Neuroprotection in Parkinson's disease: modafinil and Δ^9-tetrahydrocannabinol

The research reported in this thesis was financed by and carried out at
TNO Defence, Security and Safety,
Business Unit Biological and Chemical protection

Cover design	Martijn Pieck, Utrecht University
Printing	Gildeprint bv.
ISBN	978 903 934 585 6

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Neuroprotectie bij de ziekte van Parkinson:
modafinil en Δ^9-tetrahydrocannabinol

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op donderdag 12 juli 2007 des middags te 2.30 uur

door

Sarah Anna Maria van Vliet

geboren op 15 mei 1979 te Pijnacker

Promotor: **Prof. dr. B. Olivier**

Co-promotor: **Dr. I.H.C.H.M. Philippens**

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van:

J.E. Jurriaanse stichting

Harlan Netherlands bv.

Parkinson Patiënten Vereniging

PsychoGenics Inc., USA

Van Leersum fonds

Mystery creates wonder and wonder is the basis of man's desire to understand.
Neil Armstrong

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Chapter 1

General introduction

Parkinson's disease

Background

The first description of the clinical features of Parkinson's disease (PD) appeared in James Parkinson's 'Essay on the Shaking palsy' in 1817. A century later the central pathology of PD, the loss of neurons in the substantia nigra pars compacta (SNpc) and the presence of intraneuronal cytoplasmic inclusions, named Lewy bodies, was discovered. The three classical major signs of PD are rest tremor, rigidity and akinesia, which is expressed as absence of normal unconscious movements, such as arm swing in walking. Other symptoms are bradykinesia, which is slowness of movement, hypokinesia, which is reduction in movement amplitude, paucity of normal facial expression, stooped posture, reduced normal postural reflexes and the inability to begin a voluntary movement such as walking. Besides these motor-related behavior disabilities, impaired cognition and psychiatric disorders, like depression and dementia are also significantly more frequent among PD patients (Dauer and Przedborski 2003). Most patients suffer from considerable motor disabilities 5-10 years after diagnosis of the disease, despite treatment with the available symptom suppressing medications.

PD has a mean onset age of 55, and the incidence increases markedly with increase of age from 20/100,000 overall to 120/100,000 at an age of 70. Age is the most consistent risk factor and, with the increasing age of the general population, the prevalence of PD will rise steadily in the future. Expectations are that the worldwide population of 4.1 million people will grow to 8.7 million people in 2030. The disease occurs throughout the world, in all ethnic groups and affects males slightly more than females (Morgante et al. 2000). The mortality rate is 2-5 times higher in the Parkinson patients than among age-matched controls (Louis et al. 1997).

Neuropathology

The pathological hallmarks of PD are the loss of nigrostriatal dopaminergic (DAergic) neurons and presence of intraneuronal protein inclusions named Lewy bodies. PD-associated loss of DAergic neurons has a characteristic topology, distinct from the pattern seen in normal aging (Fearnley and Lees 1991). Pathologic studies suggest that DAergic neuronal death in the SNpc has to exceed a critical threshold of 50-60% reduction before the cardinal parkinsonian signs occur (Jellinger 2001) and is preceded by a presymptomatic window of about 6 years (Fearnley and Lees 1991).
In contrast to what is often thought, the pathology is not restricted to the SNpc and the DAergic system as, in a lesser extent, noradrenergic, cholinergic and serotonergic systems are also affected (reviewed by Hornykiewicz and Kish 1987).

Lewy bodies are also found throughout the brain. Recently, it has been shown that distribution of these morphological abnormalities starts in the lower part of the brain stem and in time it topographically spreads throughout the brain (Braak et al. 2003). These observations are confirmed by reports about non-motor symptoms like depression and reduced olfaction before occurrence of the cardinal PD symptoms

(Tandberg et al. 1996, Harding et al. 2002).

Nevertheless, the SNpc is more vulnerable for neuronal death in PD compared to other brain areas. Suggestions regarding the cause of this vulnerability are that these neurons have a higher sensitivity for endogenous and extrinsic toxins, increased metabolic stress, higher physiologic rates of protein oxidation, selective generation of potential toxins or failure to detoxify or dispose them (Lang and Lozano 1998a).

Basal ganglia

The basal ganglia are involved in control, planning and execution of motor behavior. The striatum together with globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra (SN) form the basal ganglia. The striatum, consisting of putamen and caudate nucleus, is the most important input nucleus of the basal ganglia. It receives, besides DAergic input from the SNpc, glutamatergic projections from motoric and sensoric cortical areas, limbic structures and thalamus. The most important output nuclei of the basal ganglia are the globus pallidus interna (GPi) and the substantia nigra pars reticulata (SNpr), which tonically inhibit the thalamus. Two parallel γ-aminobutyric acid (GABA)-ergic pathways run from the striatum to the output nuclei, i.e. the direct and indirect pathway (Fig. 1, Wichmann and DeLong 1998, Blandini et al. 2000). The indirect pathway first runs to the external layer of the GP and from there has a GABAergic projection to the STN and from this nucleus to the GPi and SNpr with glutamatergic efferents. The direct pathway runs directly to the GPi/SNpr. From the GPi/SNpr there are glutamatergic projections to the thalamus. Activation of the direct pathway results in disinhibition of the thalamic tone and facilitates movements, whereas activation of the indirect pathway inhibits the output nuclei and therefore movement. The striatal neurons projecting to the nuclei involved in the direct pathway express dopamine D_1 receptors, whereas the striatal neurons projecting to the indirect pathway express dopamine D_2 receptors. Therefore, stimulation of dopamine D_1 receptors results in activation of the direct pathway and stimulation of dopamine D_2 receptors in inhibition of the indirect pathway.

Reduction of the DAergic neurons in the SNpc in PD results in reduced DAergic striatal input. This causes overactivity of the indirect pathway, resulting in excessive glutamatergic drive to the GPi/SNpr and causes reduced activity of the inhibitory GABAergic direct pathway, together resulting in an increased activity of the GPi/SNpr. The increased GABA release by the GPi/SNpr results in excessive inhibition of the thalamus and brain stem nuclei. The excessive thalamic inhibition leads to suppression of the cortical motor system, possibly resulting in akinesia, rigidity and tremor, whereas the inhibitory descending projection to brain stem locomotor areas may contribute to gait and posture abnormalities (Wichmann and DeLong 1998, Blandini et al. 2000). The model of the basal ganglia is a simplification as more connections between the nuclei exist, but this working theory allowed generation of hypotheses about the PD pathology and the response to therapy.

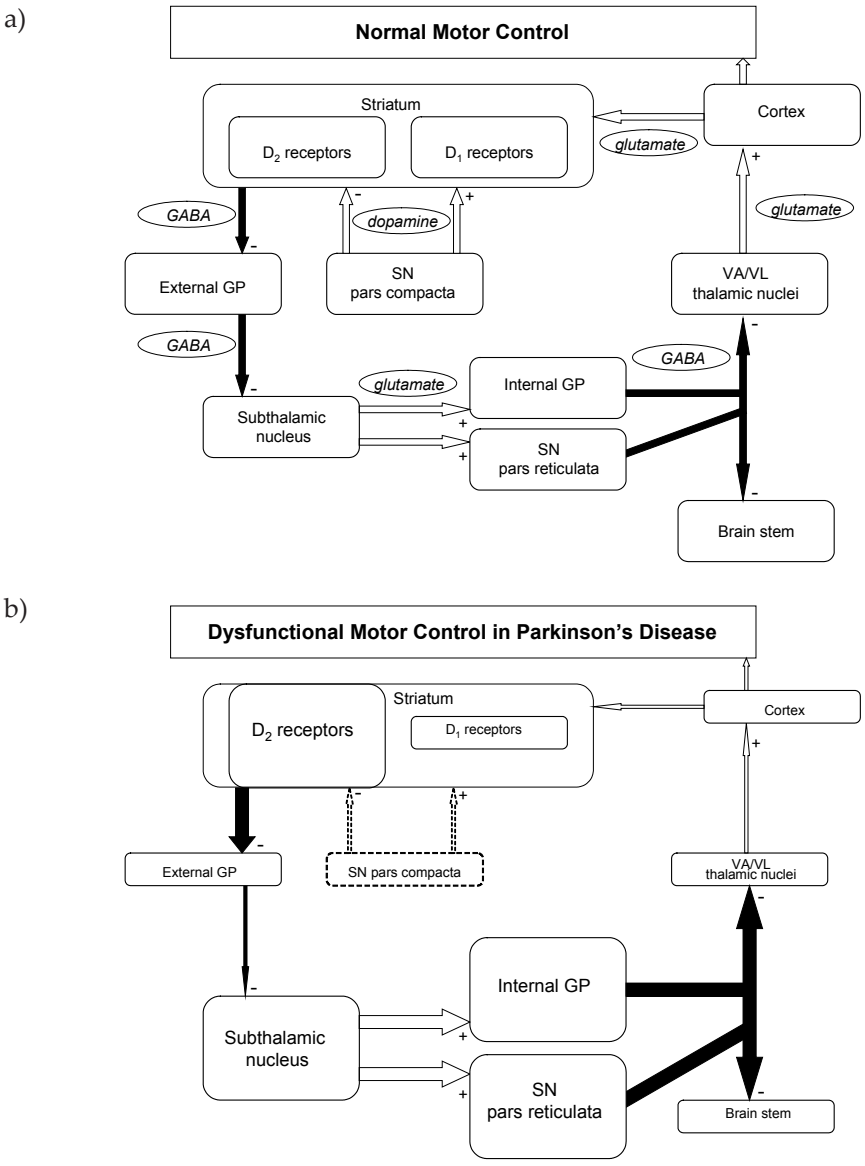


Fig. 1 Functional model of the basal ganglia in persons with a) normal motor control and b) patients with Parkinson's disease. For the purposes of clarity, the neuroanatomy and interconnections shown are incomplete. White arrows indicate excitatory pathways and black arrows inhibitory pathways. The width of the arrows in panel b indicates the degree of overall functional change in the activity of each pathway (changes in neuronal firing rates) as compared with the normal state (panel a), and the size and outlining of each box indicate the activity of the brain region as compared with the normal level of activity (panel a). Dashed lines and arrows indicate the dysfunctional nigrostriatal dopamine system in Parkinson's disease. GP: globus pallidus; VA/VL: ventral anterior and ventrolateral. Plus signs indicate excitation, and minus signs inhibition. (Adapted from Lang and Lozano 1998b)

Etiology of PD

In most cases, the cause of PD is unknown. Presumably, both environmental and genetic factors play a role. Therefore, it seems to be a complex interplay between these factors. In 95% of PD cases there is no genetic linkage. These types of PD are named idiopathic PD (Gasser 2005). The environmental factor theory posits that PD results from exposure to neurotoxins. For example, rural areas have been associated with PD as relations were found with the use of herbicides, like paraquat, and pesticides, like rotenone or well water and development of the disease. However, data are inconsistent and limited to a small group within the PD population (Semchuck et al. 1992, Gorell et al. 1996). Another example is the finding that people exposed to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) developed a syndrome similar to PD (Langston et al. 1983).

The 5% of PD cases with a genetic linkage are mostly of the autosomal dominant or recessive inheritance pattern. These people often develop young onset PD, which starts before the age of 40. Single gene mutations are now identified at 10 loci and six genes, among others SNCA (α-synuclein), PINK1, DJ-1, LRRK2 coding for proteins involved in protein phosphorylation, protein misfolding, oxidative stress, mitochondrial dysfunction and impairment of the ubiquitin proteasome system, processes that are closely interlinked (reviewed by Wood-Kaczmar et al. 2006).

Cell death processes

The mechanisms responsible for cell death in PD are largely unknown. There are two theories, which could mutually exist and do not have to exclude each other. The first theory suggests that mitochondrial dysfunction, oxidative stress, excitotoxicity, neurotrophic support deficiency and immune mechanism result in degeneration of the neurons. The second one suggests that the degeneration is caused by protein mishandling and aggregation (Fig. 2, Dauer and Przedborski 2003). It appears that the extent to which each of these factors contribute to the neuronal degeneration varies in the individual patient (Schapira 2006).

Mitochondrial dysfunction In PD patients there is a 30-40% decrease of complex I activity of the mitochondrial electron transport chain in the SNpc, which could contribute to energy failure of the cell and predispose it to other toxic or genetic insults or increase its susceptibility to apoptosis (Mann et al. 1992). The role of mitochondria in PD are confirmed by the identification of genes encoding for mitochondrial proteins, e.g. PINK1 and DJ-1 and the environmental toxins MPTP and rotenone, which inhibit complex I of the mitochondrial electron transport chain (see animal models section).

