the peripheral benzodiazepine receptor (PBR). Activated microglia are distinguished by the exceptional abundance of PBRs. The availability of specific ligands to these PBRs, such as [^{11}C]-PK11195, paved the road to *in vivo* animal and human PET studies measuring neuroinflammation in neurological injury.

MPTP and 6-OHDA animal PD models and [^{11}C]-PK11195 PET showed an immediate inflammatory reaction to the toxic insult, but also a reaction at later stages, enhancing the neurodegenerative process [199]. Inhibition of the inflammatory reaction leads to decreased dopaminergic degeneration [11].

Microglial activation has been investigated *in vivo* in PD patients. Ouchi et al studied 10 early-stage drug-naive PD patients with [^{11}C]-(*R*)-PK11195 and a dopamine transporter marker [^{11}C]-CFT. Midbrain [^{11}C]-(*R*)-PK11195 binding potential (BP) correlated inversely with [^{11}C]-CFT BP in the putamen and correlated positively with clinical motor scores[200]. In healthy subjects, the [^{11}C]-(*R*)-PK11195 BP in the thalamus and midbrain showed an age-dependent increase. Gerhard et al. found increased [^{11}C]-(*R*)-PK11195 binding in the pons, basal ganglia and frontal and temporal cortical regions in PD patients[201]. Eight PD patients were examined longitudinally, and their [^{11}C]-(*R*)-PK11195 signal remained stable over 2 years [201]. In their study, in contrast with the findings of Ouchi et al., levels of microglial activation did not correlate with clinical severity or putamen [^{18}F]-dopa uptake. They suggested that microglia are activated early in the disease process and levels then remain relatively static. The same group also studied eleven Huntington disease patients at different disease stages. They observed a significant increase in striatal PK11195 binding, which correlated with disease severity as reflected by striatal reduction in [^{11}C]-raclopride binding, clinical scores and CAG index [202]. Also detected were increases in prefrontal and cingulate cortex. In a study with four patients with progressive supranuclear palsy (PSP), two of the patients were rescanned after 6 to 10 months and showed a stable level of microglial activation [203]. Thus, when rescanning individual patients, levels of microglia activation seem to remain stable, while in patient groups, correlation with clinical disease stage and progression may be found. It is not clear yet whether early PD shows a different pattern of microglia activation than more advanced patients.

Studies of PD and atypical parkinsonism reveal a certain degree of overlap. Increased PK11195 binding has been measured in the SN and the globus pallidus in PD patients [142], while MSA patients show more widespread increases including in SN, pons, pallidum, striatum and dorsolateral prefrontal cortex [204]. However, another study in PD patients also showed increases in pons, basal ganglia, frontal and temporal regions [201]. PSP patients showed increased binding in the basal ganglia, midbrain, frontal lobe and cerebellum [203]. Four patients with corticobasal degeneration (CBD) showed increased binding in striatum, pons, pre- and postcentral gyrus and frontal lobe [205].

Cagnin et al. performed a PK11195 PET study in Alzheimer disease patients and found increased signal in temporal cortex, inferior parietal cortex, posterior cingulate and amygdala [206]. They also detected active neuroinflammatory pathology in specific areas, such as the inferior temporal cortex, in patients with minimal cognitive impairment. Symptoms did not change over 23 months, while these subregions underwent atrophic changes on MRI scan, supporting the view that the pathological process can remain unrecognised for several years before reaching the threshold of clinical manifestation. Microautoradiographic studies using [^{3}H]-(*R*)-PK11195 PET have also demonstrated binding by activated microglia remote from the primary lesion in regions that are connected via retrograde and anterograde neuronal projections [13]. Secondary neuroinflammatory changes may thus also occur throughout a neuronal circuitry before structural damage can be seen.

Thus, [^{11}C]-(*R*)-PK11195 PET provides a measurement of disease location and may help in characterising *in vivo* the underlying disease activity as expressed by activated microglia in neurodegenerative disorders. Moreover, it may be applied when monitoring the efficacy of putative neuroprotective agents in these progressive disorders.

Concluding remarks

According to our current knowledge, PD neurodegeneration results from multiple events and interactive mechanisms. Synergistic actions of endogenous and exogenous neurotoxic compounds, perhaps on the basis of genetic susceptibility, can be the start of a toxic insult with consequent neurodegeneration. The neuroinflammatory reaction in PD most likely arises secondary to different more upstream neuronal insults, but can be an important factor in ongoing neurodegeneration, as chronic inflammatory processes have overtly harmful consequences for the cellular environment. Neuroinflammatory processes seem to form a self-propelling cycle driving the neurodegenerative process. This could have important therapeutic implications. Experimental models showed decreased neurodegeneration after targeting

neuroinflammatory-related deleterious mechanisms. The time has come to transfer this knowledge into the field of human disease. *In vivo* PET studies give the opportunity to monitor neuroinflammatory activity in the brain and study the potential clinical relevance of drugs targeting neuroinflammation in PD and other neurodegenerative diseases. These studies can and should now be done.

Chapter 7

[^{11}C]-PK11195 PET: quantification of neuroinflammation and a monitor of anti-inflammatory treatment in Parkinson's disease?

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Abstract

[^{11}C]-PK11195-PET has been used for *in vivo* brain imaging of microglia activation in Parkinson's disease (PD) patients. COX-2 inhibition has been shown to reduce neuroinflammation and neurodegeneration in animal models of PD. This pilot study assessed the use of [^{11}C]-PK11195 PET to quantify neuroinflammation and evaluate the ability of COX-2 inhibition to reduce neuroinflammation in PD patients.

Methods: Fourteen PD patients and eight healthy, age matched controls underwent a [^{11}C]-PK11195 PET and MRI scan. Five PD patients were scanned before and after one month of celecoxib treatment 200 mg/day. Arterial plasma sampling and metabolite analysis were performed to create plasma input curves. A 2-compartment model and Logan analysis were applied and parametric DV images were compared using t-test in SPM2. In addition a simplified reference region model (SRTM) was applied, with both the cerebellum and a reference region derived from cluster analysis.

Results: Using the cluster analysis, PD patients showed higher contralateral putamen BP and midbrain BP compared to controls, although considerable overlap was seen and differences were not statistically significant. Unexpectedly, BP and DV after celecoxib were slightly higher. Cerebellum as reference region resulted in lower BP values and k_3/k_4 gave 10-fold higher BP values. Linearization of the data did not show differences between PD patients and controls.

Conclusions: In current practice, [^{11}C]-PK11195 seems an unsuitable tracer for accurate or reliable quantification of neuroinflammation. Refinement of [^{11}C]-PK11195 uptake analysis and, more importantly, further development of better tracers is necessary to enable accurate measurement of neuroinflammation and effects of anti-inflammatory treatment in patients.

Introduction

The cause of Parkinson's disease (PD) is unknown. Inflammation, oxidative stress and mitochondrial impairment are thought to play key roles in the progression of dopaminergic neurodegeneration. An increased number of activated microglia has been found in the substantia nigra of PD brains [180]. Animal studies have suggested the relevance of microglia activation to the nigral cell death [192]. This may signify that activated microglia form an important mechanism by which PD progresses. Furthermore, epidemiological data indicate that anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAIDs) have a protective effect on PD [10]. Increased expression of cyclooxygenase-2 (COX-2) and production of prostaglandin E2 synthesis have been implicated in neurodegeneration in several parkinsonian animal models[11;196]. An important question is, whether COX-2 inhibition can reduce neuroinflammation in PD patients, to inhibit progression of the disease.

Positron emission tomography (PET) with [^{11}C]-PK11195, a peripheral benzodiazepine receptor (PBR) ligand, has been studied for *in vivo* imaging of microglia activation in PD patients. Recently, using [^{11}C]-PK11195 PET in PD patients increased inflammation was found in the midbrain [200]and also in the basal ganglia and cortical regions[201]. From these studies it is not possible to correlate disease stage with the amount of neuroinflammation.

We conducted a pilot study to investigate whether [^{11}C]-PK11195 PET provides a sensitive method for evaluating the effect of anti-inflammatory treatment by COX-2 inhibition in PD patients. We studied neuroinflammation *in vivo* in both de novo and more advanced PD patients. As different methods of analysis have been published of [^{11}C]-PK11195 PET in patient studies, we applied both reference tissue models and plasma input models to analyze the data.

Methods

Fourteen PD patients of 62 ± 11 years (criteria of Gelb et al) and eight healthy volunteers aged 58 ± 12 years participated. The study was approved by the Medical Ethics Committee of the University Medical Centre Groningen and all subjects gave informed consent. Six de novo PD patients with symptom duration of less than one year were untreated. More advanced PD patients had Hoehn and Yahr stage 2 and used combination therapy of either ropinirole or pramipexole with levodopa. Control subjects were not on any drugs. Five PD patients were re-scanned after one month taking celecoxib 100 mg. bid. There is no known effect of celecoxib on tracer metabolism, however to exclude any possible interaction, celecoxib (with half-elimination time 8-12 hours) was stopped one day before the [^{11}C]-PK11195 PET scan, which was performed in the afternoon.

PET scans were carried out with an ECAT EXACT HR+ positron camera (Siemens/CTI, Knoxville, TN).After an injection of 400 ± 30 MBq [^{11}C]-PK11195 subjects underwent one hour dynamic PET scanning consisting of 21 frames Serial arterial blood sampling to define the input function for [^{11}C]-PK11195 was done using a computerised sampling programme. In five scans (four subjects, in one of the PD patients twice) plasma sampling failed due to technical problems; however, these scans were included in the reference tissue model analysis. Metabolite fraction in plasma was measured using high-pressure liquid chromatography analysis in samples taken by hand at 15, 45 and 60 minutes, to generate a metabolite corrected plasma input curve.

After coregistration of the MRI scan to the PET scan (summated frames 1-10 as blood flow-like image for better anatomical demarcation) using Statistical Parametric Mapping (SPM2), the coregistered MRI scans were normalised to the SPM MRI template and parameters were used for spatial normalisation of the individual dynamic PET scans. The regions of interest (ROI's) of the SPM brain ROI tool were used, and in addition a midbrain and cerebellar ROI were drawn manually on the spatially normalised MRI scan. The

time activity curve (TAC) of each ROI was calculated. Both the cerebellum and a reference cluster were applied in a simplified reference tissue model (SRTM). Software to perform the cluster analysis was kindly provided by the MRC Clinical Sciences Centre (Faculty of Medicine, Imperial College, London, UK). To produce results comparable with previous studies, ten clusters were extracted from each scan [207]. The first cluster, representing the largest cortical regions with 20 to 25 % of all voxels, was taken as reference input function.

Furthermore, using Matlab (The Mathworks Inc., Natick, U.S.A.) and a linearization according to Logan, starting at t=10 minutes, distribution volume (DV) images were constructed for the ROI's as well as on a voxel by voxel basis to obtain parametric DV images. The DV for the ROI's obtained with Logan analysis was compared to a 2-tissue compartment model calculating k_1, k_2, k_3 and k_4, in which the ratio k_3/k_4 was taken as binding potential (BP). For voxel by voxel comparison of regional differences between the groups, the parametric DV images were analysed using SPM2. A between-group comparison was done with two-sample t-test contrasts set at $p< 0.01$, and $p<0.05$ after correction for multiple comparisons for statistical significance. Comparison was performed both with and without proportional scaling to a global mean, as scaling was reported to account for differences in blood-brain barrier transport (k_1/k_2 ratio) and non-specific binding of [^{11}C]-PK11195 [9].