Oxidative stress In PD brains markers of free radical damage are elevated and levels of glutathione, an anti-oxidant enzyme protective against hydrogen peroxide, are decreased (Riederer et al. 1989, Alam et al. 1997). Under normal circumstances there is a balance between production and removal of powerful oxidants formed as by-products of mitochondrial respiration. Reaction of these molecules with DNA, proteins and lipids alters their structure and results in cellular damage. Furthermore, the metabolism of DA is involved in the generation of superoxides and auto-

oxidation of DA produces DA-quinone, a protein damaging molecule (Drukarch and Van Muiswinkel 2000).

Excitotoxicity Excitotoxicity occurs after persistent activation of the N-methyl-D-aspartic acid (NMDA) receptor resulting in increased intracellular levels of Ca^{2+} ions. This leads to activation of proteases, endonucleases and nitric oxide synthase, which result in generation of oxidative stress (Beal 1998). There is no direct evidence of excitotoxicity in PD, but the preservation of DAergic neurons with the calcium-binding protein calbidin in PD supports this theory (Hirsch et al. 1992). Furthermore, the increased glutamatergic drive from the STN due to the disturbed balance in the basal ganglia in PD is also suggested to be a source of excitotoxicity (Rodriquez et al. 1998).

Complexity and interlinking The three mechanisms leading to cell death (mitochondrial dysfunction, excitotoxicity and oxidative stress), described above, can act separately or cooperatively to cause neurodegeneration. It is almost impossible to unravel the strands since each process appears to be intimately connected to the others. For example, metabolic injuries result in loss of mitochondrial function leading to depletion of ATP, loss of intracellular calcium buffering capacity causing excitotoxicity and an increase in damaging oxygen and nitrogen radicals causing oxidative stress (Alexi et al. 2000).

Neurotrophic factors Neurotrophic factors such as glial-derived neurotrophic factor (GDNF) have an important role in neuronal survival and differentiation during development and after injury. Inadequate levels of neurotrophic support

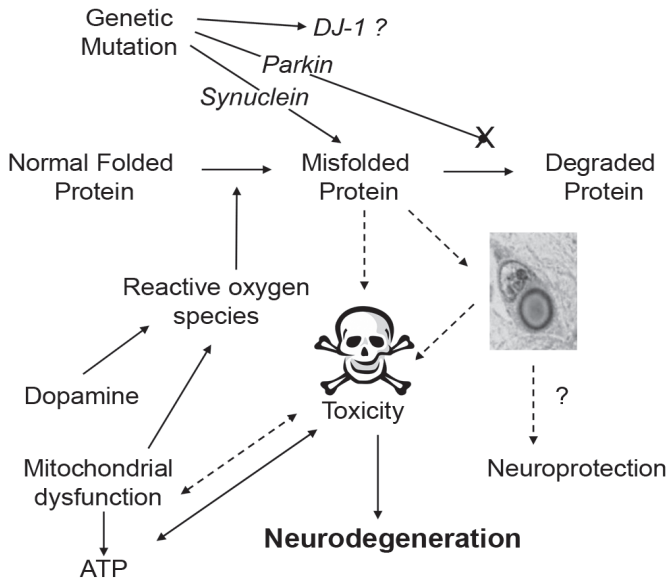


Fig. 2 Mechanism of neurodegeneration in Parkinson's disease. (Adapted from Dauer and Przedborski 2003)

lead to apoptotic neuronal death (Lang and Lozano 1998a).

Immune factors Immune factors, probably secondarily, may contribute to progressive nigral cell loss based on increased levels of microglial activation and expression of proinflammatory cytokines (McGeer et al. 1988, Boka et al. 1994, Hunot et al. 1999).

Protein mishandling and aggregation The evidence pointing to proteasomal dysfunction in PD comes from direct and indirect analyses of proteasomal enzymatic activity, levels of its subunits and expression of its activators in the brains of patients with the disorder (Olanow and McNaught 2006). The ubiquitin-proteasome system has a critical role in the maintenance of cellular function and viability by clearance of misfolded proteins. The cause of proteasomal dysfunction is presently unknown. Possible causes are gene-related encoding changes, oxidative damage, ATP depletion and the direct and indirect actions of toxins (Olanow and McNaught 2006). There is still a debate whether the protein aggregation is neurotoxic or a result of neuroprotective actions of the cell (Dauer and Przedborski 2003).

Therapy

Therapy for PD can be subdivided in three categories: symptomatic therapy, protective or preventive therapy and restorative or regenerative therapy.

Symptomatic therapy

Dopaminergic treatment Levodopa, a DA precursor, is the most commonly used drug for the motor symptoms of PD. However, major disadvantages are reduction of efficacy within 5-7 years due to developing pathology and the development of motor complications like 'off' periods (greater severity of parkinsonian symptoms when medication is wearing off) and dyskinesias (uncontrollable choreic movements at the peak of levodopa serum levels) (Jankovic 2005). The mechanisms by which these effects develop are not completely understood but include pulsatile stimulation of DA receptors by short-acting agents such as levodopa and the degree of striatal deinnervation (Obeso et al. 2000). Co-administration of catechol-O-methyltransferase (COMT) inhibitors, entacapone or tolcapone, enhance bioavailability of levodopa by prevention of the breakdown of DA (Shapira 2005).

DA agonists like bromocriptine, pramipexole and ropinirole have also significant benefits for patients over time and are without induction of motor complications. However, other side-effects as sleepiness, cognitive disturbances and leg edema are induced and at some point in the disease levodopa supplementation is needed (Foley et al. 2004). The DA agonists, cabergoline and apomorphine, are used as adjunct to levodopa therapy to reduce the motor fluctuation by their more continuous DAergic stimulation (Goetz et al. 2005). Monoamineoxidase (MAO)-B inhibitors, which prevent breakdown of DA, such as selegiline and rasagiline are also prescribed as they improve motor function in early and advanced PD (Morgan and Sethi 2006).

Non-dopaminergic treatment Anti-cholinergics and amantadine were used before the introduction of levodopa. Nowadays, it is mainly used for patients with tremor dominant PD, although their benefit compared to dopaminergics is disputable (Goetz et al. 2005). Novel non-DAergic targets as adenosine A_{2a} receptor antagonists (modulation of GABA release) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA) receptor antagonists (modulation of excessive glutamate release) are currently under investigation in a clinical phase II study (Wu and Frucht 2005). The non-motor symptoms of PD, like depression, dementia and sleep disturbances, are mostly treated with the existing medication for these symptoms (Morgan and Sethi 2006).

Surgical treatment The proposition of the basal ganglia model resulted in development of current surgeries as thalamotomy, pallidotomy and subthalamotomy. The data about the efficacy is still insufficient, but pallidotomy is most beneficial and results in significant improvement in contralateral tremor, rigidity, bradykinesia, and dyskinesias. However, long-term efficacy is more variable (Walter and Vitek 2004).

Deep brain stimulation The recently developed deep brain stimulation method is more routinely used. The stimulation of the GPi or STN results in improvement of the cardinal PD, whereas the latter is most effective (Siegfried and Lippitz 1994, Goetz et al. 2005). Complications directly related to stimulation are slight and are generally reduced or eliminated with adjustment of the stimulation variables (Breit et al. 2004).

Neuroprotective therapy

Symptomatic therapies are not able to prevent progression of the disease. Therefore, strategies to counteract neurotoxic processes and/or improvement of function of affected neurons seem more favorable. Current neuroprotective strategies are based on evidence concerning cell death progression in the pathogenesis of PD. Therefore neuroprotection in PD may be achieved through anti-oxidant effects, mitochondrial stabilization, glutamate antagonism, anti-apoptosis, trophic factors and/or anti-inflammatory effects (Mandel et al. 2003).

Recently, potential candidates for further clinical trials were selected from 59 suggested neuroprotective compounds by the National Institute of Neurological Disorders and Stroke (NINDS) (Ravina et al. 2003). The candidates were the bioenergetic compounds, coenzyme Q_{10} and creatine to improve mitochondrial function, the DA receptor agonists, pramipexole and ropinirole for their anti-oxidant mechanisms, MAO-B inhibitors selegiline and rasagiline for their actions via anti-oxidant and anti-apoptotic mechanisms, the trophic factors GPI 1485 and GM-1 ganglioside, the anti-inflammatory compound minocycline and based on their epidemiological efficacy: caffeine, nicotine and 17β-oestradiol.

Few randomized clinical trials have been performed with some of the suggested compounds. Symptomatic use of selegiline was reported to reduce mortality in PD. A neuroprotective trial showed that there was a 9-month delay in the need of levodopa, but after the 10-year follow-up there was no difference in mortality indicating that the positive results were caused by symptomatic effects of

the drug (Koller et al. 1993, Olanow et al. 1995). In a small neuroprotective clinical trial with coenzyme Q_{10} there was a trend towards a positive correlation between the dose and reduction in PD symptoms. This trial has been extended to a larger trial, which is still going on (Shults et al. 2002). The anti-oxidant vitamin E failed to produce positive outcome (Koller et al. 1993).

Neurorestorative therapies

GDNF and its homologue neurturin are neurotrophic factors and neuroprotective when administered intraventricularly or into the nigrostriatal system in primate models of PD. Open label clinical studies showed positive results, but double-blind studies could not confirm the observations (Sherer et al. 2006). The raised safety issues such as neutralizing antibodies and cerebellar damage have changed the focus towards development of GDNF delivery via gene therapy (Björklund et al. 2000, Kordower et al. 2000).

Neural transplantation of embryonic nigral tissue has been reported to restore striatal DAergic neurotransmission and symptomatic relief in patients, although the response is highly variable with, on the negative side, severe off-phase dyskinesias. Suitable donor material is limited and therefore studies are focused towards application of stem cells. Hurdles in this area of research are the limited knowledge about proliferation and differentiation of adult stem cells and the ethical debate about the use of embryonic stem cells (Björklund et al. 2003, Roybon et al. 2004).

In the treatment of PD, a clinically working compound that could prohibit progression of the disease has not yet been found despite extensive investigations in animal models. Therefore, the search for an effective neuroprotective compound continues. Ideally, a neuroprotective compound should have multiple mechanisms of action as they can act on different processes involved in the cell death processes. Literature indicated that modafinil and Δ⁹-tetrahydrocannabinol (Δ⁹-THC) have potential to protect neurons against degeneration and were suggested to act via multiple mechanisms on cell death processes. Therefore, these two compounds are subject of research in this thesis. Another question of interest was whether the potential neuroprotective compounds also have value in symptomatic treatment or were at least not acting pro-parkinsonian. Further on in this introduction the background of modafinil and Δ⁹-THC is described as well as their symptom control and neuroprotective properties.

For practical and ethical reasons the effects of the compounds had to be tested in an experimental PD model. In this thesis the MPTP model is used. An introduction in this MPTP model in the mouse and marmoset monkey as well as comparison with other PD models and its value in neuroprotection research are described below.

Animal models of PD

Parkinsonism can be induced in a number of species ranging from fruit flies to monkeys. Most of these animal models are based on the concept that parkinsonian signs are related to DAergic nigral cell loss. However, none mimics the complete range of the complex chronic neurodegenerative features of human PD (Emborg 2004).

MPTP

MPTP was discovered to be a DAergic neurotoxin in the early 1980s. Several young Californian drug users developed parkinsonian symptoms after use of synthetic heroin injections contaminated with the substance (Langston et al. 1983). MPTP is able to selectively damage DAergic neurons, mostly in the SNpc, which leads to impaired DAergic neurotransmission. The compound is highly lipophilic and after systemic administration rapidly crosses the blood brain barrier. Subsequently MPTP is converted into 1-methyl-2,3-dihydropyridinium ($MPDP^+$) in non-DAergic cells (e.g. astrocytes) by MAO-B and spontaneously oxidizes into 1-methyl-4-phenylpyridinium (MPP^+). Thereafter MPP^+ is released in the extracellular space by unknown mechanisms. MPP^+ enters the DAergic neurons via the DA transporter (DAT). Inside the neuron MPP^+ can be stored by the vesicular monoamine transporter (Liu et al. 1992) or accumulates in the mitochondria. In the mitochondria MPP^+ impairs mitochondrial respiration by inhibiting complex I of the electron transport chain leading to energy failure and cell death (reviewed by Smeyne and Jackson-Lewis 2005).

MPTP represents the most important and most frequently used neurotoxin in animal models (Przedborski et al. 2001). It has advantages over other models as it causes specific intoxication of DAergic structures and induces symptoms identical to PD in humans. MPTP is mainly used in mice and non-human primates. Rats are resistant to systemic MPTP due to the low concentrations of MAO-B (Schmidt and Ferger 2001). The C57BL/6 mouse strain is sensitive after systemic administration, but high doses are needed. The mouse MPTP model is useful in studies on neuropathological and neurochemical changes (Schmidt and Ferger 2001), whereas the non-human primate MPTP model seems to be more suitable for behavioral tests. MPTP treated non-human primates express behavioral disturbances comparable to humans, and mice behavior tends to be reversed within a couple of days.

Commonly used regimes of MPTP administration in non-human primates are multiple systemic injections or intracarotid administration. With these regimes a 95% loss of tyrosine hydroxylase (TH) mRNA in the SNpc is found, representing the end stage of PD. TH is the first and rate-limiting enzyme in the synthesis of catecholamines. An alternative dosing regime is administration of low doses of MPTP for many weeks or month. This approach mimics chronic degeneration and mirrors human PD pathological more appropriately. A disadvantage of the chronic regime is the substantial spontaneous behavioral recovery after cessation of the toxin delivery (Emborg 2004).

The MPTP models do not include one of the important characteristic features of PD: the Lewy bodies, although sometimes inclusions resembling Lewy bodies have been described in non-human primates (Forno et al. 1986, Purisai et al. 2005). A newly developed combination of α-synuclein viral vectors inducing overexpression of the protein in the MPTP model or continuous infusion of MPTP can cover this lacuna (Kirik et al. 2003, Fornai et al. 2005). Other shortcomings of the MPTP models include the lack of the progressive nature of the symptomatology, the lack of psychological disturbances and other non-motor symptoms (Gerlach et al. 1991).

Other PD models

6-hydroxydopamine (6-OHDA) was the first neurotoxin discovered to induce selective cathecholaminergic cell death (Senoh et al. 1959). 6-OHDA is unable to cross the blood brain barrier and must be administered via injections into the medial forebrain bundle, the SNpc or striatum. An advantage in the use of unilateral injection of 6-OHDA is that the other hemisphere serves as control. Disadvantages are that it is time consuming, that other neurotransmitter systems are affected (Annett et al. 1992) and that there is absence of development of levodopa-induced dyskinesias.

Agricultural chemicals, such as rotenone, maneb and paraquat if administered systematically also induce specific features of PD (Dauer and Przedborski 2003). Disadvantages of these models are the variability in induction efficacy and lack of specificity, although the models are of interest as α-synuclein-positive inclusions were found. The development of gene-based PD models and transgenic or gene-deficient mice or flies also contribute to the research on protein dysfunction (Dauer and Przedborski 2003).

MPTP model and neuroprotection

The MPTP model seems to be a good model to test neuroprotective effects as it induces a replicable nigral lesion, stable DAergic cell loss over time and there is a window of opportunity allowing the neuroprotective compound to work (Emborg 2004). The use of the non-human primate MPTP model is most preferable as the anatomy and parkinsonian behavior of non-human primates are more comparable to the human situation. The non-human primate and human striatum comprises discrete structures separated by the internal capsule, whereas in rodents it is a single structure. Furthermore, comparability in striatal function and distribution of DA neurons in the SNpc are in favor of the non-human primates (Hardman et al. 2002). Marmoset monkeys *(Callithrix jacchus)* are most often used for the MPTP model as they are small in size, therefore better to handle and require less space and food than rhesus monkeys.

Furthermore, a good neuroprotective compound should be able to prevent the behavioral impairments, neurochemical deficits and pathologic degeneration.

Behavioral impairments Parkinsonian behavior of MPTP non-human primates is comparable to human PD symptoms (Przedborski et al. 2001). Clinical assessment of PD symptoms is mostly done with the UPDRS (unified Parkinson's disease rating scale). Adapted rating scales are developed for MPTP non-human primates. For

instance, the 'clinical score', a rating scale described in this thesis (e.g. chapter 6) includes items for the cardinal parkinsonian symptoms and some marmoset specific items. Additionally quantitative assessment of animal behavior is an commonly used tool. In the MPTP non-human primate models it is shown that measurement of changes in general activity has direct application to the clinic as hypokinesia is a common feature of PD. Furthermore, the fine motor skills of patients are reduced due to a combination of tremor, slowness of movement and disturbed motor planning. Assessment of these fine motor skills in MPTP non-human primates, e.g. hand-eye coordination are also affected (reviewed by Emborg 2004).

Neurochemical deficits The neurotoxic activity of MPTP leads to a variety of neurochemical changes, which is characterized by a reduction in concentration of DA and of its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum, but also effects on other monoamines, noradrenaline (NA) and serotonin, are found (Gerlach et al. 1991).

Pathologic degeneration MPTP induces selective lesions in the DAergic neurons of the SNpc. Staining of TH is often used as a quick and sensitive measure method for visualization of surviving DAergic neurons (Pearson et al. 1983).

Modafinil

Background

Modafinil (d,1-2-[(diphenylmethyl)sulfinyl]acetamide, Fig. 3) is a vigilance-enhancing compound and available for clinical use since 1998 under the trade names Modiodal® (Europe) and Provigil® (USA). Modafinil is approved as treatment for excessive daytime sleepiness in narcolepsy (Bastuji and Jouvet 1988, Green and Stillman 1998), shift work sleep disorder (Czeisler et al. 2005) and obstructive sleep apnea/hypopnea (Black and Hirschkowitz 2005).

Modafinil (100-600 mg/day) produces a unique spectrum of pharmacological effects in human subjects, the most prevalent are enhanced vigilance, arousal and wakefulness (Bastuji and Jouvet 1988). It is generally well tolerated, with

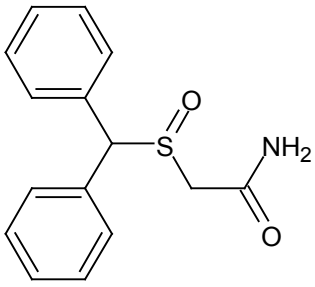


Fig. 3 Chemical structure of modafinil.

most prevalent adverse events being headache, nausea, nervousness, anxiety and insomnia (Robertson and Hellriegel 2003). Furthermore, modafinil does not produce withdrawal and tolerance that are associated with traditional stimulants such as chronic amphetamine use (Jasinski and Kovacevic-Ristanovic 2000, Rush et al. 2002, Myrick et al. 2004). Therefore, modafinil is currently categorized as a Schedule IV drug (low abuse potential) by the Federal Food and Drug Administration (FDA) in the USA. Modafinil also differs from traditional stimulants as it has no effect on sleep architecture, including REM rebound after sleep deprivation (Hermant et al. 1991, Buguet et al. 1995).

Mechanism of action

Modafinil's precise mechanism of action is not known. Modafinil's actions are limited to specific brain regions, in contrast to the stimulant amphetamine which has actions in a wide variety of brain regions as reported in a glucose utilization study (Engber et al. 1998). Morphological distribution of neural active areas confirm the involvement of modafinil in promotion of wakefulness (Lin et al. 1996, Engber et al. 1998, Scammell et al. 2000). No activity in the basal ganglia after modafinil administration was initially reported (Lin et al. 1996, Engber et al. 1998). However, when the sleep-wake cycle of rodents was taken into consideration, a similar dose did induce neural activation in the striatum (Scammell et al. 2000).

Modafinil is associated with increased adrenergic, glutamatergic and hypocretin activity and decreased GABA activity in specific parts of the brain (reviewed by Ballon and Feifel 2006). This will be explained in the following paragraphs.

Noradrenaline One of the first suggestions was that modafinil indirectly stimulated α_1-adrenoceptors. Adrenergic signaling in the central nervous system (CNS) plays a prominent role in the timing of sleep states and in regulation of physiological and behavioral phenomena (Wisor and Eriksson 2005). Evidence showed that modafinil's wake-promoting behavioral effects in mice were blocked by α_1- and β-adrenoceptor antagonists (Duteil et al. 1990, Lin et al. 1992, Stone et al. 2002). Although modafinil was not as effective as other α_1-adrenoceptor agonists in treatment of cataplexy in dogs and indicates an indirect modulation of the NA release (Shelton et al. 1995).

GABA & glutamate The effects of modafinil on GABA and glutamate release were revealed by microdialysis studies of Ferraro et al. (1997, 1998). They reported a decrease in GABA release in striatum and sleep-related areas and an increase of glutamate release in hippocampal formation and ventromedial and -lateral areas of the thalamus and sleep-related areas. This evidence points to a contribution of GABA and glutamate release in the vigilance-enhancing properties of modafinil.

Hypocretin Hypocretin is a neuropeptide implicated in the regulation of appetite and wakefulness (Chemelli et al. 1999). Modafinil activates the hypothalamic waking system and suppresses sleep-promoting neurons in the ventrolateral preoptic nucleus (Scammell et al. 2000). Hypocretin regulates arousal via stimulation of glutamatergic nerve firing in the hypothalamic circuit (Li et al. 2002).

Dopamine Recently modulatory effects on presynaptic activation of DA release are suggested as part of the mechanism of action of modafinil. It was mitigated because of the differences with amphetamine, a DAT inhibitor, and the inability of haloperidol, a dopamine D_1 and D_2 receptor antagonist to prevent the behavioral effects of modafinil (Simon et al. 1995). New findings have given the debate around of the DAergic involvement in the mechanism of action of modafinil a new impulse. These findings were the lack of stimulative properties of modafinil in the DAT knockout mouse (Wisor et al. 2001) as well as the low affinity for the DAT (>1 μM affinity), but not for other DA subtypes or other receptors (Mignot et al. 1994). Furthermore, positron emission tomography scans of living brains of rhesus monkeys have shown occupancy of the DA and NA transporters by effective modafinil doses (Madras et al. 2006). A new theory suggests that DAergic stimulation of adrenergic receptors is an explanation for the effects of modafinil (Wisor and Eriksson 2005).

Therapeutic and neuroprotective aspects

Modafinil has a potential to be a versatile therapeutic and neuroprotective compound because of its broad range of potential targets. Modafinil has, besides the already approved uses, also potential non-approved clinical uses because of its superior pharmacologic profile compared to other stimulants like amphetamine and methylphenidate. Modafinil treatment reduces impulsivity and hyperactivity in attention deficit hyperactive disorder patients (Taylor and Russo 2000, Turner et al. 2004), counteracts sedative effects of anti-psychotic, anti-depressant and mood stabilizing agents or sedation after general anesthesia (Makela et al. 2003, Webster et al. 2003, Larijani et al. 2004) and supports and augments the effects of anti-depressants (Menza et al. 2000, Ninan et al. 2004). Modafinil is also off-label used in the treatment of excessive daytime sleepiness in PD (Happe et al. 2001, Nieves and Lang 2002, Adler et al. 2003), one of the common non-motor symptoms in PD. The striatal activation by modafinil (Scammell et al. 2000), the modulation of DA release and the modulation of GABA and glutamate release in parts of the basal ganglia (Ferraro et al. 1997, 1998) indicates that modafinil could have anti-parkinsonian effects on the motor symptoms of PD.

Modafinil also has a potential in protection against neuronal death in *in vitro* and *in vivo* models of mechanic, neurotoxic and ischemic injury. Modafinil prevents glutamate toxicity in cultured cortical cells (Antonelli et al. 1998), it prevents increases in toxic aspartate and glutamate levels after striatal ischemic injury caused by endothelin-1 in rats (Ueki et al. 1993b) and it prevents development of lesions in the hippocampus induced by the neurotoxic nerve gas soman (Lallemant et al. 1997). Modafinil also has shown to have a neuroprotective potential in the treatment of PD as it could prevent degeneration of the nigrostriatal pathway in MPTP models and mechanical injury of the nigrostriatal pathway (Fuxe et al. 1992, Ueki et al. 1993a, Jenner et al. 2000). It seems that the neuroprotective abilities of modafinil are independent of brain area or neurotransmitter type. These studies suggest therefore that the neuroprotective actions of modafinil besides its effects on neurotransmitters also could be generated via direct interference with cell death processes.

Cannabinoids

Background

In humans, marijuana, an extract of the leaves and flower tops of the hemp plant *(Cannabis sativa)*, exhibits clear euphorigenic properties, but also alterations in cognition and memory, anxiety, analgesia, hypothermia, increased food intake, anti-emetic effects and vasorelaxation (Chaperon and Thiebot 1999). The cannabinoid system includes at least two cannabinoid receptors (CB_1 and CB_2) and endogenous cannabinoids like anandamide and 2-arachidonylglycerol (Devane et al. 1992, Mechoulam et al. 1995, Howlett et al. 2002), which are suggested to be neuromodulatory (Wilson and Nicoll 2002). The cannabinoid system can be manipulated exogenously with among others Δ^9-THC (Fig. 4), a principal active ingredient of marijuana, and highly selective and potent receptor agonists, like CP55940 and WIN 55212-2, and antagonists, like rimonabant (SR141716A) and LY320135 (Howlett et al. 2002).

Localization and function of cannabinoid receptors

Cannabinoid CB_1 receptors are located mainly in the CNS, but also in small amounts in the periphery, e.g. in the pituitary gland and immune cells. A high density of these receptors is found in areas associated with higher cognitive functions, areas associated with control of motor and sensory functions of the autonomic nervous system and in areas associated with control of movement, mainly basal ganglia and cerebellum (Glass et al. 1997). In nuclei of the basal ganglia, namely GPi, SNpr and STN cannabinoid CB_1 receptors are mainly localized presynaptically, whereas in the striatum high levels of pre- and postsynaptic cannabinoid CB_1 receptors are found (Herkenham et al. 1990, 1991, Mailleux and Vanderhaeghen 1992, Hohemann and Herkenham 2000). The cannabinoid CB_2 receptor is found in tissues involved in immune cell production and regulation and their function is generally suppressive (Ameri 1999).