Results

PD patients showed higher putamen and midbrain BP compared to controls, although overlap was seen and none of the differences were statistically significant. In all PD cases except one, highest putamen BP was contralateral to the clinically most affected body side. The more advanced PD group showed higher mean putamen and midbrain BP values compared to the group with de novo PD (table 1). Several BP had to be discarded due to fitting errors resulting in negative or unrealistically high BP. Cerebellum SRTM gave fewer fitting

errors but resulted in lower BP values, especially in the PD group. BP from k_3/k_4 partly correlated with SRTM results but gave about 10-fold higher BP values. Logan analysis did not show differences between PD patients and controls.

Region	Method	Con	PD de novo	PD adv	Scan 1	Scan 2
MB	ceRT	0,17 (0,10)	0,09 (0,07)	0,14 (0,10)	0,09 (0,07)	0,11 (0,05)
	clRT	0,16 (0,11)	0,15 (0,05)	0,17 (0,09)	0,10 (0,03)	0,15 (0,02)
	k3/k4	1,57 (1,50)	1,09 (0,59)	2,66 (2,51)	0,68 (0,08)	0,86 (0,08)
	Logan DV	0,48 (0,05)	0,40 (0,11)	0,41 (0,06)	0,34 (0,15)	0,50 (0,02)
Put mas	ceRT	0,12 (0,07)	0,11 (0,01)	0,13 (0,05)	0,13 (0,03)	0,14 (0,05)
	clRT	0,15 (0,04)	0,21 (0,12)	0,28 (0,13)	0,23 (0,17)	0,19 (0,06)
	k3/k4	1,61 (0,75)	1,70 (0,73)	1,88 (0,79)	1,14 (0,12)	1,34 (0,08)
	Logan DV	0,73 (0,11)	0,86 (0,17)	0,65 (0,09)	0,74 (0,44)	0,83 (0,44)

Table 1: BP and Logan DV mean and standard deviation (SD) values of different ROI based analysis methods in the control group (n=8), de novo PD (n=6) and advanced PD (n=8) groups. The last two columns show results of the PD group with scan 1 and 2 pre- and post-treatment (n=5). We obtained plasma data in both scan 1 and 2 in only two patients, for comparison of Logan DV and k3/k4 values. SRTM analysis results could be compared between scan 1 and 2 of the 5 patients. MB=midbrain. Put mas = putamen contra lateral to the clinically most affected side; in controls the highest putamen BP is taken. ceRT = cerebellum reference tissue model, clRT = cluster reference tissue model. None of the comparisons between the PD and control group reached statistical significance ($P<0,05$) using t-test.

Fig. 1 shows a DV image of a PD patient scan pre- and post treatment, with higher DV after celecoxib. Due to the sampling errors in 3 of the 5 PD patients in this group, we could evaluate Logan results of pre- and post treatment scans in only two patients. The reference tissue models were applied to all 5 patients and showed similar or even higher BP values after celecoxib, without significant changes in k_1. BP changes were not significant in this small group. No changes in PK11195 metabolite activity were seen after celecoxib.

Fig. 1

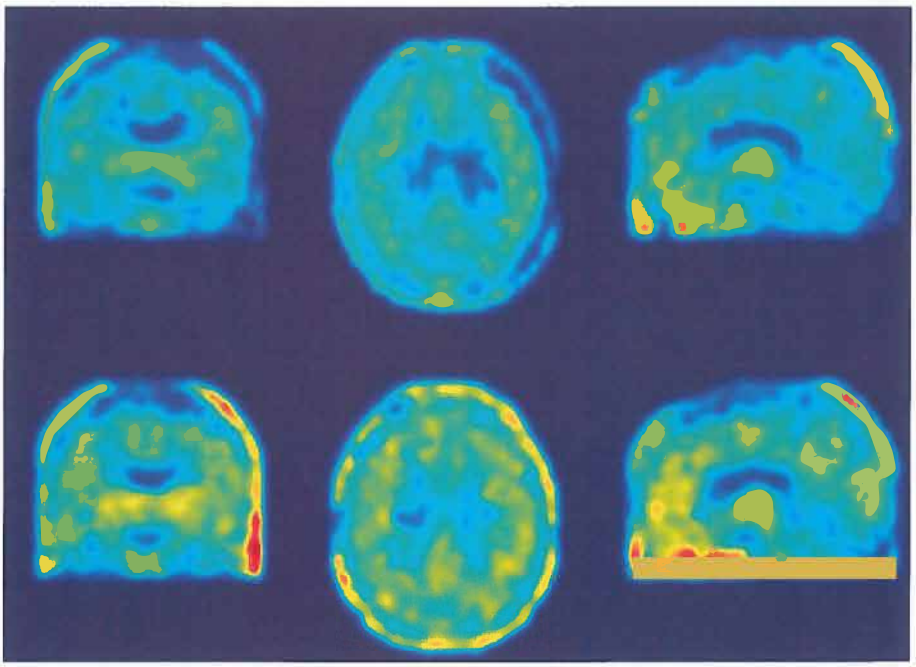

Figure 1: Logan DV image of a PD patient before celecoxib treatment (upper row) and after one month of celecoxib. Scaling of the images is fixed for visual comparison. At visual inspection, DV is higher after celecoxib treatment. ROI values confirmed this finding although no statistically significant differences were found.

Discussion

In our study, simplified reference tissue models showed higher mean [^{11}C]-PK11195 BP in the contralateral putamen and in the midbrain in PD patients compared to healthy control subjects. BP values were higher in the more advanced PD group compared to the *de novo* PD group. Neuroinflammation may be a chronic process that intensifies during progressive neurodegeneration. However, differences between PD subjects and healthy controls were not statistically significant, and variable results were obtained with different methods of analysis. Previous studies also show variable results with several analysis methods using different adjustments to obtain the reference region [102;200;201;207]. The cerebellum has previously been demonstrated to be an optimal reference region in a [^{11}C]-PK11195 PET study with healthy controls and AD subjects[102]. In a large study with [^{11}C]-PK11195 PET in PD patients, however, the cerebellum showed increased tracer uptake in the PD patient group [201] and thus might not be an optimal reference region in PD patient studies. In that study cluster analysis was applied to extract and identify a normal reference input function. In the study of Ouchi *et al.* [200], the reference region for SRTM was composed of the cerebellum TAC adjusted to a control mean cortical region input function. This may indeed bypass the fact that PD patients possibly show increased [^{11}C]-PK11195 uptake in multiple regions, including the cerebellum. However, exact individual quantification of [^{11}C]-PK11195 in several brain regions cannot be adequately performed with these adjustments. Thus, in our opinion analysis methods of [^{11}C]-PK11195 PET studies do not provide solid data to quantify neuroinflammation in patients with different stages of neurodegenerative diseases.

Secondly, contrary to the expectation in this pilot study, a slightly higher [^{11}C]-PK11195 BP after celecoxib was found both with Logan and SRTM analysis. Although no conclusions can be drawn from this small pilot, it may raise concern against possible differential effects of COX-2 inhibition in PD patients. Exacerbation of chronic neuroinflammation with COX-2 inhibition was seen in different animal inflammation models, and this concern has also been expressed in humans [208]. Thus, it is important to determine

whether COX-2 inhibition in humans with a chronic inflammatory process will attenuate inflammation or might have differential effects. Radiotracer studies that can monitor neuroinflammatory processes will be of great value for the translation of potential effective treatments from animal disease models to human disease, since they give the possibility of direct *in vivo* measurement of treatment effects in animals as well as in patients.

In conclusion, the current analysis methods of [^{11}C]-PK11195 uptake in brain did not provide solid data to assess changes in specific tracer uptake by anti-inflammatory treatment in our patient group. The simplified reference tissue model may be biased by the amount of non-specific binding. Consequently, tracers with higher levels of specific binding in the brain and better capacity to quantify PBR expression are being developed. Several tracers show promising results in animal studies and need to be further evaluated for use in patient studies[95;96].

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Chapter 8

Neuroinflammation and blood-brain barrier P-glycoprotein function after striatal 6-hydroxydopamine lesion and COX-2 inhibition

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Submitted.

Abstract

Background: Neuroinflammation and blood-brain barrier (BBB) P-glycoprotein (P-gp) function are proposed to be involved in the progression of neurodegeneration in Parkinson's disease (PD). We hypothesize that COX-2 inhibition reduces neuroinflammation and subsequently improves expression and function of P-gp.

Methods: Using striatal 6-OHDA lesions in rats followed by either celecoxib or vehicle treatment, we have measured the resulting microglia reaction in the substantia nigra (SN) by histological quantification and uptake of [^{11}C]-PK11195 using microPET. Furthermore, we used [^{11}C]-verapamil and microPET for *in vivo* measurement of P-gp function, together with histological quantification of P-gp expression.

Results: 6-OHDA lesion caused increased microglia activity in rat substantia nigra, however [^{11}C]-PK11195-microPET did not prove a sensitive measure of neuroinflammation in these small regions. Our study did not confirm decreased microglia activity when COX-2 inhibition was started after the 6-OHDA lesion. COX-2 inhibition, on the other hand, prevented P-gp up-regulation after lesioning.

Discussion: COX-2 inhibition does not mitigate microglia activation after 6-OHDA lesion. On the other hand, COX-2 inhibition decreases the up-regulation of P-gp expressing vasculature which normally occurs after the induction of dopaminergic lesioning by 6-OHDA.

Introduction

Parkinson's disease (PD) is a major neurodegenerative disorder, characterised by the progressive loss of dopaminergic neurons in the substantia nigra (SN) and of other catecholaminergic systems projecting from the brainstem. It is currently unknown how the various patterns of pathology develop, but certainly local tissue influences such as inflammation, oxidative stress and mitochondrial impairment play a role in the progression of dopaminergic neurodegeneration. In recent years, the involvement of neuroinflammatory processes in PD neurodegeneration has gained increasing attention. Regardless of etiology, nigral neuronal damage may set in motion a persistent and progressive neurodegeneration in PD, through release of aggregated alpha-synuclein and subsequent production of proinflammatory mediators by activated microglia. Although the chronic inflammatory process may attempt to clear released alpha-synuclein as well as neuromelanin, several studies suggested that persistent inflammation leads to the release of pro-inflammatory mediators that enhance dopaminergic neurotoxicity [11;186;192].