The control of movement by cannabinoids seems to be generated via actions on GABA, DA and glutamate release due to reduction of intracellular Ca^{2+}

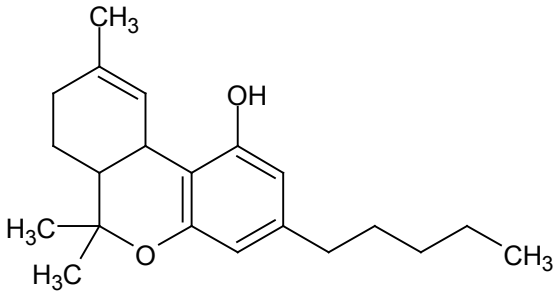


Fig. 4 Chemical structure of Δ⁹-tetrahydrocannabinol.

concentration, reduction of adenylyl cyclase and modulation of ion channels (Howlett et al. 2004). The presynaptic localization of the cannabinoid receptors results in modulatory actions on GABA and glutamate release (Szabo et al. 2000, Howlett et al. 2004). The cannabinoid system influences DA release as cannabinoid CB_1 receptors are co-localized with dopamine D_1 and D_2 receptors in striatal projection neurons (Herkenham et al. 1991) and cannabinoid CB_1 receptors are upregulated during reduced nigral DA release (Lastres-Becker et al. 2001).

Subtle differences exist between the cannabinoid CB_1 receptor distribution of the basal ganglia in rodents and primates. The most striking differences in the basal ganglia are the higher receptors levels in the SNpc and striatum and the lower level in the GP and SNpr in the primate brain compared to the rat brain (Tsou et al. 1998, Ong and Mackie 1999). This may affect the interpretation of experimental behavioral results from rodents to man.

Therapeutic and neuroprotective aspects

Cannabinoids and Δ⁹-THC in particular have great potential in the treatment of a variety of clinical disorders, because of their broad range of effects on different physiological systems. Analgesic properties of cannabinoids are found effective in spinal cord injury and post-operative pain, but also spasticity in multiple sclerosis is reduced. Furthermore, cancer and HIV patients benefit from the anti-emetic and appetite stimulating effects of cannabinoids (Croxford 2003).

Cannabinoids have potential for application in PD as patients experience some beneficial effects of cannabis as it reduces parkinsonian symptoms and levodopa-induced dyskinesia (Sieradzan et al. 2001, Venderova et al. 2004). Furthermore, the cannabinoid system seems to be involved in the PD pathology since an increased number of cannabinoid CB_1 receptors in the basal ganglia of parkinsonian humans, non-human primates and rats is found compared to healthy brains (Silverdale et al. 2001, Lastres-Becker et al. 2001). The upregulation of the cannabinoid CB_1 receptors is suggested to be a compensatory mechanism to counteract the unbalance in the basal ganglia physiology (Van der Stelt and DiMarzo, 2003). Therefore, intervention via cannabinoid CB_1 receptors to modulate the disturbed balance of the basal ganglia is often suggested as a possible therapy against PD symptoms (Brotchie 2003).

Cannabinoids are also implicated in the control of cell survival/death decision in the CNS and periphery (Guzmán et al. 2001). This finding is based, among others, on the observation that cannabinoids protect neurons from toxic insults such as glutamatergic and ouabain-induced excitotoxicity (Shen and Thayer 1998, Van der Stelt et al. 2001a, b), ischemic stroke (Nagayama et al. 1999), oxidative stress (Hampson et al. 1998, Marsicano et al. 2002), and others (reviewed in Grundy et al. 2001). Cannabinoid CB_1 receptor activation could lead to neurotrophic effects, reduction of nitric oxide, modulation of ion channel currents and decrease of glutamate release. Activation of cannabinoid CB_2 receptors in microglial cells, but also peripheral cells could modulate the immune response. Furthermore, receptor-independent mechanisms like anti-oxidant properties are suggested to mediate cannabinoid neuroprotection (Grundy 2001). These findings indicate that Δ⁹-THC

could be a potent neuroprotective compound, especially in PD as in the pathological state changes in the cannabinoid system are observed. This was illustrated by a recent report about the neuroprotective abilities of Δ^9-THC in the 6-OHDA rat model (Lastres-Becker et al. 2005). The positive findings in this first study stimulate the exploration of the neuroprotective effects of Δ^9-THC in other PD models.

Δ^9-THC is the cannabinoid of choice in this thesis as it is the main component of marijuana, which is often used by PD patients to relieve their symptoms (Venderova et al. 2004). Furthermore, besides its affinity for the cannabinoid CB_1 receptor, Δ^9-THC has also anti-oxidant properties making the neuroprotective abilities of this compound more versatile (Hampson et al. 1998).

Aim of the thesis

The aim of this thesis is to test potential neuroprotective compounds for the treatment of PD. The focus is on the compounds modafinil and Δ^9-THC because of their ability to interfere with cell death processes. Furthermore, these compounds were suggested to have multiple mechanisms of action which would potentially improve their neuroprotective capacity. From this, we derived the hypothesis that modafinil and Δ^9-THC could also be neuroprotective in animal models of PD.

As the test compounds are available clinically and an animal model close to man is used in the studies, positive outcome will bring the application of these compounds one step closer to therapeutic use. Furthermore, the outcome of these studies, positive or negative, will contribute to more knowledge of effective neuroprotective compounds for PD and their mechanisms of action. This will lead to a more focussed approach and may eventually lead to a higher clinical efficacy in the treatment of PD.

To assess the capability of the proposed treatment, we have used extensive behavioral tests as well as measurements of pathology such as immunohistochemistry, neurochemical analysis and magnetic resonance scanning. The neuroprotection studies are preceded by behavioral assessment of the compounds in naïve and parkinsonian animals to assess respectively the side-effects and the symptomatic effects of the compounds, which will contribute to the interpretation of behavioral outcome of the neuroprotection studies.

Outline of the thesis

Part I describes the behavioral effects of modafinil and Δ^9-THC in naïve marmoset monkeys. In *chapter 2* the dose-dependent behavioral effects of modafinil were tested using observational methods and behavioral tests measuring locomotor activity, hand-eye coordination, response to a threat situation and startle response. In *chapter 3* the effects of modafinil on fatigue of sleep deprived animals and their ability to execute a task were tested and compared to another stimulant, caffeine. In *chapter 4* the dose-dependent effects of Δ^9-THC on behavioral outcomes are described similar

to chapter 2.

Part II consists of *chapter 5*, which describes the characteristics of Δ^9-THC and modafinil to control the symptoms of parkinsonian marmoset monkeys measured with observational methods, behavioral tests measuring locomotor activity and hand-eye coordination.

Part III describes the neuroprotective effects of modafinil and Δ^9-THC in the MPTP model. In *chapter 6* are the effects of modafinil to prevent neurodegeneration in the MPTP marmoset model on behavioral and neurochemical outcome reported. Furthermore, the sensitivity of behavioral measures for MPTP intoxication is noted. *Chapter 7* elaborates the neuroprotection data of modafinil described in chapter 6 with data on pathology. The neuroprotective effects measured with immunohistochemical and magnetic resonance techniques and their relation to behavioral outcome are described. In *chapter 8* two exploratory studies on the mechanism behind the neuroprotective characteristics of modafinil are reported. In the MPTP mouse model it is tested whether modafinil is neuroprotective when given hours prior to disease induction. Furthermore, the effect of modafinil on gene expression in astrocytes is described. In *chapter 9* the ability of Δ^9-THC to prevent neuronal damage in the MPTP-treated marmoset is explored. *Chapter 10* summarizes and integrates the results, also the clinical implication of neuroprotection is discussed.

Part I

Behavioral effects

Chapter 2

Behavioral effects of modafinil in marmoset monkeys

Sanneke van Vliet, Marjan Jongsma, Raymond Vanwersch, Berend Olivier, Ingrid Philippens

Psychopharmacology (2006) 185(4): 433-40

Abstract

Rationale: Modafinil is increasingly used in sleep disturbances in general and in neurodegenerative diseases and is recently being used in healthy people for attentional control. However, the application of modafinil is possibly not only restricted to alertness-enhancing effects. More insight in this compound may lead to new applications. Not all behavioral aspects have been studied sufficiently; therefore, more detailed investigations on modafinil's positive and aversive behavioral effects are addressed in this paper.

Objectives: Determination of effects of modafinil in marmoset monkeys with observational methods and with behavioral tests measuring locomotor activity, hand-eye coordination, response to a threat situation and startle response.

Methods: Two hours after oral administration of modafinil in doses of 50, 100, 150 and 225 mg/kg, animals were observed and tested in the behavioral test systems.

Results: Locomotor activity was increased after 100 mg/kg modafinil in the Bungalow test and after 100, 150 and 225 mg/kg as found in the movement parameters of the human threat test. Moreover, modafinil showed anxiolytic-like effects in the human threat test. No other side-effects were observed, nor were the hand-eye coordination and startle response affected.

Conclusions: Besides psychostimulation, modafinil has no aversive effects in the doses used in the domains measured. The potential anxiolytic-like effects of modafinil may create new possibilities for the therapeutic use of modafinil.

Introduction

Modafinil is a vigilance-enhancing compound, approved as treatment for excessive daytime sleepiness (EDS) in narcolepsy (Bastuji and Jouvet 1988, Green and Stillman 1998). Modafinil is also effective in many other neurological diseases associated with EDS (Rammohan et al. 2002, Happe 2003) including reduction of fatigue in multiple sclerosis (Rammohan et al. 2002) and Parkinson's disease (PD; Happe et al. 2001, Nieves and Lang 2002, Adler et al. 2003).

Besides effects on alertness, modafinil has other putative therapeutic applications. The sedative effects of anti-psychotics and opiates or sedation after general anesthesia can be reduced (Makela et al. 2003, Webster et al. 2003, Larijani et al. 2004). Modafinil also supports and augments the effects of antidepressants (Menza et al. 2000, Ninan et al. 2004). Furthermore, modafinil treatment reduces impulsivity and hyperactivity in attention deficit hyperactive disorder patients (Taylor and Russo 2000, Turner et al. 2004). Positive effects on cognition were also found in healthy humans (Turner et al. 2003).

As an alertness enhancer, modafinil is preferred over amphetamine and amphetamine-like stimulants, because of its lower abuse and dependency potential (Jasinski and Kovacevic-Ristanovic 2000) and a favorable side-effect profile. In humans, modafinil is well tolerated with side-effects reported as mild to moderate headache, nausea, nervousness, anxiety and insomnia (Robertson and Hellriegel 2003).

The exact mechanism of action of modafinil is still unclear. The morphological distribution of neural active areas after modafinil implicates involvement in sleep wake regulation, like the anterior hypothalamic nucleus and adjacent areas, but not in motor function areas (Lin et al. 1996, Engber et al. 1998, Scammell et al. 2000). Modafinil modulates neurotransmitter function such as noradrenaline (NA) indirectly, since modafinil-induced locomotor activity is prevented by an α1-adrenoreceptor antagonist (Duteil et al. 1990). It also affects γ-aminobutyric acid (GABA), reducing its release in striatum, sleep-related areas and the outflow of the cortex (Tanganelli et al. 1995, Ferraro et al. 1996, 1998).

The functional effects of modafinil should be more intensively studied because of the increased application of modafinil in therapeutic areas like PD, depression and sleep, but also its extended therapeutic use and possible attention-enhancing effects in healthy people. To approach the human situation as much as possible, a non-human primate model was selected with a high level of similarity to human sleep architecture: the marmoset monkey. This model also has a high level of similarity to human PD after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication. The marmoset monkey is a good model for testing modafinil because its closer resemblance to man compared to rodent. Whereas many of the physiological and pharmacological questions can be (and have been) addressed in rodent models and tests, behavioral and cognitive parameters can be assessed more accurately in animals closer to man. Furthermore, in primate models similar test methods can be used in humans. For example, the abnormal involuntary movement scale (AIMS),

a clinical rating scale for dyskinesia, can be applied without modification to PD patients (Di Monte et al. 2000) and sleep electroencephalogram measurements also show high similarity (Philippens et al. 2004).

In the present study, the effects of modafinil in doses of 50, 100, 150 and 225 mg/kg were assessed using observation scales and behavioral test systems. Direct observation with extensive observation scales, measuring effects on general and dyskinetic behavior, quantified the effects of modafinil. The quality of movement was measured with behavioral test systems assessing the locomotor activity, the hand-eye coordination and startle response. Measurement of the effects of modafinil on the hand-eye coordination is unique. Also little research is done concerning the effects of modafinil on anxiety. Therefore, in this study, the reaction to the human threat has been tested.