Furthermore, the blood-brain barrier (BBB) plays an important role in the protection of neurons against the accumulation of toxic substances in neurodegenerative disease. It is known that environmental toxins influence PD onset [113]. Recently, we have found that the BBB P-glycoprotein (P-gp) efflux function decreases with age [209] and decreases in progressed PD neurodegeneration [210]. P-gp was also shown to be involved in brain efflux of Amyloid-ß and progression of Alzheimer's disease [109;131]. P-gp regulation has furthermore been associated with neuroinflammatory processes. An important feature of inflammatory-related processes in PD is the marked increase in cytokine levels, including TNF-alpha, in the striatum and cerebrospinal fluid of PD patients [187]. Studies regarding the functionality of P-gp report a depressed functionality related to TNF-alpha treatment or to LPS-induced inflammation [145]. Most studies demonstrated that the function and expression P-gp in different cell lines is (reversibly) inhibited by several inflammatory cytokines [145;146].

Several animal PD studies argue for a role of microglial activation and COX-2 activity in the progression of neurodegeneration [11;12;188;198]. The precise role of COX-2 inhibition on microglia activation, however, is not yet clear. Furthermore, the effect of mitigation of inflammation on P-gp function has not been studied in a PD model before.

The aim of this study was, to investigate whether COX-2 inhibition after striatal 6-OHDA lesion mitigates microglia activation and thereby improves BBB P-gp function, as measured by histological quantification as well as *in vivo* by animal PET imaging. Inflammation through activated microglia can be measured directly *in vivo* using PET and the radiolabeled isoquinolone PK11195 [91-93], which is a ligand for the peripheral benzodiazepine receptor (PBR). Using striatal 6-OHDA lesions in rats followed by either celecoxib or vehicle treatment, we have measured the resulting microglia reaction in the SN by histological quantification and uptake of [^{11}C]-PK11195. Furthermore, we used [^{11}C]-verapamil and PET for *in vivo* measurement of P-gp function, together with histological quantification of P-gp expression. Tyrosine hydroxylase (TH) staining of the SN was used to confirm the effect of the striatal lesion at the level of the SN.

Methods

Animals

The study was carried out in compliance with the national law on animal experiments and was approved by the animal ethics committee of the University of Groningen. Healthy male Wistar rats were purchased from Harlan (Horst, the Netherlands). The rats were housed in groups under a 12-hour light-dark regimen at a constant temperature (21± 2°C). Standard laboratory chow (RMHB, Hope Farms, the Netherlands) and water were available ad libitum. Between arrival and stereotactic operation the rats had at least seven days to acclimatize.

Four groups of rats were investigated: four sham-operated control animals with PET scan and termination after one week, six 6-OHDA lesioned animals with

PET scan and termination after one week ("OHDA-group"), six 6-OHDA lesioned animals receiving polyethylene glycol (PEG) vehicle treatment for three weeks before PET scan and termination ("PEG-group"), and eight 6-OHDA lesioned animals receiving celecoxib treatment for three weeks before PET scan and termination ("COX-group").

6-OHDA lesion model

To create progressive degeneration of the nigrostriatal dopaminergic system, male Wistar rats (200-250 g, Harlan, the Netherlands) received unilateral stereotaxic 6-OHDA (Sigma, the Netherlands) injections with a 10 µl Hamilton syringe in the right striatum. Animals were anaesthetized by ip injection with a mixture of 25mg/kg ketamine and 0.2 mg/kg medetomidine, and placed in a stereotactic frame. A concentration 3.0 µg/µl free base 6-OHDA dissolved in 0.2 % ascorbic acid/saline (Sigma) was injected into three locations with 2.5 µl per location. Coordinates of the location (calculated from bregma) were defined as described before (Sanchez-Pernaute et al 2004); with site 1, AP +1.3, L -2.8, DV -4.5, IB -2.3; site 2, AP +0.2, L -3.0, DV -5.0, IB -2.3; site 3, AP -0.6, L -4.0, DV -5.5, IB -2.3 mm. Rate of injection was 1 µl/min and the syringe was left in place for an additional 3 min before withdrawal. Starting the day after surgery, one group (n=8) was treated via oral intubation with celecoxib 20 mg/kg/day (Pfizer, The Netherlands) dissolved in polyethylene glycol (PEG) for three weeks, another group (n=6) received vehicle (PEG).

microPET imaging

One ore three weeks after lesioning, animals were anaesthetized by ip injection with a mixture of 25mg/kg ketamine (Pfizer, The Netherlands) and 0.2 mg/kg medetomidine (Orion Corp., Finland). Tail vein was catheterized for infusion of the labelled ligand, alternatively the ligand was infused through the penis vein. Animals were scanned using a Siemens/Concorde microPET camera (Focus 220). Animals were placed in imaging position on a bunk bed. The camera was started during [^{11}C]-PK11195 injection. Dynamic data were acquired for an hour. At least two hours after [^{11}C]-PK11195 injection, [^{11}C]-verapamil was injected for the second scan, with data acquisition for an hour.

Data sets were fully corrected for random coincidences, scatter and attenuation. A separate transmission scan was acquired for attenuation correction. PET images were overlaid on a cyclosporine-blocked [^{11}C]-verapamil scan with clear brain delineation to obtain equal positions of the heads and brains in all scans, using standard software (AsiPro VM 6.2.5.0, Siemens-Concorde, Knoxville, TN). Regions of interest (ROIs) were drawn in ECAT program with the help of a [^{11}C]-raclopride-PET scan, serving as overlay for orientation of the striatum. Using CAPP5 software, ROIs were drawn that included the left and right striatum and the left and right SN and activity per unit volume was calculated. Ratios for activity per unit volume were defined as right over left striatum and right over left SN. Standardized uptake values (SUV) were defined as activity per unit volume*body weight (g) / injected dose (MBq). Group comparisons were performed using independent samples t-test and SPSS software. Results were considered significant with $p<0.05$ in two-sided t-test.

Immunohistochemistry (IHC)

After the scan, the animals were kept under anaesthesia and were terminated by intracardial perfusion with 4% paraformaldehyde (PFA, Merck; 4005, Darmstadt, Germany). The brains were removed and postfixed in 4% PFA. Before sectioning, brains were immersed in 20% sucrose PBS for 24 hrs. Brains were cryosectioned at 16 μm, collected on polylysine coated glass slides and stored at -80°C. Glass slides containing sections which included striatal or nigral part of the brain were randomly selected. Sections were air-dried. To exhaust endogenous peroxidases, sections were pre-treated with 0.5% hydrogen peroxide in PBS for 30 min.

In the case of P-gp staining, it turned out that antigen retrieval methodology was necessary to uncover P-gp epitopes. In accordance to Shi [211], both length and temperature of incubation, as well as buffer pH was optimized to achieve maximum retrieval. Within the range of conditions tested, 2 x 15 min autoclave heating of the sections at 115°C in a 0.1 M sodium citrate distilled water solution set to pH 9 yielded the best result and was applied.

Sections were washed 3 times in PBS, followed by incubation in PBS containing 5% normal goat or rabbit serum, 2% bovine albumin serum and

0.1% Triton X-100 for 30 min. Sections were subsequently incubated overnight at 4°C in PBS containing 1% normal goat or rabbit serum, 2% bovine albumin serum and 0.1% Triton X-100 and the primary antibodies, all at 1:100 dilution. Anti-IBA-1 primary antibody (rabbit polyclonal Anti-Ionized calcium Binding Adaptor molecule 1 (IBA-1) (50µg/100ul) IgG, Wako Chemicals GmbH, Germany) was used to identify microglia as was done before [212]. Anti-P-gp primary antibody (goat polyclonal Anti-Multi Drug Resistance 1 (P-gp) IgG (C-19, sc-1517, 200ug/ml), Santa Cruz Biotechnology, Inc., Germany) was used to identify endothelial residing P-gp proteins in accordance to practice by colleagues [213]. Anti-TH primary antibody (polyclonal rabbit Anti-Tyrosine Hydroxylase (TH) (AB152) (80µg/ml), Chemicon International Inc., California, USA) was used to identify dopaminergic soma and fibres in the SN and striatum. Primary antibody was omitted in selected sections to verify the specificity of staining, thus serving as negative controls. After rinsing the sections 3 times for 5 min. in PBS, biotinylated secondary anti-rabbit/goat antibodies were diluted 1:250 in PBS containing 0.1% Triton X-100 and applied, followed by room temperature incubation for 2 hrs. Next, sections were rinsed 3 times in PBS and incubated at room temperature in PBS diluted Horseradish peroxidase-conjugated avidin (Vector labs, CA, USA), 16 µl/ml of reagents A and B for 30 min. Following thorough rinsing with PBS, 3,3-diaminobenzidine (20 mg/ml Tris-HCl) was used as chromogen to visualize staining during 10 min. of incubation. Rinsing of sections in Tris-HCl and distilled water was followed by dehydration. Finally sections were mounted and coverslipped.

Quantitative assessment of TH- and IBA stained cells and P-gp-stained vessels

TH-stained cells were counted on three representative slices containing the SN at 20x magnification. Ratios between the number of TH positive cells in the left (normal) and right (lesioned) side were determined and percentage loss was calculated. For IBA stained cells, SN left and right cell counts were compared using t-test with $p<0,05$ to evaluate significant increase in activated microglia. The amounts of P-gp stained microvessels in the different groups/hemispheres

were quantified using a method described by Hoffmann and colleagues [214]. Grids of 50 μm squares were drawn on microphotographs from the SNc using the same magnification in every instance. Counts were taken of crossings of stained vessels between these squares (see Figure 1). The number of counts was considered a good estimate for the amount of specific P-gp staining. Right hemisphere counts were standardized using the counts of the corresponding left hemispheres (i.e. right/left ratios were calculated).

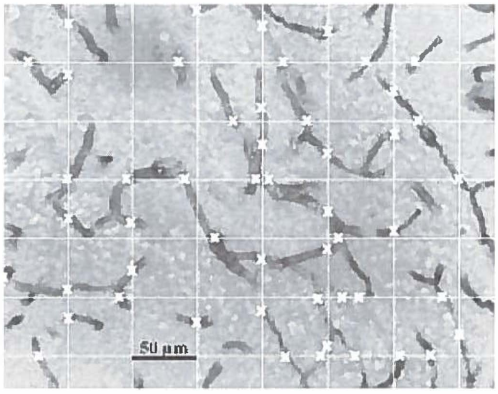

Fig. 1:
Quantitative assessment of P-gp stained microvessels in the rat substantia nigra pars compacta. The number of border crossings by fully stained vessels within a 650 x 850 μm region which was divided into 221 squares was counted and provided an estimate for P-gp.

Results

Striatal TH immunoreactivity was used to evaluate the success of the 6-OHDA lesion. All 6-OHDA lesioned groups displayed clear focal loss of DA fibres in the right striatum. Three weeks after the lesion, both COX and PEG groups showed TH-expressing cell loss in the SN at the side of the striatal lesion (fig. 2), while in the 6-OHDA group one week after lesion clear TH-positive cell loss (70%) in the SN was seen in only one rat and smaller loss (40%) in another one. On TH-positive cell counting, the celecoxib treated group showed a mean cell loss in the right SN of 61% (SD ± 24), which was 65% (SD ± 10) in the vehicle treated group. Also in the rats with no apparent SN TH lesion one week after 6-OHDA lesion, the SN did already show increased activated microglia

(fig. 3). The 6-OHDA lesioned SN showed increased microglia counts after IBA staining in all groups, although this increase was not significantly different in the 6-OHDA lesioned group after one week in comparison to the sham-operated group ($p=0,17$) (see fig.4).

[^{11}C]-PK11195-PET showed increased tracer uptake in the lesioned striatum in all groups ($P<0.05$) (fig 5), but no difference was seen in [^{11}C]-PK11195 binding between left and right SN in any of the groups (see fig.4). Notably, the striatal uptake at the lesioned side was visually more compelling than after quantification. Striatal areas of high ^{11}C-PK11195 binding showed high IBA-1 reactivity (data not shown).

Furthermore, P-gp staining of capillary density was assessed using anti-P-gp immunohistochemistry and ratios were calculated for left and right SN. The PEG treated rats showed increased P-gp expression in the lesioned SN, whereas the COX group showed decreased P-gp expression (Fig. 6A), although no statistical significance was reached ($p=0,09$). Finally, right-left ratios for striatal [^{11}C]-verapamil accumulation were compared between COX and PEG groups. Unfortunately, in the PEG group only two verapamil scan succeeded, so the unbalanced design with 2=8 in the COX group and n=2 in the PEG group prohibited statistical analysis. Higher accumulation of [^{11}C]-verapamil at the right side was seen in the PEG group but in the COX group (Fig. 6B).

Discussion

Unilateral 6-OHDA-induced lesioning in the striatum produced a clear loss of TH-expressing cells in the SN, already visible in two rats after one week, but most prominently in the other rats at three weeks after lesioning. The mild increase in the number of activated microglia in the SN in the rats without apparent SN TH cell loss at one week after the striatal 6-OHDA lesion, suggest that microglia activation precedes neuronal cell demise. Microglia can indeed be activated at projection sites of injured neurons. Several studies suggest that axonal transport disruption and axonal damage precede neuronal death, and may be causal to disease progression in neurodegenerative diseases [215]. A recent

study showed changes in proteins involved in axonal transport coupled with neuroinflammation, preceding α-synuclein-mediated neuronal death [216]. Microglia activation thus seems to be an early event in relation to progression of neurodegeneration.

While an increase in activated microglia in the SN at the 6-OHDA lesioned side was detected with IHC, [^{11}C]-PK11195 PET imaging did not show asymmetry at the level of the SN, neither visually nor quantitatively. Possibly, high aspecific tracer uptake in the nearby pituitary gland prevents sensitive detection of smaller increases in activated microglia in the SN. However also in the striatum, where visually a very clear increase in activated microglia was seen with IHC, increased tracer uptake was visually clear but only moderate on quantification. We therefore had to conclude that [^{11}C]-PK11195 is not a reliable tracer for quantification of microglia activation in small regions of rat brain like the SN, while assessment of pathological changes in this region is necessary for investigation of models for PD neurodegeneration.

In the current study we did not find evidence for a celecoxib-mediated effect on microglial activation after lesioning of the nigrostriatal projections, as measured by the extent of activated microglial cell count in the SN. Other studies suggest that the effect of COX modulation may also be independent from microglia activity [197;198;217]. Teismann et al. showed that the selective COX-2 inhibitor rofecoxib blocked ventral midbrain prostaglandin production in MPTP injected mice and attenuated neuron and fiber loss, demonstrating the crucial enzymatic function of COX-2 to its neurotoxic effects on dopaminergic neurons. The authors suggested that the neuroprotective effect of COX-2 inhibition was related to the blockade of COX-2-mediated dopamine oxidation and not to decreased microglial activation [196]. Another explanation for the different findings concerning neuroprotective effects in several studies of neuroinflammation and neurodegeneration may be the timing of treatment. Attenuation of microglial activation may be insufficient to modulate neurotoxicity whereas early, transient activation of microglia may suffice to initiate neurodegeneration. Other studies that showed mitigation of inflammation by COX-2 inhibition had started treatment at the time of lesioning [11;218] and not after lesion formation, as was done in our study to

obviously mimic a clinically relevant order. As far as a neuroprotective effect, we were unable to detect attenuation of TH-positive cell loss in the SN in the celecoxib treated group. It should be noted that immunohistochemical detection of a clear unambiguous neuroprotective effect in the SN is extremely arduous; such an effect can only be reliably demonstrated in an extensive detail stereological evaluation of the entire SNc region in a large number of rats to exclude the inevitable variability in size and location of the striatal 6-OHDA lesion and the likewise subsequent extent and location of retrograde SN degeneration.

We had hypothesized that COX-2 inhibition would reduce inflammation and according to literature [112] would subsequently improve expression and function of P-gp. This showed not to be the case. However, notwithstanding the (absence of) effect on neuroinflammation, celecoxib showed a direct influence on P-gp expression. Our study showed increased P-gp expressing vessels at the side of 6-OHDA lesions. Disruption of the BBB after 6-OHDA with increased expression of P-gp has been described before [219]. However our study showed that [^{11}C]-verapamil, being a substrate for P-gp efflux from the brain, was not increasingly expelled upon P-gp up-regulation. This may suggest that the up-regulation of P-gp after 6-OHDA lesion is accompanied by increased permeability of the BBB, resulting in increased rather than decreased brain penetration of [^{11}C]-verapamil, or that dysfunctional P-gp is expressed. Importantly, however, in the celecoxib-treated animals a decrease of P-gp expressing vessels, without changes in [^{11}C]-verapamil accumulation was seen. Thus, COX-2 inhibition seems to prevent up-regulation of P-gp-expressing vasculature. In the literature, overexpression of COX-2 was associated with up-regulation of P-gp expression [220;221]. Furthermore, in treatment-refractory epilepsy it was demonstrated that glutamate can cause localized up-regulation of P-gp via COX-2 and that this phenomenon can be prevented with COX-2 inhibitors [222]. Thus, instead of improving P-gp function by mitigating inflammation, COX-2 inhibition may show an opposite direct effect by inhibition of P-gp up-regulation. On the other hand, increased P-gp expression after the 6-OHDA lesion could also relate to neomicroangiogenesis, which was described before in a toxic PD model. In MPTP intoxicated

monkeys, an increased expression of the angiogenic factor VEGF has been observed [223]. Furthermore, an increased number of endothelial cells in the blood vessels in the SN were found in PD patients [224]. The increased number of blood vessels may be involved in compensatory, neuroprotective mechanisms by increasing the elimination of toxic compounds from the brain. However, an increase in blood vessels may also increase the transfer of putatively toxic compounds into the brain, and has, for example, been associated with the rise in nigral iron content described in PD [225]. Moreover, in another study intranigral injections of VEGF resulted in increased blood vessel density with BBB disruption, and caused degeneration of nigral dopaminergic neurons [226]. In 6-OHDA treated neurons, low doses of VEGF displayed neuroprotective effect, while higher doses resulted in more angiogenesis and brain oedema [227]. Finally, it remains to be determined whether changes in vascularisation have a detrimental or beneficial effect in parkinsonian neurodegeneration. The effects of COX-2 inhibition on neovascularisation need to be further investigated in PD models.

In conclusion, our study did not confirm decreased neuroinflammation and microglia activity when COX-2 inhibition was started after a toxic 6-OHDA lesion. COX-2 inhibition, on the other hand, seems to prevent up-regulation of P-gp-expressing vasculature. It should be further investigated whether the latter may be an unwanted effect of COX-2 inhibition in progressive neurodegenerative disease, where decreased P-gp function may play an important role in the clearance of neurotoxic proteins, or whether it may be beneficial to inhibit neovascularisation in PD neurodegeneration.

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We gratefully thank I. Manting-Otter for the histotechnical assistance.

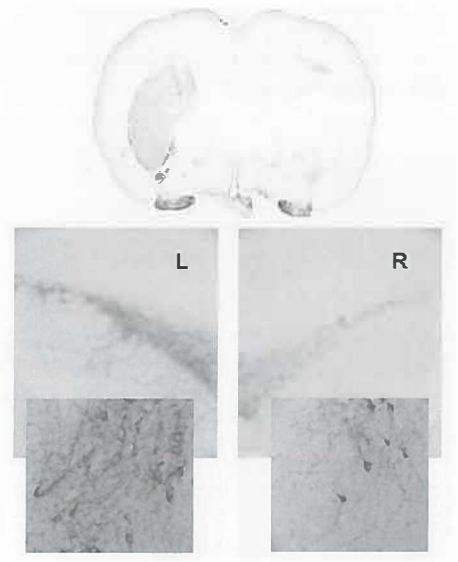


Fig.2:
TH staining of the striatum and SN 3 weeks after right striatal 6-OHDA lesion and celecoxib treatment, showing clear striatal and SN TH loss. Cell counting in this example revealed 60 % cell loss in the right SN.

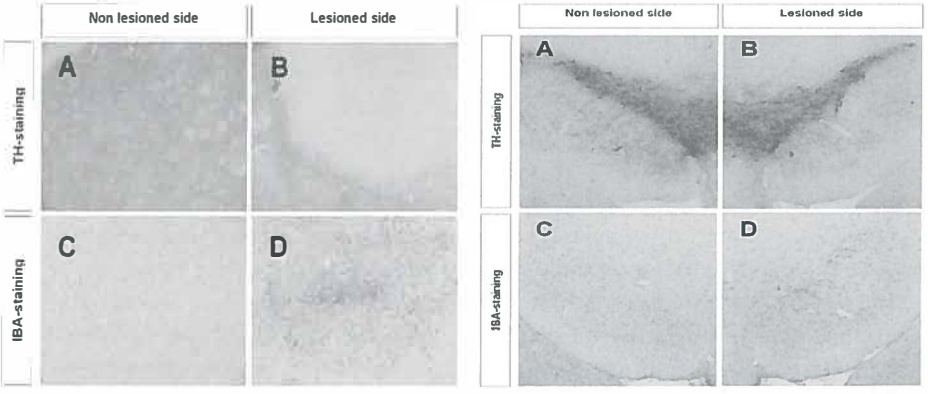


Fig.3: TH staining shows clear striatal TH loss 1 week after 6-OHDA lesion (left B), accompanied by large increase in striatal microglia (left D). At the level of the SN, after 1 week no obvious TH loss was seen (right B) in most rats, while already an increase in microglia in the SN was observed (right D).