Materials and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus),* aged 2-6 years with body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. Marmosets are social animals, but due to practical purposes among others the validation of observational tests in single housed marmosets, the animals were kept one to a cage. Moreover, the marmosets were supplied as individuals; therefore it was less stressful for these animals to keep them singly housed. The ambient temperature was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

Modafinil was administrated orally by gavage in doses of 50, 100, 150 and 225 mg/kg. The doses chosen were based on earlier studies in marmosets, rats and mice (Duteil et al. 1990, Engber et al. 1998, Jenner et al. 2000). The peak activity of modafinil in marmoset monkeys was two hours after administration with effects lasting over a 10-hour period (Jenner et al. 2000). Consequently, the appearance of symptoms was observed and the human threat test was carried out at 120 minutes after administration followed by the hand-eye coordination task at 130 minutes post-dose. At 145 minutes post-dose, the locomotor activity was assessed in the Bungalow test, followed by the startle response at 170 minutes post-dose. To reduce the number of animals, each animal received a maximum of two different doses of modafinil in a randomized design. The number of animals used in the test systems (hand-eye coordination, locomotor activity and startle response) is five (50 mg/kg), nine (100

mg/kg), eight (150 mg/kg) and six (225 mg/kg). The startle response is tested in five animals after 225 mg/kg modafinil due to a technical failure. To extend the reliability of the data, extra observations with the human threat test and the behavior scales of five animals (50 mg/kg (n=1); 100 mg/kg (n=4)) were added. Control values were obtained in the same animals without treatment. No differences between control and vehicle values were found (data not shown).

Drug administration

Modafinil (Modiodal®, d,1-2-[(diphenylmethyl)sulfinyl]acetamide) was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds such as lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in a 10% sugar solution in a dose volume of 1.5 ml/kg. The used doses of modafinil were 50, 100, 150 and 225 mg/kg.

Behavioral assessment

Observation of signs and symptoms: For the observation of signs and symptoms two rating scales were used. 1) A general clinical scoring list in which the condition of the animal was rated. The following symptoms were registered: inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility and presence of tremors. The degrees of severity were coded from 0 (normal) to 4 (severe). 2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976). The AIMS is widely used clinically for qualification of involuntary movements, occurring among others in PD, schizophrenia and elderly (Woerner et al. 1998, Beasley et al. 1999, Katzenschlager et al. 2004). These scales have successfully been applied in monkey research in our institute for more than 10 years. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity, and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occurred due to stimulation by the observer were rated one step lower than those observed spontaneously. The observations were performed in a blinded matter. Besides the rating scales, the deviation from the normal behavior not mentioned in the above-mentioned scales was registered.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video

tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Hand-eye coordination task: The hand-eye coordination task is a sensitive task for measuring controlled motor movements. An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed one minute to grasp the non-moving reward. Each type of trial was presented 14 times per session. At the beginning of each trial a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

Human threat test: The human threat test is a non-human primate putative model of anxiety. Marmosets will exhibit fear-related behavior in the presence of a human observer in front of the cage (Costall et al. 1988). The most pronounced behavior would be retreating to the back of the cage and showing characteristic postures. The behavior was assessed in the home cage (40 x 60 x 60 cm) with a hanging basket in the back of the cage, a wooden board (20 x 10 cm, 30 cm above cage floor) on the left side in the back and on the other side a perch, at the same height, positioned from the back to the front of the cage. To assess the behavior, the observer stood approximately 30-100 cm from the cage front and made eye contact with the marmoset throughout a 2-minute test period. During this period the movements, behavior and position of the marmoset in the cage were recorded by video registration.

A range of parameters was obtained according to Carey et al. (1992) based on Stevenson and Poole (1976): 1) The number of characteristic postures exhibited: tail posture (tail raise to present the genital region), scent marking (the anal and genital area is pressed against the substrate to be marked with excretion of the glands), arched pilo-erection (arched back posture with full body pilo-erection), slit stare (stare with the eyes half closed in combination with tufts flattened and exposure of the teeth), rearing (upright position with flexed paws), twisting (head and torso movement from side to side), 2) The time spent in the front of the cage, 3) The number of position changes in the cage, 4) The number of movements from the back of the cage to the front, 5) The number of jumps from the left side of the cage to the right side or vice versa.

Auditory startle response: The auditory startle reflex is a motor response following an intense sound stimulus. The apparatus to measure startle response in marmoset monkeys has been described earlier and has been validated by Philippens et al. (2000). The animals were placed in a transparent plastic tube on a pressure transducer in an illuminated sound attenuated box. Twenty startle stimuli (20 ms, 120 dB, white noise) were delivered in random order (inter stimulus interval: 14 ± 4 seconds). For the duration of 200 ms, directly after the stimulus presentation, the force exerted by the animal was registered. The startle reflex was represented by the amplitude of the response.

Statistics

No statistical analysis was performed on the data of the behavioral observation scales because the changes can be regarded as minimal.

The remaining results are presented as mean ± SEM and parametric statistical analysis was applied. The study can be considered as a semi-within design, since some animals received multiple doses, however not all doses. Therefore, the statistical analysis is based on a between subjects design. First, an overall repeated measures (RM) analysis was applied where the dose-group (the animals belonging to a certain dose with a certain treatment) was the between-factor and the treatments, control and modafinil, were included as the within-factor. If $p<0.05$ or a clear trend was seen, a paired t-test was applied to test each dose-group for a difference between the treatment groups. For this analysis, the significance level of $p<0.05$ was used.

Results

Observation of signs and symptoms

Administration of 50, 100, 150 and 225 mg/kg modafinil resulted in none or minimal changes in the rating scales (clinical symptoms and AIMS). In Table 1 the mean scores of the affected animals and the number of affected animals are shown. Most changes on both rating scales were within normal ranges (score of 1 means normal behavior with the parameter seen in a higher frequency). Only scores above '1' are defined as affected behavior. Therefore, only in the lowest dose group (50 mg/kg) three parameters were rated as affected (Table 1). By observation, an increased activity after high doses of modafinil was seen. After 150 mg/kg, some stability problems were seen. After the highest dose some animals were hyperactive showing fast and repetitive-, but not stereotypic- movements, increased distraction and escape attempts.

Quality of movements

Modafinil affected the locomotor and exploratory activity measured with the Bungalow test (RM, $p<0.001$) and the hand-eye coordination (RM, $p<0.01$). More specifically, the locomotor activity after 100 mg/kg modafinil was increased compared

to the normal activity (paired t-test, $p<0.05$, Fig. 1). The difference in number of compartment changes between the control value and 100 mg/kg modafinil was 115 ± 46. The hand-eye coordination task, measured as the number of correctly taken rewards, was still at maximum performance after 50 (n=5), 100 (n=9), 150 (n=8) mg/kg modafinil. Only after 225 mg/kg modafinil 2 of the 6 animals showed decreased performance on the task (Fig. 1).

Startle response

The startle response was not affected after any of the doses of modafinil (Fig. 1).

Human threat test

Modafinil affected the body postures (RM, $p<0.001$, Table 2). More specifically, the body postures were lowered after 50, 150 and 225 mg/kg modafinil (paired t-test, $p<0.05$) and with a trend after 100 mg/kg (paired t-test, $p=0.053$). A statistical increase was seen in the effects of modafinil on the 'time spent in front of the cage' (RM, $p=0.052$). This was due to an increase in time spent in front after 100 and 225 mg/kg (paired t-test, $p=0.065$ and $p=0.051$).

Modafinil increased the 'position changes in the cage' (RM, $p<0.001$), the related 'movements forward' (RM, $p<0.001$) and the 'jumps from side to side' (RM,

Table 1 Clinical score and AIMS. Overview of the mean scores of the *affected* animals on the items of the clinical score and AIMS after administration of 50, 100,150 and 225 mg/kg modafinil.

		Modafinil			
		50 mg/kg	100 mg/kg	150 mg/kg	225 mg/kg
Clinical score					
Grooming		1 (1/6)	1 (4/13)	1 (6/8)	1 (3/6)
Apathy		1 (2/6)	1 (3/13)	1 (1/8)	1 (3/6)
Immobility		1.5 (2/6)	0	0	0
Tremors		0	0	0	0
Total affected animals		*2/6*	*4/13*	*6/8*	*5/6*
AIMS test					
Facial and oral mov.	Facial expression	2 (1/6)	0	0	0
	Lips, peri-oral area	1 (2/6)	1 (1/13)	1 (1/8)	1 (2/6)
	Jaw	0	0	1 (2/8)	0
	Tongue	0	1 (1/13)	0	0
Extremity mov.	Upper	0	1 (1/13)	0	0
Trunk mov.	Lower	1.5 (2/6)	0	0	1 (2/6)
	Neck, shoulder, hips	1 (2/6)	0	1 (3/8)	1 (1/6)
Global judgment	Severity	0	0	0	0
	Incapacitation	1 (1/6)	0	0	1 (1/6)
Total affected animals		*5/6*	*3/13*	*5/8*	*6/6*

Score of symptoms 0: normal; 1: minimal, but extreme normal; 2: mild; 3: moderate; 4: severe (# of affected animals/ total group size). Total affected animals: # of animals with ≥ 1 symptom/ total group size. Mov. indicates movements

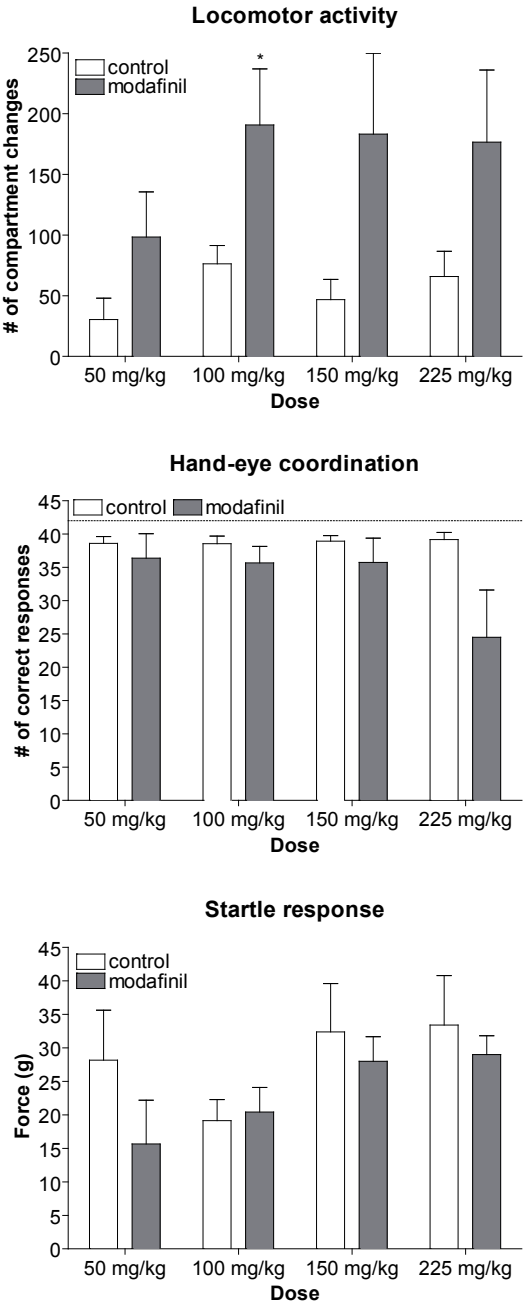


Fig. 1 Behavioral tests. Bars show mean (+ SEM) of control values (before administration) and after 50, 100, 150 and 225 mg/kg of modafinil. *p<0.05 vs. control. Dotted line indicates maximum number of responses.

p<0.001). More detailed, the 'position changes in the cage' increased after 100, 150 and 225 mg/kg (paired t-test, p<0.05) with a trend after 50 mg/kg (paired t-test, p=0.053). The 'movements forward' increased after 100 and 150 mg/kg with a trend after 50 mg/kg (paired t-test, p=0.055). The 'jumps from side to side' increased after 100 and 225 mg/kg (paired t-test, p<0.05).

Discussion

In view of the increasing applications of modafinil in various therapeutic areas, including PD and sleep disorders, we assessed putative positive and aversive effects of modafinil in marmoset monkeys using observational methods and various behavioral test systems. Modafinil was tested in doses of 50, 100, 150 and 225 mg/kg in a time interval at two and three hours after administration.

Increased activity was measured in the Bungalow test and during the human threat test on the parameters 'position changes', 'movements forward' and 'sideward jumps'. This increased activity was reported earlier in other species (Jenner et al. 2000, Simon et al. 1995, Ward et al. 2004). However, the increase in activity is not reported in humans, except related side-effects such as nervousness and insomnia (Robertson and Hellriegel 2003).

Other psychostimulants such as caffeine and amphetamine also increase locomotor activity (Eden et al. 1991, Fredholm et al. 1999). These compounds affect dopaminergic (DAergic) and NAergic systems, which are mainly involved in the control of locomotor activity (Anden et al. 1973). Modafinil does not modulate the DA system, as modafinil lacks pronounced effects on motor function areas (Engber et al. 1998, Lin et al. 1996). Indeed, the dopamine D_2 receptor antagonists haloperidol and sulpiride did not prevent the modafinil-induced locomotor activity (Duteil et al. 1990). However, modafinil can increase DA transmission by binding weakly to the dopamine transporter (Mignot et al. 1994, Wisor et al. 2001) and the modafinil-induced increased c-fos in certain brain areas like striatum and amygdala seems moderated by substantial dopaminergic afferents (Scammell et al. 2000). Modulation of the NA

Table 2 Human threat test. Overview of the difference (mean ± SEM) between the parameters of the human threat test before and after administration of 50, 100, 150 and 225 mg/kg modafinil. *p<0.05 vs. control.

	Modafinil			
	50 mg/kg	100 mg/kg	150 mg/kg	225 mg/kg
# of body postures	-5.2 ± 1.2 *	-2.8 ± 1.3	5.8 ± 2.3 *	-4.8 ± 1.0 *
Time spent in front (sec)	-8.3 ± 5.3	21.7 ± 11.7	2.3 ± 8.8	29.7 ± 11.6
# of position changes	8.6 ± 3.4	7.1 ± 1.9 *	7.1 ± 2.8 *	16.7 ± 9.0 *
# of forward movements	3.3 ± 1.3	3.3 ± 0.7 *	4.8 ± 1.8 *	7.0 ± 5.2
# of sideward jumps	5.2 ± 3.9	3.0 ± 1.3 *	5.4 ± 2.6	9.8 ± 2.9 *

system by modafinil seems more likely. Modafinil-induced locomotor activity was prevented by the α_1-adrenoceptor antagonists, prazosin and phenoxybenzamine (Duteil et al. 1990). Catecholamine depletion by reserpine also prevented modafinil-induced locomotor activity, but inhibition of tyrosine hydroxylase by α-Methyl-p-tyrosine had no effect. This indicates an indirect effect of endogenous NA stores stimulating the α_1-adrenoreceptors (Duteil et al. 1990). Besides effects on the NA system, modafinil reduces GABA release from striatal and pallidal areas of the basal ganglia without affecting glutamate release (Ferraro et al. 1998). It is quite probable that locomotor activity can be increased as a result of reduction of GABA release from the globus pallidus to the thalamus. Where the increased glutamate levels result in excitatory output from the basal ganglia.

The increased locomotor activity was not accompanied by stereotypic behavior or other observable side-effects, except at the highest doses. Hand-eye coordination was not changed after any dose of modafinil, although an overall effect was found. The setup of the hand-eye coordination task used could not reveal improvement of hand-eye coordination, because the maximum number of correct responses had already been reached during training of the task. In healthy volunteers modafinil did not affect the reaction time. On the other hand, the response latency was lower resulting in reduced impulsive responding (Turner et al. 2003). The lack of stereotypic behavior and minimal effects on hand-eye coordination, as tested in this study, seem to support the idea that modafinil does not exert its effects via the dopaminergic system.

Two parameters of the human threat test, the 'number of body postures' and 'time spent in front', are most sensitive to anxiolytic effects of drugs (Carey et al. 1992). This was also validated in the marmoset colony of our institute with the anxiolytic agent diazepam (0.25 mg/kg i.m.). Diazepam lowered the number of characteristic body postures and increased the time spent in the front of the cage (Van Vliet et al. 2005). In this study, modafinil reduced the 'number of body postures' and a trend for an overall increased 'time spent in front' after 100 and 225 mg/kg modafinil was observed. This can be due to increased activity, which is responsible for a reduced total time spent on a specific position. However, despite this, the ratio of place preference (front vs. back of the cage) increased with 43 ± 5% after 100 and 225 mg/kg modafinil. The shift in place preference indicates that more time is spent in an aversive place after modafinil. Therefore, it can be concluded that the anxiolytic-like effects of modafinil are independent of the stimulative properties of modafinil.

Anxiolytic-like effects of modafinil have not been reported earlier. Simon et al. (1994) found no effects on anxiety in mice, but their tests were primarily designed to measure anxiogenic effects. Although anxiogenic effects have been mentioned as reported side-effects in human studies (Robertson and Hellriegel 2003). However, in this review it is not mentioned what this conclusion of anxiogenic effects was based on and the reference mentioned was only about narcolepsy patients.

As the body postures were reduced after modafinil, it was expected that effect on the 'time spent in front' would be clearer. Whereas the latter parameter is very sensitive to anxiolytic effects and is the most frequently affected behavior in other

studies (Barros and Tomaz 2002). A reason can be that no selection on animals with anxious behavior was carried out beforehand. Prior screening of the animals is fairly common in applications of the human threat test in order to reduce the inter-individual variability (Costall et al. 1988). The omission of pre-selection resulted in a higher baseline level (24 ± 5%) of the 'time spent in front'. Therefore, our expectation is that putative anxiolytic effects of modafinil would be more distinct after pre-selection and animals less habituated to their environment and observer.

Anxiety can be reduced by benzodiazepines, like diazepam, which are acting on $GABA_A$-receptors (Nemeroff 2003). The anxiolytic-like effects of modafinil cannot be explained by a direct effect on $GABA_A$-receptor systems, although modafinil reduces GABA levels in hypothalamus and cerebral cortex outflow (Tanganelli et al. 1995, Ferraro et al. 1999). A potential advantage of modafinil over benzodiazepines in the treatment of anxiety is the lack of sedation, often seen after benzodiazepines.

In conclusion, modafinil increases locomotor activity in marmosets, probably the human counterpart of increases in nervousness and agitation. Besides that, modafinil shows minimal aversive effects, even at higher doses. Therefore, the effects of modafinil found in the marmoset monkey support the advantageous characteristics of this compound over other psychostimulants. The potential anxiolytic-like effects of modafinil in marmosets point to further examination of this new characteristic and may create new possibilities for the therapeutic use of modafinil.

Chapter 3

Efficacy of caffeine and modafinil in counteracting sleep deprivation in the marmoset monkey

Sanneke van Vliet, Marjan Jongsma, Raymond Vanwersch, Berend Olivier, Ingrid Philippens

Manuscript submitted

Abstract

The effects of sleep deprivation are a burden in our 24-hour society. The use of wake-promoting compounds could improve the performance in situations where sleep cannot be allowed. In this study the efficacy of the wake-promoting compounds, modafinil and caffeine, in counteracting the effects of 24-hour sleep deprivation in the marmoset monkey were tested. As caffeine is habitually used, the efficacy of both compounds after short- and long-term use was investigated.

After a normal active day, the animals were kept awake and received wake-promoting compounds during the whole night. Three times during the sleep deprived night, putative fatigue was assessed with an activity test and the vigilance and ability to execute a task was assessed with a hand-eye coordination task. Both compounds were able to counteract to some extent the decline in performance. Modafinil was able to keep the activity at baseline performance, but performance on the hand-eye coordination task was not improved. Caffeine was able to keep performance in the hand-eye coordination task at a level just below daytime level, but was not able to keep activity at daytime levels during the last part of the night. Caffeine and modafinil administration for two weeks showed a comparable effect on activity as acute use. The performance on the hand-eye coordination task was similar after chronic caffeine and improved after chronic modafinil.

It is therefore concluded that modafinil and caffeine were both able to postpone or prevent the decline in vigilance and psychomotor performance and increase in fatigue induced by sleep deprivation.

Introduction

Sleep is essential for survival. Regular and good quality sleep is vital for proper performance and a healthy life. In our 24-hour society many professions, like aviators, soldiers or shift workers are exposed to disturbed and deprived sleep. In such situations sleepiness can have detrimental consequences caused by declined cognitive performance resulting in reduced productivity and safety (Folkard and Tucker 2003). In situations where sleep is not allowed, wake-promoting drugs to postpone sleepiness could be a realistic option to decrease chances on detrimental consequences. Single use of wake-promoting drugs or in combination with strategic naps, with or without hypnotics, can improve the quality of wakefulness.

Wake-promoting drugs are effective in operational situations (reviewed by Bonnet et al. 2005). For example after forty wakeful hours helicopter pilots do perform better on nearly all of their tasks after intake of dexedrine compared to placebo (Caldwell and Caldwell 1997). Amphetamine has been applied successfully to keep soldiers awake during tactic operations in Desert Shield and Storm (Emonson and Vanderbeek 1995). However, amphetamines are known to induce negative cardiovascular, autonomic and neurological effects including modification of sleep recovery, withdrawal effects and have a high abuse potential (Schedule II of the Controlled substance act of the Drug Enforcement Administration (DEA)).

The wake-promoting compounds modafinil and caffeine have a less aversive side-effect profile and could be an alternative for amphetamine use. First, modafinil (Modiodal®, d,1-2-[(diphenylmethyl)sulfinyl]acetamide) is often prescribed as treatment of narcolepsy and excessive daytime sleepiness (Bastuji and Jouvet 1988). Other studies found that modafinil improved psychomotor vigilance after sleep deprivation in humans (Wesensten et al. 2004, 2005, Caldwell et al. 2004) and also maintained cognitive performance at well-rested levels for approximately 44 hours after sleep deprivation, but not 60 hours after sleep deprivation (Lagarde et al. 1995). Modafinil induces a prolonged vigilance state due to its long half life of 12-15 hours (Robertson and Hellriegel 2003). Furthermore, the lack of effect on sleep architecture can be of value in unexpected sleep opportunities e.g. during military operations (Caldwell and Caldwell 2005). A disadvantage is that medical prescription is needed although modafinil has a low abuse potential (Schedule IV, controlled substance act of the DEA).

Caffeine (1,3,7-trimethylxanthine) is often used as an alertness enhancer as it is widely available in coffee, tea, caffeinated beverages and chocolate. After sleep deprivation in humans, caffeine improved vigilance and psychomotor performance (eg. Wesensten et al. 2002, McLellan et al. 2004, 2005) although higher doses were needed in prolonged periods of sleep loss (Bonnet et al. 2005). Caffeine is considered a safe drug without disturbing physiological consequences. Furthermore, caffeine has a fast onset of action, which makes it useful in situations where direct increase of alertness is needed (reviewed in Caldwell and Caldwell 2005, Bonnet et al. 2005). A disadvantage of caffeine is its widespread use on a daily basis which may lead to adaptation to the stimulating effects and diminish its efficacy as a wake-promoting

agent (Fredholm et al. 1999).

The efficacy of modafinil and caffeine after long periods of sleep deprivation (24-60 hours) are well studied in human (Bonnet et al. 2005). In most studies the efficacy of the stimulants were tested after sleep deprivation. The use of the wake-promoting compounds at the start of and during sleep deprivation are more of interest as short-term sleep deprivation occurs more often, e.g. during shift work rather than extreme sleep deprivation. Therefore, this study focuses on the efficacy of caffeine and modafinil in directly counteracting sleep deprivation effects. Another characteristic to be investigated is the ability of the wake-promoting compounds to keep performance during sleep deprivation at a normal level.

To investigate effects of drugs, animal studies are in some aspects preferable over human studies. Besides ethical and practical problems in studying drug effect on sleep in human volunteers, animals can be tested under standard and rigidly controlled conditions. These conditions are needed to increase the comparability between acute and long-term use of wake-promoting compounds as human caffeine intake is often on a daily basis and in varying amounts. The experimental animal used in this study mimics human sleep as close as possible: the marmoset monkey. The sleep of the marmoset is quite analogous to the human sleep, e.g. in duration, number and ratio of non-REM and REM cycles (Crofts et al. 2001, Philippens et al. 2004).

In order to select an optimal wake-promoting drug for the management of sleep and wakefulness, the efficacy of such a drug in counteracting the effects of sleep deprivation on fatigue and performance needs to be investigated. Therefore, the effects of acute and chronic caffeine and modafinil on behavioral performance were determined in the marmoset monkey during a night of sleep deprivation. Fatigue was measured with the Bungalow task, a locomotor activity test and the psychomotor performance was measured with the hand-eye coordination task.

Materials and methods

Animals

Seven adult male marmoset monkeys (*Callithrix jacchus*), aged 2-6 years with initial body weights between 350-550 g were obtained from Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was maintained at >60%. A 12-hour light-dark cycle was maintained with exception of the sleep deprived nights, when the light was continuously on during the night. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Communities Counsel Directive (86/609/EEC).

Study design

The study was based on a cross-over design in which every animal received each

compound tested. The baseline values were obtained during daytime in a non-sleep deprived state prior to the sleep deprivation nights.