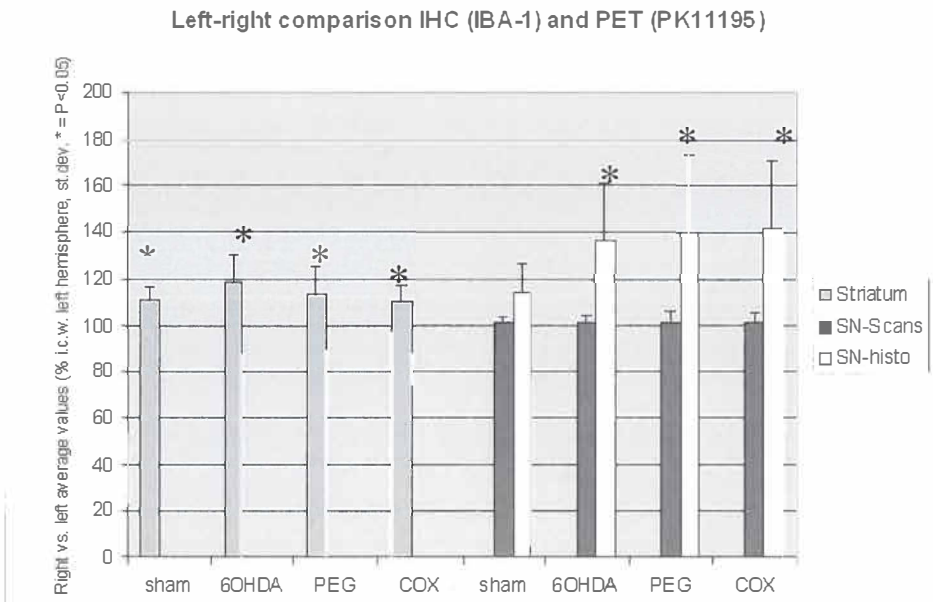


Fig. 4: Using both PET and immunohistochemistry we measured *in vivo* the inflammatory response 7 days (sham and 6-OHDA groups) and 21 (COX and PEG groups) days after 6-OHDA/Saline injection. Right hemispheres of rats were compared to the corresponding left hemisphere. The grey and black bars represent the percentage of ^{11}C-PK11195 binding in the striatum and substantia nigra, respectively, as determined by PET, and the white bars represent the amount of microglia (number/square measure) in the substantia nigra as determined by immunohistochemistry and manual counting. The percentages presented are compared relative to 100%, that is, to the binding/counts of the corresponding left striatum. ^{11}C-PK11195 uptake ratio was significantly higher (* = $P<0.05$) in all groups with highest uptake ratios in the 6-OHDA (7 days) group. No significant deviation from left side substantia nigra ^{11}C-PK11195 binding was found in either of the groups ($P<0.05$).

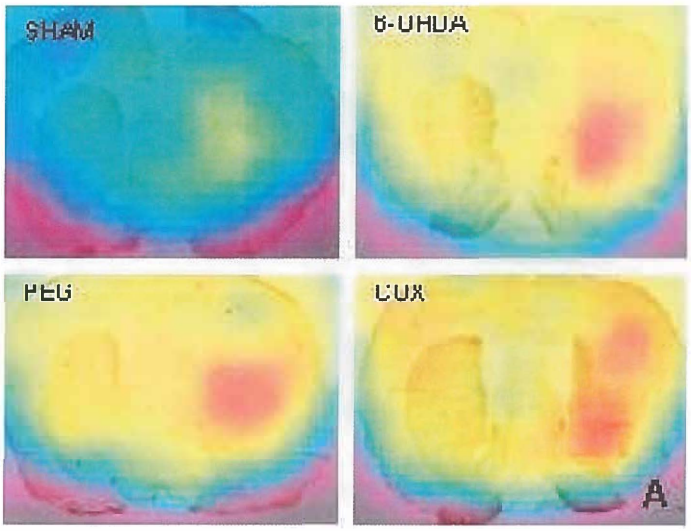
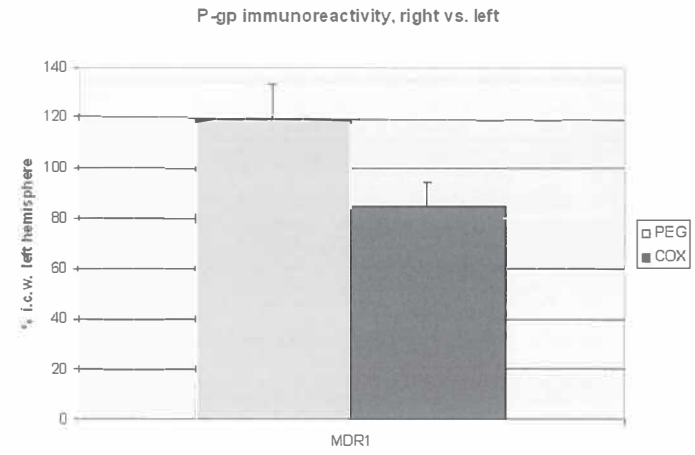
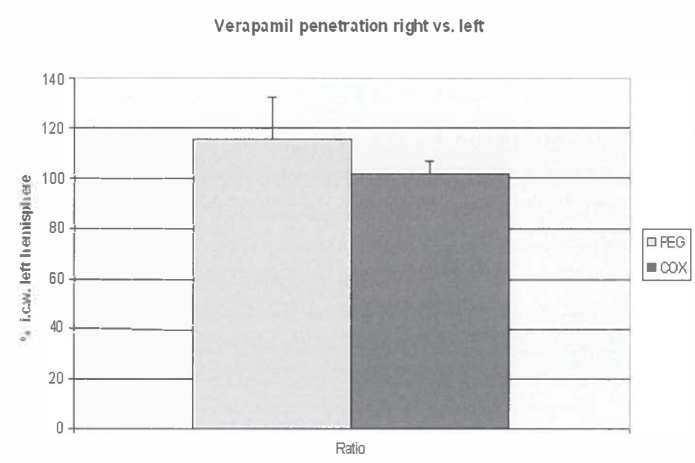


Fig. 5: Striatal ^{11}C- PK11195 binding in PET analysis. PET images are shown overlaid on a striatel TH staining image. Increased ^{11}C- PK11195 binding is seen in the right (i.e. lesioned) hemispheres, especially in the 6-OHDA lesioned animals (i.e. COX, 6-OHDA and PEG groups).



6A



6B

Fig. 6A: Comparison of right-left ratios for P-gp stained capillary density in the SNc between PEG and COX groups. The right side SNc in PEG group showed a (non-significantly) higher density (120 %) in P-gp immunoreactive capillaries compared to the left SNc region, whereas in the COX group the density in P-gp immunoreactive capillaries in the right side SNc was lower (80 %) in comparison to the left SNc region. The comparison of the PEG and COX right-left ratios show a non-significant ($p=0.09$) difference between the groups.
B: Overview of left-right ratios of striatal [^{11}C]-verapamil accumulation between PEG and COX groups (n=2/8). The small group size prohibited statistical analysis. The relatively high accumulation in the PEG right side striatum was not seen in the COX group.

Chapter 9

General Discussion

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting approximately 1 % of the population by the age of 65. With increasing age, neurodegenerative diseases such as PD become increasingly prevalent. Therapies to retard or prevent neuronal degeneration remain a major unmet need of this disorder. The key to development of successful disease modifying therapy lies in a better understanding of the pathogenesis of PD. Research on the aetiology of Parkinson's disease (PD) has resulted in much information on neurodegenerative processes, but still little is known about the events causing the initiation and also the progression of the disease. Several possible causes of PD neurodegeneration have been proposed, such as exogenous toxins, inflammation, genetic mutations and combinations of these factors [107].

In this thesis, using PET imaging in PD patients two mechanisms that may be involved in PD development and progression of neurodegeneration were investigated, namely blood-brain barrier (BBB) P-glycoprotein (P-gp) dysfunction and neuroinflammation.

Blood-brain barrier P-glycoprotein function

The classical hallmarks of idiopathic PD are the loss of dopaminergic cells in the substantia nigra and formation of proteinaceous inclusion bodies that contain fibrillized α-synuclein. However, other catecholaminergic neurons in the midbrain are also damaged. In particular the olfactory system and the vagal nucleus are suggested to be affected first, at a later stage followed by deeper structures, such as the substantia nigra [19]. This sequence could point to environmental factors penetrating the nervous system, possibly via the nose, or via the vagal nerve through myenteric neurons [228], or via the blood-brain barrier. From epidemiological studies as well as animal PD models, it is known that exposure to environmental toxins may contribute to neuronal loss in PD.

Decreased BBB P-gp efflux function was proposed as a possible causative link between toxin exposure and PD neurodegeneration [6;7]. Dysfunction of the blood-brain barrier (BBB) P-glycoprotein (P-gp) efflux system could allow more, potentially neurotoxic, endogenous or exogenous substances to accumulate in the brain, over time damaging sensitive neuronal cells e.g. dopamine neurons in the mesencephalon.

We hypothesized that BBB P-gp functional impairment may be a primary vulnerability factor for the development of PD. In the first study, described in **chapter three**, BBB P-gp function was investigated *in vivo* in 10 early stage PD patients and 8 healthy control subjects using [^{11}C]-*(R)*-verapamil-PET. Cerebral volume of distribution (DV) of verapamil was used as measure of P-gp function. In addition, MDR1 genetic polymorphism was assessed to identify possible genetically determined lower P-gp function as a "vulnerability factor" for PD development [6;82]. A larger variation in DV of [^{11}C]-*(R)*-verapamil was seen in the early stage PD group as compared to the control group. However, decreased BBB P-gp function in the midbrain of PD patients, which was found in a previous pilot study performed by Kortekaas et al.[7] was not seen in our study with early stage PD patients. Also MDR1 polymorphisms that could be involved in decreased P-gp function were not specifically found in the PD patients, and the polymorphisms were not correlated to differences of *in vivo* P-gp function. Since we could not confirm decreased P-glycoprotein function in de novo and early stage PD patients, we concluded that BBB P-gp dysfunction does not play a primary causative role in the pathogenesis of idiopathic PD.

Another possibility would be that P-gp shows a reactive response to the pathologic process, which might lead to diminished P-gp function, as has also been described in Alzheimer's disease (AD) [109]. For the second study, described in **chapter four**, we used [^{11}C]-verapamil-PET to study functional P-gp at the BBB in de novo PD patients as well as patients in more advanced disease stages and in patients with progressive supranuclear paly (PSP) and multisystem atrophy (MSA), who also show parkinsonism but on the basis of different patterns of neurodegeneration with faster decline. De novo PD patients showed lower uptake in midbrain and frontal regions, which could indicate a regional up-

regulation of P-gp function as possible compensatory mechanism early in the course of the disease. Advanced PD patients and PSP patients had increased [^{11}C]-verapamil uptake, reflecting decreased P-gp efflux function, in mostly white matter frontal regions compared to controls. PSP and MSA patients showed increased [^{11}C]-verapamil uptake in the basal ganglia. Regionally decreased P-gp function could thus occur not only in more advanced PD, but may also play a role in other neurodegenerative diseases with parkinsonism, and likely plays a role in advancing neurodegenerative processes in general.

The involvement of P-gp may relate to protein misfolding and accumulation as part of the pathologic process in neurodegenerative diseases. Intracellular protein misfolding/aggregation are features of many late-onset neurodegenerative diseases, called proteinopathies, including for example Alzheimer's disease, Parkinson's disease, MSA and PSP. The genetic mutations causing several forms of proteinopathies confer novel toxic functions on the specific protein. Thus, the factors regulating the synthesis and clearance of these aggregate-prone proteins are putative therapeutic targets. In Parkinson's disease, the proteasome complex is involved in the clearance of aggregated α-synuclein. P-gp was found to interact actively with the proteasome complex, and a role for P-gp in the transport of proteasome-derived peptides was suggested [138]. Decreased cerebrovascular P-gp may thus impair the ability of the brain to expel excess proteins. The resulting accumulation of protein in cells could overwhelm cellular ubiquitin-proteasomal degradation, a pathway that is involved in the development of PD pathology. Furthermore, it is recognised that the prevalence of dementia is higher in PD patients than in age-matched controls, which often is found to be defined by Alzheimer's disease (AD) pathology in conjunction to the Lewy bodies [140]. Decreased P-gp function to expel proteins in advanced PD could also play a role in the co-existence of α-synuclein and β-amyloid in PD with dementia (PDD), a patient group that was not investigated in the present study. As transport of β-amyloid by P-gp has been shown [131], future studies should also aim to investigate functional P-gp *in vivo* in AD patients and in PD patients with dementia.