The first experiment evaluated whether a steady state level of modafinil or caffeine was sufficient to counteract the effects of a 24-hour sleep deprivation. The animals were kept in their home cage without their sleep box. The animals were actively kept awake during the night by human intervention, like making noise and eye contact and removal of the sleep boxes. At different time points during the night the animals were tested on the hand-eye coordination task followed by the Bungalow test. Evening tests were performed between 20:30 h and 23:00 h, night tests between 1:00 h and 3:30 h and early morning tests between 5:30 h and 8:00 h. Animals were tested in the same order within and between the nights.

The doses of caffeine and modafinil were based on a behavioral study in our laboratory (partially described in chapter 2) and literature (Howell 1995). To achieve a steady state concentration of the compounds during the experiment, repeated administration of the compounds was necessary. The dose and timing of the administration was based on the pharmacodynamics of compounds used (Modafinil (100 mg/kg) peak-concentration: 10.7 μg/ml at 279 minutes, half-life: 277 minutes; Caffeine (30 mg/kg) peak-concentration: 16 μg/ml at 79 minutes, half-life: 595 minutes). Calculations indicated that caffeine had to be administered three times during the night in the doses of 30, 5 and 10 mg/kg to reach a steady state level during the night. Time of administration was every 4.5 hours starting 30 minutes before the first behavioral test. Modafinil had to be administered in a dose of 100 mg/kg 3 hours before the first behavioral test and in a dose of 25 mg/kg 9 hours later.

The second part of the study evaluated the efficacy of long-term administered modafinil or caffeine to counteract the effects of sleep deprivation. Therefore, modafinil (100 mg/kg, 2 hours before lights off) or caffeine (30 mg/kg, 1 hour before lights off) were administered daily for 14 days followed by an experimental night as described before in the first experiment. Furthermore, during the chronic administration period the hand-eye coordination and locomotor activity of the animals were also tested 2 hours before lights off at day 4, day 8 and day 12 during chronic administration.

Drug administration

All compounds were administered orally by gavage. Modafinil was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds: lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in water in a volume of 2 ml/kg. Caffeine (Sigma chemical Co. St. Louis, MO, USA) was dissolved in warm water. Caffeine was given in a volume of 1 ml/kg and freshly prepared before each administration

Behavioral assessment

Hand-eye coordination task: The hand-eye coordination task is a sensitive task for measuring coordinated motor movements and vigilance. An automated robot-guided apparatus using positive reinforcement as a motivating stimulus for the animals (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al., 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. The task during the nightly test sessions consisted of 46 trials. Three trial types were executed, namely a non-moving reward in the middle of the window and two horizontally moving rewards at different speeds (0.04 and 0.08 m/s). The non-moving rewards were presented 14 times and each type of moving reward 16 times in one session. The animal was allowed one minute to grasp a non-moving reward. The task during the daytime test sessions consisted of 42 trials with three trial types: one using a non-moving reward in the middle of the window and two horizontally moving rewards at different speeds (0.08 and 0.32 m/s). Higher speeds were used during daytime to investigate performance improvement. Each type of trial was presented 14 times in one session. Each trial was preceded by a sound signal, to alert the animal. A pressure detector on the robot and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before start of the study, all animals were trained to successfully grasp minimally 80% of the presented rewards.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Statistics

The data were analyzed using Repeated Measures (RM) ANOVA and paired t-test procedures in SPSS (SPSS Inc, Chicago, USA). Differences were considered to be statistically significant if $p<0.05$.

Results

Acute effects

Vehicle: Sleep deprivation leads to a reduction of activity found in the vehicle treated animals (Fig.1; RM, $p<0.001$]). The reduction in activity of these animals was clear during the night sessions and morning sessions compared to daytime values (paired t-test, $p<0.05$). No effects were found during the evening sessions. The reduction in the morning sessions was even more pronounced as the activity was significantly lower than the evening sessions (paired t-test, $p<0.05$). No effects were found during the evening session.

Sleep deprivation also negatively affected the performance on the hand-eye coordination task (Fig. 2; RM, $p<0.05$]). A decline in correct responses was found during the night and morning sessions (paired t-test, $p<0.05$) and not during the evening sessions.

Caffeine and modafinil: Caffeine and modafinil treated animals were more active during the sleep deprivated night than the vehicle treated animals (Fig. 1a, b; RM, drug*session, $p<0.01$]). During the night sessions the activity after both compounds was comparable to daytime activity. However, during the morning sessions a reduction of activity of the caffeine treated animals was found (paired t-test, $p<0.05$). Modafinil treatment showed opposite effects as the animals were more active than in the two other groups (paired t-test, $p<0.05$). Although they became more restful in the morning (paired t-test, $p<0.05$ vs. evening session), the activity was still comparable to daytime values.

The performance on the hand-eye coordination task after caffeine and modafinil treatment was also better than after vehicle treatment (Fig 2a, b; RM, drugs, $p<0.05$). During the night sessions the performance on the hand-eye coordination task was comparable to daytime level after treatment with caffeine or modafinil. This was also the case during the morning sessions after caffeine. However, the performance of the modafinil treated animals during the morning sessions was affected (paired t-test, $p<0.05$ vs. daytime values)

Table 1 Behavioral effects daytime chronic treatment. Effects of chronic treatment with modafinil and caffeine on the daytime activity and hand-eye coordination task performance tested before daily administration as a percentage of the daytime values (mean ± SEM) on day 4, 8 and 12.

		Modafinil	Caffeine
Locomotor activity	Day 4	32.6 ± 26.3	75.7 ± 11.9
	Day 8	109.2 ± 20.7	82.8 ± 17.9
	Day 12	104.8 ± 25.3	72.1 ± 16.2
Hand-eye coordination task	Day 4	100.9 ± 6.5	98.4 ± 8.8
	Day 8	109.6 ± 8.7	105.1 ± 5.5
	Day 12	105.6 ± 8.0	104.2 ± 6.1

The correct responses after caffeine treatment were comparable with daytime values. However, the response on the different speeds of reward presentation could be changed. This is indeed the case during the evening and night sessions after caffeine. There were more correct responses on rewards presented with the highest speed (speed 2) than on the non-moving rewards (speed 0) (Fig. 2a; paired t-test, $p<0.05$). During the morning session after caffeine as well as the other treatments the performance on the different speed was equally distributed.

Chronic effects

The chronic administration of modafinil or caffeine for 14 days was followed by a sleep deprivation night comparable with the protocol of acute effects. The chronic use of alertness enhancers before the sleep period did not affect locomotor activity and performance on the hand-eye coordination task during daytime (Table 1).

Caffeine: Chronic caffeine could not fully prevent the decline in activity during the sleep deprivation, as also observed after acute caffeine (Fig. 1a, RM, $p<0.05$). Despite this reduction, no significant differences were found between chronic caffeine and daytime activity.

Performance on the hand-eye coordination task during chronic treatment was comparable to daytime values as also noticed after acute treatment (Fig. 2a). After acute caffeine treatment changes of performance at different speeds of reward presenting were not observed.

Modafinil: Chronic treatment with modafinil showed the same positive effects on activity as acute treatment (Fig. 1b). The performance on the hand-eye coordination task of the chronic modafinil treated animals was comparable to the daytime values before sleep deprivation after all three sessions (Fig. 2b). More specifically, the chronic use of modafinil improved the performance during the morning session compared to the performance after acute modafinil treatment (paired t-test, $p<0.05$). Also a tendency in reduction of the performance during the evening sessions was observed, which was mostly due to reduction in response to non-moving rewards ($p<0.05$ vs. baseline of non-moving rewards).

Discussion

The marmoset monkey is not frequently used as a model for sleep deprivation. However, it was proved at our laboratory that sleep deprivation affect the homeostasis similarly in marmoset monkey compared to human, which indicate that this animal model is useful for sleep deprivation research (data not shown). Under normal conditions, the marmoset shows a distinct diurnal rhythm as the activity declines 1-2 hours before lights off, followed by a resting period until some time before lights on after 12 hours (Erkert 1989). Our findings indicate that 24 hours of sleep deprivation with constant lights on is enough to induce a time-dependent decline in vigilance, measured with the hand-eye coordination task and an increase

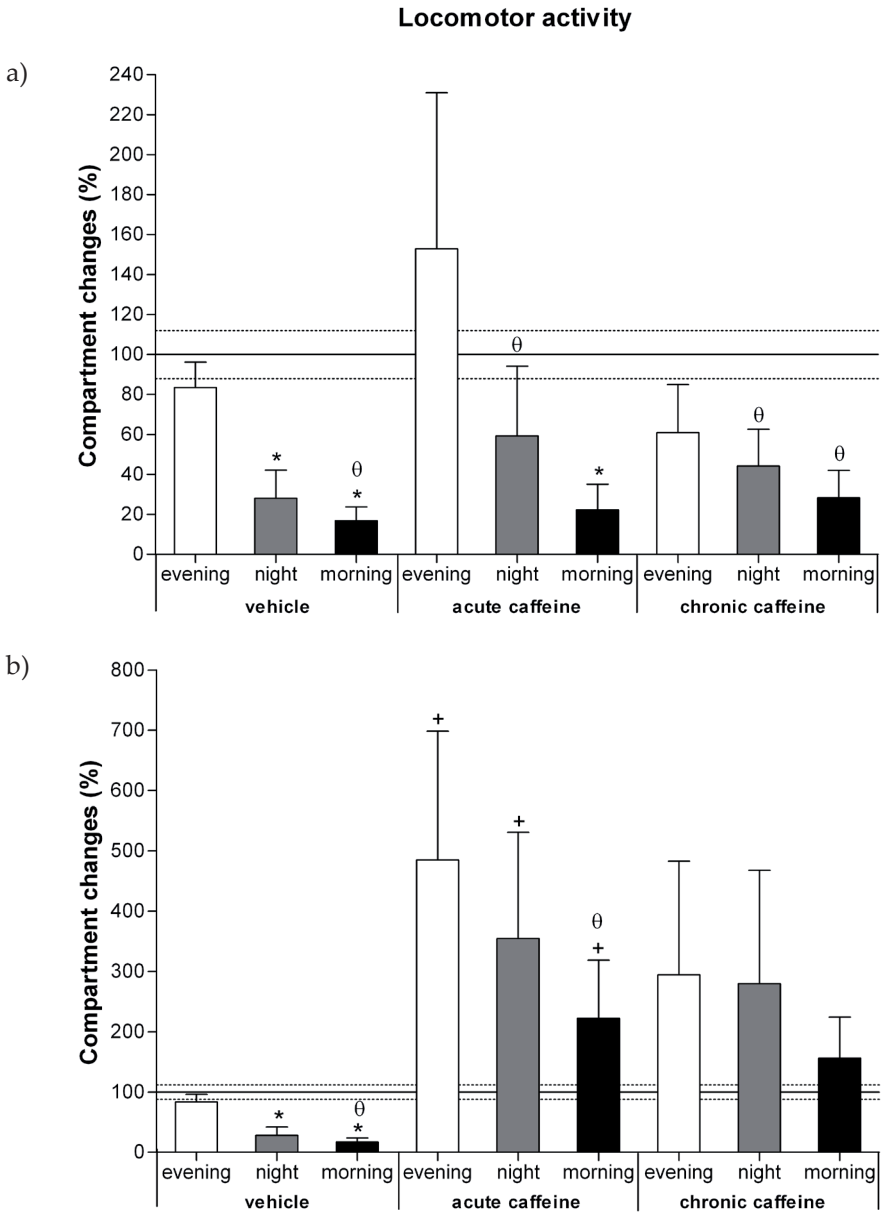


Fig. 1 Locomotor activity. Correct responses as percentage of daytime values (mean + SEM) of a) vehicle, acute and chronic caffeine treated sleep deprived animals and b) vehicle, acute and chronic modafinil treated sleep deprived animals. Solid and dotted lines indicate daytime level (mean ± SEM) without sleep disruption (100%). *p<0.05 vs. baseline values; +p<0.05 vs. vehicle; $^{\theta}$p≤0.05 vs. evening session within one treatment.