Furthermore, it was found that environmental factors causing neuronal damage, such as pesticides, are closely linked to mechanisms underlying the

formation of α-synuclein pathology and PD-like neurodegeneration. In mutant human α-synuclein transgenic mice, treatment with the pesticides paraquat and maneb resulted in increased neuronal α-synuclein pathology throughout the central nervous system [139]. P-gp has been associated with the efflux transport of several PD-related toxins, including organophosphate pesticides [229] and MPTP, although the brain efflux of the latter may be mostly effectuated by the vesicular monoamine transporter 2 (VMAT2), that has several structural features and substrates in common with P-gp [230]. Interestingly, Dinis-Oliveira et al investigated paraquat-induced lung toxicity and found that P-gp induction by dexamethasone lead to decreased lung levels and toxicity of paraquat [231]. It can thus be hypothesized that P-gp dysfunction in more advanced PD may also contribute to neuronal damage due to increased accumulation of toxins.

Finally, we have investigated changes in BBB P-gp function with aging in **chapter five**. Ageing is the main risk factor for the development of neurodegenerative diseases such as PD. Our study showed decreased cerebral P-gp function with aging, particularly in internal capsule and corona radiata white matter and in orbitofrontal regions. Decreased blood-brain barrier P-gp function in those regions could thus explain part of the vulnerability of the aging brain to white matter degeneration. Moreover, in the light of the role of P-gp to expel excess protein, decreased blood-brain barrier P-gp function with ageing could be a mechanism by which age acts as the main risk factor for the development of neurodegenerative disease.

Common final pathways for aging, environmental and genetic mechanisms can thus exist wherein decreased cerebral P-gp function could play a role in progressive neurodegeneration by accumulation of neurotoxic substances. Finally, the relation between P-gp function and brain accumulation of proteinaceous inclusions should be further investigated. In AD, this could also be investigated *in vivo* in patients by the use of PET and the tracers [11C]-verapamil for P-gp function and [11C]-PIB for Amyloid-β disposition[232]. Involvement of P-gp dysfunction in PD dementia and in dementia in Lewy Body disease (LBD), which is also associated with Amyloid-β accumulation [233], should be investigated.

Neuroinflammation and COX-2

Progressive PD neurodegeneration was previously considered to be a purely neuronal pathological process, but is now seen as the result of multiple pathogenic factors. In recent years, the cross talk between neurons and glia has become an intensive research focus for the understanding of brain pathophysiology. More specifically, neuroinflammatory processes involving an increased expression of cyclooxygenase (COX) and elevated prostaglandin E2 (PGE2) levels have been associated with several neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) [234]. We have reviewed the rationale for involvement of neuroinflammation and COX-2 in PD neurodegeneration in **chapter six**. In post-mortem PD brains, an increased number of activated microglia was found [180]. Animal PD studies have suggested the relevance of microglia activation to the nigral cell death. This may signify that activated microglia form an important mechanism by which PD progresses. Furthermore, several epidemiological data indicate that anti-inflammatory agents such as non-steroidal anti-inflammatory drugs (NSAID's) have a protective effect on Parkinson's disease [9;10]. Non-selective or mainly COX-1 selective NSAID's are widely used as anti-inflammatory analgesics. However, COX-2 has emerged as the isoform primarily responsible for prostanoid production in acute and chronic inflammatory conditions. Thus, COX-2 was suggested to specifically contribute to neurodegenerative processes. Indeed, increased expression of cyclooxygenase-2 (COX-2) and production of prostaglandin E2 synthesis have been implicated in neuroinflammation and neurodegeneration in several parkinsonian animal models [11;12]. However, in spite of intense research in the last decade, the evidence for a direct role of microglia activation or COX-2 expression in neurodegenerative disease is still controversial. In the first studies that used [^{11}C]-PK11195 PET in PD patients, increased inflammation was found in the midbrain [200] and also in basal ganglia and cortical regions [201]. From these studies, the relation of disease progression with the amount of microglia activation is not yet clear. Our aim was to investigate whether COX-2

inhibition can reduce neuroinflammation in PD, to inhibit progression of the disease. We have studied the involvement of activated microglia in PD patients and effect of COX-2 inhibition with the selective COX-2 inhibitor celecoxib, in PD patients as well as in a 6-hydroxydopamine PD rat model, using [^{11}C]-PK11195 and PET or microPET.

As described in **chapter seven**, we studied microglia activation *in vivo* in a group of de novo and a group of more advanced PD patients. Furthermore, a pilot study was done in five PD patients to investigate whether [^{11}C]-PK11195 PET provides a sensitive method for evaluation of the effect of anti-inflammatory treatment by COX-2 inhibition. As different analysis methods have been published of [^{11}C]-PK11195 PET in the patient studies, we have applied both reference tissue models and plasma input models to analyze the data. The simplified reference tissue models showed higher mean [^{11}C]-PK11195 binding potential (BP) in the contralateral putamen and in the midbrain in PD patients compared to healthy control subjects, although considerable inter-individual variance was seen and differences did not reach statistical significance. BP values were higher in the more advanced PD group compared to the *de novo* PD group, which suggests that neuroinflammation is a chronic process during progressive neurodegeneration. However, contrary to the hypothesis of this pilot study, a trend towards higher [^{11}C]-PK11195 BP after celecoxib was found, although no statistically significant difference was revealed in this small group. Exacerbation of a more chronic inflammatory reaction with COX-2 inhibition was seen in different animal inflammation models, and this concern has also been expressed in humans [235]. Thus, it will be important to determine whether COX-2 inhibition in the human situation with a chronic inflammatory process will attenuate inflammation or might have differential effects. From our study we had to conclude, however, that the current analysis methods of [^{11}C]-PK11195 uptake in brain do not provide solid results to assess changes in specific tracer uptake by anti-inflammatory treatment. The simplified reference tissue model, which gave the most consistent results, may be biased by the amount of non-specific binding. Consequently, tracers with higher levels of specific binding in the brain and better capacity to quantify PBR expression are needed and being developed.

Several tracers showed promising results in animal studies [95;96] and need to be further evaluated for use in patient studies.

Because [^{11}C]-PK11195 was the tracer that was most well known for imaging microglia activation at the time of this thesis, this tracer was also used in the 6-hydroxydopamine (6-OHDA) rat study, described in **chapter eight**. We had hypothesized that COX-2 inhibition would reduce neuroinflammation and subsequently improve expression and function of P-gp. P-gp regulation has been associated with neuroinflammatory processes. An important feature of inflammatory-related processes in PD is the marked increase in cytokine levels, including TNF-alpha, in the striatum and cerebrospinal fluid of PD patients [187]. Studies regarding the functionality of P-gp report a depressed functionality related to TNF-alpha treatment or to LPS-induced inflammation [145]. Most studies demonstrated that the function and expression P-gp in different cell lines is (reversibly) inhibited by several inflammatory cytokines [145;146]. Using striatal 6-OHDA lesions in rats followed by either celecoxib or vehicle treatment, we have measured the resulting microglia reaction in the substantia nigra (SN) by histological quantification and uptake of [^{11}C]-PK11195 using microPET. Furthermore, we used [^{11}C]-verapamil and microPET for *in vivo* measurement of P-gp function, together with histological quantification of P-gp expression. The 6-OHDA lesion caused increased microglia activity in the lesioned striatum as well as retrograde in the SN. However [^{11}C]-PK11195-microPET showed only increased tracer binding in the striatum and did not prove a sensitive measure of neuroinflammation in smaller regions of rat brain. Furthermore, we did not find evidence for a celecoxib-mediated effect on microglial activation after lesioning of the nigrostriatal projections.

An explanation for the different findings concerning anti-inflammatory and neuroprotective effects of COX-2 inhibition in several studies may be the timing of treatment. Other studies that showed mitigation of inflammation by COX-2 inhibition had started treatment at the time of lesioning [11;218], while in our study celecoxib treatment was started the day after the 6-OHDA lesion. Possibly, attenuation of microglial activation may be insufficient to modulate neurotoxicity whereas early, transient activation of microglia would be sufficient to initiate neurodegeneration. On the other hand, other studies suggest that the

effect of COX modulation may also be independent from microglia activity [197;198;217]. Teismann et al. suggested that the neuroprotective effect of COX-2 inhibition is related to the blockade of COX-2-mediated dopamine oxidation and not to decreased microglial activation [196]. In concordance with this hypothesis, we did not see decreased microglia activation by celecoxib. However, notwithstanding the (absence of) effect on neuroinflammation, celecoxib showed a direct influence on P-gp expression. We found increased P-gp expressing vessels at the side of 6-OHDA lesions. Disruption of the BBB after 6-OHDA lesion with subsequent increased expression of P-gp has been described before [219]. Our study showed that [^{11}C]-verapamil, being a substrate for P-gp efflux from the brain, was not increasingly expelled upon P-gp up-regulation. This may suggest that the up-regulation of P-gp after 6-OHDA lesion is accompanied by increased permeability of the BBB, resulting in increased rather than decreased brain penetration of [^{11}C]-verapamil, or that dysfunctional P-gp is expressed. In the celecoxib-treated animals however, our study showed down-regulation of P-gp expression after 6-OHDA lesioning, which was accompanied by unchanged [^{11}C]-verapamil accumulation compared to the non-lesioned side. Thus, COX-2 inhibition seems to prevent up-regulation of P-gp. Several other studies have associated overexpression of COX-2 with up-regulation of P-gp expression [220;221]. Furthermore, in treatment-refractory epilepsy it was demonstrated that glutamate can cause localized up-regulation of P-gp via COX-2 and that this phenomenon can be prevented with COX-2 inhibitors [222]. Thus, instead of improving P-gp function by mitigating inflammation, COX-2 inhibition may show an opposite direct effect by inhibition of P-gp up-regulation. On the other hand, increased P-gp expression after the 6-OHDA lesion could also relate to neomicroangiogenesis, which was described before in a toxic PD model [223] and alos observed in the SN in PD patients [224]. The increased number of blood vessels may be involved in compensatory, neuroprotective mechanisms by increasing the elimination of toxic compounds from the brain. However, an increase in blood vessels may also increase the transfer of putatively toxic compounds into the brain, and has, for example, been associated with the rise in nigral iron content described in PD [225]. Moreover, in another study intranigral injections of the angiogenic

factor VEGF resulted in increased blood vessel density with BBB disruption, and caused degeneration of nigral dopaminergic neurons [226]. In 6-OHDA treated neurons, low doses of VEGF displayed neuroprotective effect, while higher doses resulted in more angiogenesis and brain oedema [227]. In conclusion, it remains to be determined whether changes in vascularisation have a detrimental or beneficial effect in parkinsonian neurodegeneration. The effects of COX-2 inhibition on neovascularisation need to be further investigated in PD models.

Furthermore, it should be borne in mind that interactions between apoptotic neurons and microglia can also lead microglia to acquire an anti-inflammatory phenotype with potential neuroprotective properties. Minghetti's group has provided evidence that under chronic stimulation a progressive downregulation of glial pro-inflammatory molecule expression is seen, while the synthesis of other products with potential protective activities is stimulated [236]. Relating to COX-2 activity, it has been found that genetic deletion of the PGE2 receptor exacerbated neuronal damage [237]. Moreover, it has been demonstrated that COX-2 induction in inflammation is expressed chronically and is also observed during the resolution of inflammation and during healing of wounds [208]. In chronic inflammation, COX-2 inhibition may lead to exacerbation instead of resolution of inflammation [238]. Finally, concerning the use of celecoxib in human subjects, it should be noted that clinical trials in Alzheimer's disease that used COX-2 inhibitors were stopped because of association of these compounds with increased cardiovascular risk[239]. Although this association was not confirmed for celecoxib use, it is important to consider the potential side effects with long-term use.

When these experimental findings have to be translated to the human disease, questions that remain are whether COX-2 inhibition could be effective to diminish neurodegeneration when started in early clinical disease stages, and whether indeed undesired effects can be seen relating to inhibition of inflammatory processes in neurodegeneration. First, it needs to be investigated whether microglia activation is involved in disease progression in PD patients. In vivo PET imaging studies with [^{11}C]-PK11195 have shown increased microglia activation in PD patients; although its relation to disease progression

is still unclear [200;201]. In this thesis we found that these methods are inadequate to quantify microglia activation and measure possible effects of anti-inflammatory treatment in patients. New PET tracers for quantification of microglia activation are being developed for use in patients [94-96] and may help to further elucidate these issues in the human disease situation. On the other hand, the epidemiological studies may also point at involvement of COX-1 inhibition in the progression of neurodegeneration, since non-selective or mainly COX-1 selective analgesics were used. Since COX-1 forms an element in the early glial inflammatory response, the potential protective effects of COX-1 inhibition in neuroinflammation in PD should be further investigated. Furthermore, NSAIDs might exert neuroprotective actions through COX-unrelated mechanisms, involving inducible NO synthase (iNOS), activation of peroxisome proliferator-activated receptor (PPARγ), suppressing the formation of dopamine quinones and scavenging free radicals, that should be further investigated in experimental models.

Concluding remarks

In this thesis, we have investigated whether BBB P-glycoprotein function plays a primary causative role in the pathogenesis of PD. The results of our studies did not confirm dysfunctional P-gp in early PD, but merely suggest P-gp dysfunction to be an epiphenomenona in neurodegeneration. Secundary dysfunctional P-gp at the BBB may lead to progressive neurodegeneration through accumulation of neurotoxic substances, such as pesticides or proteins such as β-amyloid. The role of P-gp in the development of PD dementia with accumulation of β-amyloid as well as in Alzheimer's disease should be further investigated. However, the detection of subtle changes in P-gp function, particularly possible upregulation of P-gp function as a possible early compensatory mechanism in pathological conditions, is problematic because of the large capacity of the pump, resulting in low brain uptake of [^{11}C]-verapamil. Novel PET probes with higher uptake will improve quantification of *in vivo* measurements of P-gp function. Moreover, the development of tracers for P-gp

function as well as expression may allow study of the relationship between expression and function in neurodegenerative diseases [240].

Neuroinflammation in Parkinson's disease is a consequence of neuronal alterations due to various causes of the disease, and it may perpetuate the neurodegenerative process. We hypothesized that a harmful substance first induces reactive microgliosis and secretion of its pro-inflammatory factors, such as PGE2 and cytokines. These may enhance COX-2 dopaminergic neuronal activity and lead to a progressive wave of neuronal damage, inducing a further cascade of events. Our studies, however, did not confirm decreased microglia activation by COX-2 inhibition. As COX-1 is mainly expressed on microglia, early damage of an insult to the SN may be diminished mainly by COX-1 inhibition to decrease reactive microgliosis. COX-1 inhibition with NSAIDs might then be beneficial as a preventive treatment rather than as a postsymptomatic cure. COX-2 inhibition may diminish neuronal damage through microglia-independent mechanisms such as COX-2 mediated dopamine oxidation. However, the potential of exacerbation of a chronic inflammatory reaction by COX-2 inhibition in the human disease situation warrants further research, which could be aided by *in vivo* PET imaging in rodent models as well as patients. Better PET tracers are necessary for adequate quantification of microglia activation. Finally, it should be further investigated whether prevention of up-regulation of P-gp expressing vasculature may be an unwanted effect of COX-2 inhibition, while decreased P-gp function may play an important role in the clearance of neurotoxic compounds, or whether it may be beneficial to inhibit neovascularisation in PD neurodegeneration. So far, the discrepant findings relating to the effect of COX-2 inhibition preclude the set-up of clinical trials in PD and warrant further investigation of the roles of the COX isomers in neuroinflammatory and neurodegenerative processes.

Nederlandse samenvatting

De ziekte van Parkinson (ZvP) is een progressieve neurodegeneratieve aandoening die voorkomt bij ongeveer 1 % van de bevolking boven de 65 jaar. Met het stijgen van de gemiddelde leeftijd van de bevolking komen neurodegeneratieve aandoeningen, zoals de ZvP, vaker voor. Behandelingen om deze progressieve aandoening te voorkomen of te vertragen zijn nog niet gevonden. Om effectieve behandelingen te kunnen ontwikkelen, is meer kennis nodig van de pathogenese van de ZvP. Onderzoek naar het ontstaan van de ZvP heeft geleid tot veel informatie over neurodegeneratieve processen, echter de processen die leiden tot het ontstaan van de ZvP en progressie van de ziekte zijn voor een groot deel nog onbekend. Verschillende oorzakelijke factoren zijn voorgesteld, zoals exogene toxines, inflammatie, genetische mutaties en combinaties van deze factoren[107].

In dit proefschrift zijn twee mechanismen, die mogelijk betrokken zijn bij het ontstaan en de progressie van de ZvP, onderzocht met behulp van beeldvorming met positron emissie tomografie (PET). De betrokkenheid van de bloed-hersen barriere (BHB) P-glycoproteine (P-gp) transportpomp, die verschillende substanties uit het brein transporteert, bij het onstaan van de ZvP werd onderzocht met [^{11}C]-verapamil-PET. Daarnaast werd de rol van neuroinflammatie bij de progressie van de ZvP onderzocht met PET en [^{11}C]-PK11195, een tracer die bindt aan geactiveerde microglia in neuroinflammatie.

BHB P-glycoproteine functie

De klassieke pathologische kenmerken van de idiopatische ZvP zijn het verlies van dopaminerge cellen in de substantia nigra (SN) en de vorming van zogenaamde Lewy bodies; inclusielichaampjes met neergeslagen fibrillen van het eiwit α-synucleine. Naast dopaminerge zenuwcellen (neuronen) zijn ook andere catecholaminerge neuronen aangedaan. Er zijn aanwijzingen dat de pathologie in de hersenen ontstaat door invloed van omgevingsfactoren, die

kunnen binnendringen in het centrale zenuwstelsel via de neus (en het olfactore systeem in de hersenen), via neuronen in de darm en de nervus vagus [41;228], of via de BHB. Epidemiologische studies en dierexperimenten hebben aangetoond, dat omgevingsfactoren zoals verschillende toxines een rol kunnen spelen bij het verlies van neuronen bij de ZvP. Een verminderde beschermende functie van de BHB door verminderde P-gp functie zou een oorzakelijke rol kunnen spelen bij neurodegeneratie bij de ZvP door blootstelling aan toxines [6;7]. De hypothese van onze eerste studie, beschreven in **hoofdstuk 3** was een verminderde BHB P-gp functie bij Parkinsonpatienten. BHB P-gp functie werd onderzocht *in vivo* met [^{11}C]-(*R*)-verapamil-PET bij 10 Parkinsonpatiënten in vroeg ziektestadium en 8 gezonde controles van vergelijkbare leeftijd. Het distributie-volume (DV) van [^{11}C]-(*R*)-verapamil in de hersenen is een maat voor de BHB P-gp functie. Daarnaast werd genetisch onderzoek gedaan naar polymorfismen van de multidrug receptor 1 (MDR1), wat de expressie van P-gp bepaalt. Een genetisch bepaalde verminderde P-gp functie door MDR1 polymorfismen is eerder geassocieerd aan de ZvP in verband met blootstelling aan toxines zoals pesticiden [6;82]. We vonden een grotere variatie van het DV in de hersenen bij de Parkinsonpatiënten in vergelijking met de controlegroep. Echter de bevinding van verminderde P-gp functie in de middenhersenen van Parkinsonpatiënten in een eerdere pilot studie met [^{11}C]-verapamil PET [7], konden we niet bevestigen in onze studie bij Parkinsonpatiënten in vroeg ziektestadium. MDR1 polymorfismen die verminderde P-gp functie kunnen geven kwamen niet vaker voor bij de Parkinsonpatiënten, en MDR1 polymorfismen toonden geen correlatie met verschillen in *in vivo* gemeten P-gp functie. Onze conclusie was, dat verminderde BHB P-gp functie geen primaire oorzakelijke rol speelt bij het ontstaan van de idiopathische ZvP.

Een andere mogelijkheid is, dat een verminderde P-gp functie kan ontstaan als reactie op het pathologische proces. Voor de tweede studie, beschreven in **hoofdstuk 4**, onderzochten we groepen van *de novo* en meer gevorderde Parkinsonpatiënten en ook patiënten met progressieve supranucleaire parese (PSP) en multipele systeem atrofie (MSA), zogenaamde parkinsonisme-plus syndromen die een verschillend neurodegeneratief proces met snellere achteruitgang tonen. De Parkinsonpatiënten in verder gevorderd

ziektestadium en ook de patienten met PSP en MSA toonden verhoogde opname in verschillende hersengebieden. Regionaal verminderde P-gp functie treedt dus op bij verschillende neurodegeneratieve aandoeningen met parkinsonisme, en speelt waarschijnlijk een rol bij neurodegeneratieve processen in het algemeen.

De betrokkenheid van P-gp bij neurodegeneratieve ziekten houdt mogelijk verband met de ophoping van verkeerd gevouwen eiwitten bij deze aandoeningen. Neurodegeneratieve ziekten zoals de ZvP, ZvA, PSP en MSA worden ook wel "proteinopathieën" genoemd, omdat het verlies van neuronen gepaard gaat met ophoping van verkeerd gevouwen eiwitten in cellen. Bij de ZvP is het proteasoom complex betrokken bij de verwerking van het eiwit α-synucleine. P-gp vertoont interactie met dit proteasoom complex, en kan dus een rol spelen bij het transport van eiwitten bij de verwerking door het proteasoom complex [138]. Verminderde P-gp functie kan dus leiden tot een verminderd vermogen tot afvoer van overtollige eiwitten uit het brein. Een verminderde P-gp functie om amyloid-eiwitten af te voeren uit het brein werd ook gevonden bij een verder gevorderd stadium van de ziekte van Alzheimer (ZvA) [109]. Verminderde P-gp functie zou ook een rol kunnen spelen bij het vaker voorkomen van dementie met Alzheimer kenmerken bij patiënten met de ZvP [140]dan bij leeftijdgelijke controles. Deze hypothese zou verder onderzocht kunnen worden met behulp van in vivo studies van P-gp functie bij patiënten met de ZvA en patiënten met de ZvP en dementie.

P-gp is ook geassocieerd met het transport van verschillende aan Parkinson gerelateerde toxines, zoals organofosfaat pesticiden [229] en paraquat [231]. Verminderde P-gp functie in de BHB bij een neurodegeneratief proces zou dus ook kunnen leiden tot verdergaande neuronale schade door ophoping van toxines.

Tenslotte hebben we veranderingen in BHB P-gp functie door leeftijd onderzocht in **hoofdstuk 5**. Hogere leeftijd is de voornaamste risicofactor voor het ontwikkelen van een neurodegeneratieve ziekte. Deze studie toonde een vermindering van P-gp functie aan in verschillende hersengebieden met oplopende leeftijd, met name in frontale gebieden en in witte stof gebieden. Een verminderde P-gp functie in deze gebieden kan bovendien een rol spelen

bij de verhoogde kwetsbaarheid van deze hersengebieden voor degeneratie bij hogere leeftijd.

Uiteindelijk bestaan er verschillende pathologische mechanismen vanuit omgevingsfactoren, genetische factoren en leeftijd en die gezamenlijk verband kunnen houden met P-gp functie, leidend tot verhoogde ophoping van neurotoxische stoffen en neurodegeneratie bij de ZvP. Vervolgonderzoek is nodig voor verder inzicht in de relatie tussen P-gp functie en eiwitophoping bij de ZvP en de ZvA en de rol hiervan bij progressie van neurodegeneratieve ziekte en dementie.

Neuroinflammatie en COX-2

Neurodegeneratie wordt beschouwd als het resultaat van multipele pathogene factoren, waar verschillende cellen bij betrokken zijn. De laatste jaren is er veel aandacht voor het samenspel van steuncellen (glia) en neuronen bij het onstaan van verschillende hersenaandoeningen. Verschillende neurodegeneratieve ziekten, zoals de ZvP en de ZvA, worden geassocieerd met neuroinflammatoire processen met geactiveerde microglia, verhoogde expressie van cyclooxygenase (COX) en verhoogde productie van prostaglandines (PGs) [143;234]. Daarnaast suggereren enkele epidemiologische studies dat anti-inflammatoire medicijnen van de groep 'non-steroidal anti-inflammatory drugs' (NSAIDs), die remmend werken op COX, een beschermend effect hebben op het ontstaan van de ZvP [9;10]. De rationale voor onderzoek naar de rol van neuroinflammatie en behandeling met selectieve COX-2 remming bij de ZvP is beschreven in een review artikel in **hoofdstuk 6**. Bij de eerste studies naar microglia activatie bij Parkinsonpatiënten werd met behulp van [^{11}C]-PK11195-PET verhoogde traceropname bij microglia activatie gezien in verschillende hersengebieden [200;201]. De relatie tussen de hoeveelheid geactiveerde microglia en de ziekteprogressie is bij deze studies niet duidelijk. Voor dit proefschrift hebben we microglia activatie *in vivo* bestudeerd met [^{11}C]-PK11195-PET bij de novo en meer gevorderde Parkinsonpatiënten (**hoofdstuk 7**). Daarbij hebben we ook een pilot studie gedaan bij 5 patiënten, om te kijken of [^{11}C]-PK11195-PET een

geschikte methode vormt om het effect van anti-inflammatoire medicatie met celecoxib, een selectieve COX-2 remmer, te meten bij Parkinsonpatiënten. We hebben verschillende scan analyse methoden toegepast. Er was een trend van hogere opname in het aangedane putamen, met name bij de gevorderde Parkinsonpatiënten. In tegenstelling tot onze hypothese werd na een maand behandeling met celecoxib een trend richting hogere [^{11}C]-PK11195 opname gezien. De mogelijkheid van exacerbatie van een ontstekingsreactie door COX-2 remming is beschreven in enkele dierexperimentele inflammatiemodellen, en de zorg om deze andere werking werd ook bij ontstekingen bij mensen beschreven [235]. Het is dus van belang om te onderzoeken of COX-2 remming bij mensen met een inflammatoir proces in het kader van een neurodegeneratieve aandoening de inflammatie verminderd, of andere effecten heeft. Uit onze studie met [^{11}C]-PK11195-PET hebben we echter moeten concluderen, dat deze meetmethode onvoldoende solide gegevens biedt voor het kwantificeren van microglia activatie en voor evaluatie van effect van anti-inflammatoire medicatie. Recent zijn verschillende tracers voor verbeterde beeldvorming van geactiveerde microglia onderzocht in diermodellen [94-96]; deze zullen ook verder onderzocht moeten worden voor onderzoek bij patiënten.

We hebben ook naar neuroinflammatie en BHB P-gp functie gekeken in een Parkinson diermodel met het toxine 6-hydroxydopamine (6-OHDA) in Wistar ratten, beschreven in **hoofdstuk 8**. Bij dit model geeft injectie met 6-OHDA in het striatum degeneratie van dopaminerge neuronen in het striatum en de substantia nigra (SN). Met behulp van een microPET scanner en de tracers [^{11}C]-PK11195 en [^{11}C]-verapamil hebben we het effect van de toxische lesie op microglia activatie en BHB P-gp functie onderzocht. De microPET resultaten werden vergeleken met weefselonderzoek met kleuringen van microglia en P-gp expressie. Daarnaast kreeg een groep ratten 3 weken behandeling met celecoxib. De hypothese was, dat COX-2 remming met celecoxib de microglia activatie door de toxische lesie zou remmen en hierdoor ook de P-gp functie kon verbeteren. Regulatie van P-gp functie wordt in de literatuur geassocieerd met neuroinflammatoire processen. [^{11}C]-PK11195-microPET beeldvorming toonde alleen verhoogde traceropname in het striatum en bleek niet sensitief genoeg voor het kwantificeren van microglia

activatie in kleinere regio's, zoals de SN bij ratten. Celecoxib gaf geen vermindering van microglia activatie, maar toonde daarentegen een vermindering van bloedvaten met P-gp expressie. Een neuroprotectief effect werd niet gezien. Enkele andere studies hebben ook een relatie gevonden tussen COX-2 remming en verminderde P-gp expressie en functie [220-222]. In tegenstelling tot verbeterde P-gp functie via vermindering van microglia activatie kan COX-2 remming dus leiden tot verminderde P-gp functie. Dit kan een ongewild bij-effect van COX-2 remmers zijn voor gebruik bij neurodegeneratieve ziekten, waarbij P-gp functie mogelijk een belangrijke rol speelt bij de afvoer van eiwitten en andere neurotoxische stoffen. Aan de andere kant kan het gunstig zijn om de nieuwvorming van bloedvaatjes bij Parkinson tegen te gaan [225].

Een mogelijke verklaring voor de verschillende bevindingen ten aanzien van het anti-inflammatoire en neuroprotectieve effect van COX-2 remming kan liggen in de 'timing'van de behandeling. Andere studies die verminderde inflammatie en neurodegeneratie toonden, hadden de behandeling met celecoxib al gestart voor het aanbrengen van de toxische lesie [11;218], terwijl wij de behandeling een dag na de lesie startten om het effect op de progressie te meten, wat meer lijkt op de situatie bij Parkinson patiënten. Aan de andere kant zijn er ook studies die aangeven dat modulatie van COX effecten heeft die onafhankelijk zijn van microglia activatie [196-198;217], bijvoorbeeld gerelateerd aan remming van COX-2 gemedieerde dopamine oxidatie [196].

Bij het vertalen van de verschillende experimentele bevindingen ten aanzien van effecten van anti-inflammatoire middelen bij neurodegeneratie bij de ZvP blijven vooralsnog enkele belangrijke vragen onbeantwoord. De rol van microglia activatie bij de voortgang van de ZvP is nog onduidelijk. Ook is nog onduidelijk of COX-2 remming bij de mens het neurodegeneratieve proces kan verminderen via andere wegen dan microglia activatie. Dit heeft ook implicaties ten aanzien van de screening van mogelijk neuroprotectieve middelen bij de ZvP. Radiotracer methoden die inzicht kunnen geven in effecten van behandeling in vivo bij zowel diermodellen als bij patiënten kunnen een belangrijke rol spelen bij de vertaling van experimentele bevindingen naar de menselijke ziektesituatie. In dit proefschrift hebben we gevonden dat [11C]-

PK11195-PET met de huidige analysemethoden onvoldoende betrouwbare data voor kwantificatie van microglia activatie en evaluatie van effect van anti-inflammatoire behandeling geeft. Nieuwe PET tracers die in ontwikkeling zijn zouden kunnen bijdragen aan een beter inzicht in de relatie tussen neuroinflammatie en neurodegeneratieve processen. Daarbij is het ook van belang de effecten van COX-1 remming, wat vooral betrokken is bij de vroege reactie van glia bij inflammatie, verder te onderzoeken, alsook de niet-COX-gerelateerde effecten van NSAID's die potentieel neuroprotectief zijn. De rol van het remmende effect van celecoxib op nieuwvorming van bloedvaatjes met P-gp expressie moet nog verder worden onderzocht.

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Publications

Bartels AL, de Klerk OL, Kortekaar R, Leenders KL: [11C]-verapamil to assess P-gp function in human brain during aging, depression and neurodegenerative disease. Submitted to Current Topics in Medicinal Chemistry; Special Issue: Imaging of ABC transporter function and expression.

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Congress presentations

Poster presentation: Blood-brain barrier P-glycoprotein function and neuroinflammation after striatal 6-hydroxydopamine lesion and COX-2 inhibition. Vascular Dementia 2009 Congress, Barcelona, Spain.

Poster presentation: Blood-brain barrier p-glycoprotein function decreases in specific brain regions with aging: a possible role in progressive neurodegeneration. Neuro Receptor Mapping congress 2008, Pittsburgh, USA.

Poster presentation: [^{11}C]-PK11195 PET: a monitor of anti-inflammatory treatment in Parkinson's disease? Neuro Receptor Mapping congress 2008, Pittsburgh, USA.

Poster presentation: Blood-brain barrier P-glycoprotein function: a pathogenetic mechanism in Parkinson's disease? The Movement Disorder Society's 10th International Congress of Parkinson's Disease and Movement Disorders 2006, Kyoto, Japan.

Poster presentation: Striatal dopa and cerebral glucose metabolism in PD patients with Freezing of Gait. International congress of Gait and Mental function 2006, Madrid, Spain.

Oral presentation: Blood-brain barrier dysfunction in Parkinson's disease: a causative mechanism? CN 2005 Meeting, Torquay, UK.