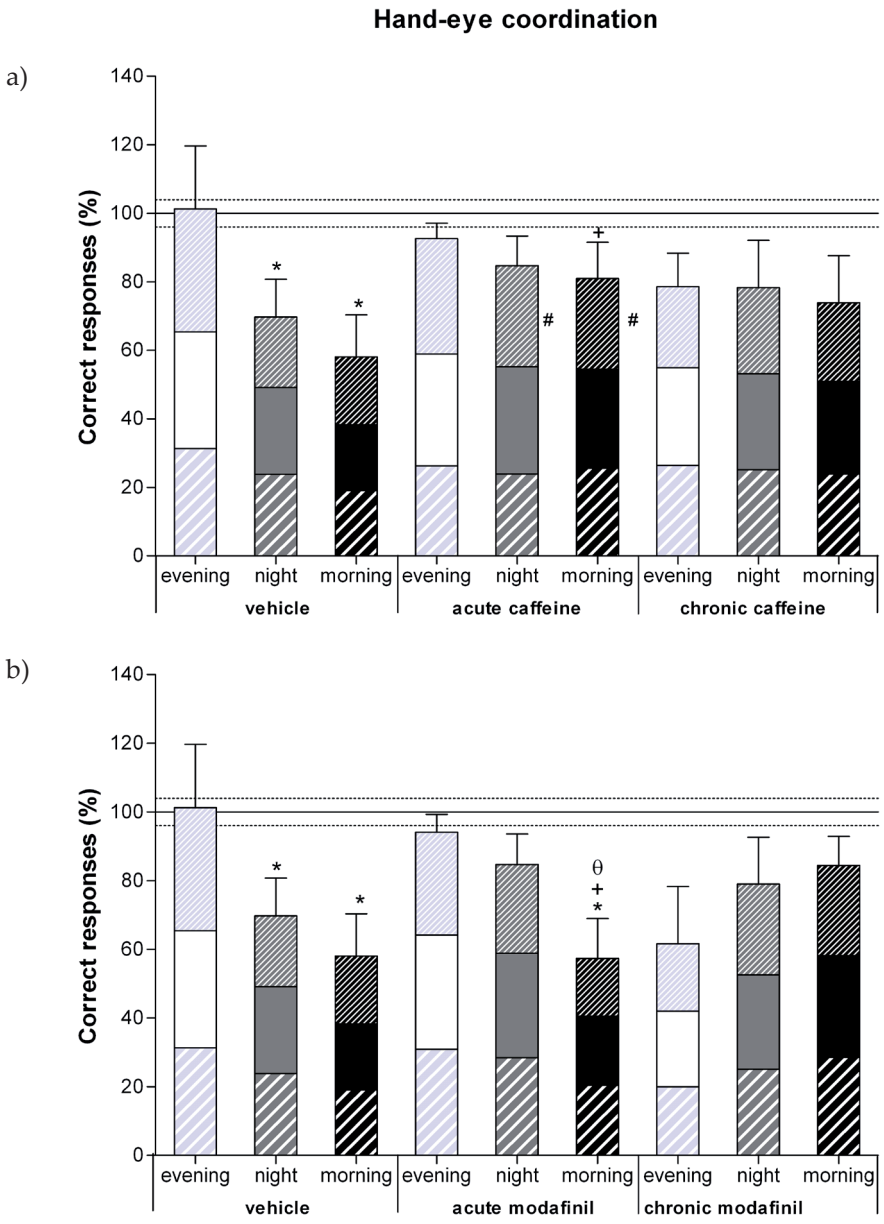


Fig. 2 Hand-eye coordination. Correct responses as percentage of daytime values (mean + SEM) of a) vehicle, acute and chronic caffeine treated sleep deprived animals and b) vehicle, acute and chronic modafinil treated sleep deprived animals. Thick diagonals indicate non-moving rewards; plain color indicates slow moving rewards; thin diagonals indicate fast moving rewards. Solid and dotted lines indicate daytime level (mean ± SEM) without sleep disruption (100%). *p<0.05 vs. baseline values; +p<0.05 vs. chronic treatment; $^{\theta}$p≤0.05 vs. evening session within one treatment; # p<0.05 vs. non-moving rewards.

in fatigue, measured with the locomotor activity test. The evening levels of fatigue and vigilance were similar to daytime values, but increased fatigue and the linked reduction in vigilance were clearly present during the night sessions with the nadir during the morning sessions. Accordingly, sleep deprivation effects on performance have been observed in other species and humans (Rogers et al. 2003, Porrino et al. 2005, Blatter et al. 2006). Animal models are often used in testing preclinical drugs. Nonetheless, investigation of the sleep deprivation counteracting effects of caffeine and the clinically approved compound modafinil in the marmoset and comparison with their effects in humans contributes to the validation of the marmoset sleep deprivation model. Therefore, the above described features in marmoset monkeys together with the availability of sensitive read out measures to asses vigilance and fatigue indicate that the marmoset monkey can be considered as a valid model for studying sleep deprivation.

In this study, we were particularly interested counteracting the effects of short-term sleep deprivation as this often occurs during shift work rather than extreme sleep deprivation. Besides the wake-promoting effects, it is also important to investigate the effects on daytime performance during chronic use of the test compounds. Therefore, the effects of chronic daily use of caffeine and modafinil before sleeping hours were measured. The chronic use of both compounds did not affect the fatigue and alertness during daytime (Table 1). Aside from absence of excessive fatigue or anxiety, no physical withdrawal effects were found during the daytime tests. This suggested that chronic use of these vigilance enhancers did not disturb normal daytime behavior.

Modafinil

Modafinil seems to be a good compound to counteract fatigue induced by sleep deprivation as the activity was clearly higher or comparable to daytime values during all sessions. Modafinil is known to increase activity in rodents and naïve marmosets (chapter 2, Simon et al. 1995, Ward et al. 2004), which is reflected in restlessness in humans (Randal et al. 2003).

Chronic modafinil had a tendency to induce a lower activity level as compared to the acute treatment. This shift in effect of chronic modafinil can be due to tolerance to the compound, although this is not reported in humans (Lyons et al. 1991, Mitler et al. 2000). Besides the positive effects on the activity, an improvement after modafinil treatment was found on the vigilance measured by the hand-eye coordination task. This effect was found after acute modafinil in the night session and after chronic use of modafinil in the night and morning sessions.

Caffeine

As caffeine is used on a daily basis, the wake-promoting efficacy of caffeine in habitual users could be diminished. Therefore, the effects between no previous caffeine use and long-term use were compared. Acute and chronic caffeine were comparably effective in counteracting the effects of sleep deprivation, although

both treatment protocols were not able to counteract the time-dependent increase in fatigue. Chronically treated animals developed some tolerance for caffeine reflected in a tendency towards a lower activity in the evening than that of the acutely treated animals, although this was not observed after the night and morning sessions.

Acute caffeine treatment resulted in an increased preference for fast-moving rewards and a decreased interest in the non-moving rewards. This can be caused by the arousal effects of caffeine. Increase in arousal improves performance on a task where relatively few sources of information have to be monitored, particularly under conditions where the need for selective attention is stressed by time pressure (Fredholm et al. 1999).

The preference for rewards at a certain speed of the hand-eye coordination task seems to be only sensitive in the first hours after acute caffeine use. During the morning sessions of the acute treatment the effects were more equal without changes in the total score and the preference was absent in the chronic caffeine treated animals. Probably the level of arousal induced by this treatment is different due to habituation to caffeine and therefore the preference for fast moving rewards is not apparent.

The present study showed that the wake-promoting compounds modafinil and caffeine were both able to postpone or prevent the effects of sleep deprivation on measurements of fatigue and vigilance. Modafinil seemed to be more effective in keeping the animals more active during the night and caffeine had a more positive effect on the psychomotor performance and vigilance. Chronic treatment did not have a negative effect on the efficacy of both compounds. Therefore, it can be concluded that the fast-acting compound caffeine seems to be the most favorable for temporarily sustaining the vigilance in habitual and non-habitual users. The slow-acting compound modafinil seems to be preferable in situations were prolonged wakefulness is necessary, but is less effective than caffeine in sustaining the performance on a hand-eye coordination task at daytime levels.

This research was supported by the Dutch Ministry of Defence. The views, opinions and/or findings contained in this chapter are those of the authors and should not be considered to reflect the views of the Dutch Ministry of Defence.

Chapter 4

Behavioral effects of Δ⁹-tetrahydrocannabinol in marmoset monkeys

Sanneke van Vliet, Marjan Jongsma, Raymond Vanwersch, Berend Olivier, Ingrid Philippens

Abstract

Cannabinoids have potential in the treatment of disorders associated with pain, nausea or loss of appetite and are also suggested to have neuroprotective characteristics. Most cannabinoid research is done in rodents, but with the emerging data on differences in the cannabinoid system between rodents and non-human primates, interest in the effects of cannabinoids in the latter species is increased. Therefore, the behavioral effects of oral Δ⁹-tetrahydrocannabinol (Δ⁹-THC) in marmoset monkeys were determined with a focus on motor-related behavior, startle response and anxiety-related behavior.

The dose response effects of Δ⁹-THC at oral doses of 2, 4, and 8 mg/kg were determined at thirty and ninety minutes after dosing by observational methods and measurements of locomotor activity, hand-eye coordination, response to a threat situation and startle response. 4 and 8 mg/kg Δ⁹-THC caused apathy, involuntary trunk and lower extremity movements, bradykinesia and affected balance during jumps. Δ⁹-THC reduced the startle response and anxiety-related behavior. After 2 and 4 mg/kg Δ⁹-THC, the activity was increased, test-dependently. No effects on task performance were found.

It can be concluded that Δ⁹-THC in the marmoset monkey dose-dependently induced effects that changed movement-related behavior, but did not affect performance in a motor coordination task. Δ⁹-THC also induced positive effects as reflected in reduction of anxiety-related behavior.

Introduction

Cannabinoids are a class of psychoactive compounds which have abundant effects in a large number of species. During the 1960s, Gaoni and Mechoulam (1964) were the first to isolate Δ^9-tetrahydrocannabinol from the leaves and flower tops of marijuana. Δ^9-THC is the major psychoactive constituent of marijuana, whereas other psychoactive constituents among a total of 66 are Δ^8-THC (less potent than Δ^9-THC), cannabinol and cannabidiol. Besides the phytocannabinoids, a range of synthetic derivatives of Δ^9-THC and antagonists and agonists of the cannabinoid receptors are produced. A third group of cannabinoids are endogenous ligands of cannabinoid receptors, anandamide and 2-arachidonylglycerol, indicative of the existence of an endocannabinoid system (Devane et al. 1992). These three groups of cannabinoids act on two receptors: first, cannabinoid CB_1 receptors located in the basal ganglia, substantia nigra pars reticulata, limbic structures and cerebellum (Herkenham et al. 1991, Glass et al. 1997), and second, cannabinoid CB_2 receptors found predominantly in the periphery on cells in the immune system and on the vasculature (Munro et al. 1993). Presynaptic cannabinoid CB_1 receptor stimulation reduces neuronal cell activity and attenuates, via retrograde signaling, the release of neurotransmitters including dopamine, noradrenaline, serotonin, γ-aminobutyric acid (GABA) and glutamate (Croxford 2003).

Because of their broad distribution through the brain and their modulatory effect on the major neurotransmitters, cannabinoids have great potential in the treatment of a variety of disorders related to the central nervous system. Spasticity in multiple sclerosis is reduced by Δ^9-THC (Petro et al. 1981) and levodopa-induced dyskinesia in Parkinson's disease (PD) is alleviated by nabilone, a cannabinoid agonist (Sieradzan et al. 2001). Analgesic properties of cannabinoids are found effective in multiple sclerosis, spinal cord injury and post-operative pain (Croxford 2003). Furthermore, dronabinol, the synthetic form of Δ^9-THC is clinically approved in cancer and HIV patients because of the beneficial anti-emetic and appetite-stimulating effects of this compound (Croxford 2003). Cannabinoids can prevent neuronal damage in a range of diseases, including cerebral ischemia, brain trauma, multiple sclerosis and Huntington's disease and against neuronal damage imposed by exposure to nerve agents (Filbert et al. 1999, Grundy et al. 2001).

Interest in the therapeutical and neuroprotective abilities of cannabinoids is increased after the relatively recent discovery of the two subtypes of the cannabinoid receptor (Howlett et al. 2002). This also resulted in data indicating differences in behavioral effects of cannabinoids in rodents and non-human primates. For example, with cannabinoid agonists catalepsy is found in rats but not in non-human primates and combined therapy with dopamine and cannabinoid ligands resulted in sedation of different levels in monkeys and rats (Meschler et al. 2000a, b, 2001). The different cannabinoid CB_1 receptor distribution in rats and non-human primates mainly in the basal ganglia can cause this discrepancy (Tsou et al. 1998, Ong and Mackie 1999). Therefore, research in the non-human primate seems appropriate, since the effects of cannabinoid agonists and antagonists in these species are probably more similar to

those in humans than in rodents (Meschler et al. 2001). Furthermore, the marmoset monkey has proven to be a good model for PD, multiple sclerosis and ischemic stroke (Marshall et al. 1999, Genain and Hauser 2001, Dauer and Przedborski 2003), diseases in which cannabinoid treatment is of potential interest. In the past cannabinoid research in monkeys, mostly rhesus monkeys, focused more on drug discrimination and self administration (e.g. Tanda and Goldberg, 2003). There has not been extensive research on the behavioral effects of cannabinoids in marmosets, except for a recent contribution of Meschler et al. (2000b).

In the present study, the behavioral and functional effects of orally administered doses of 2, 4 and 8 mg/kg Δ⁹-THC were assessed using observation scales and behavioral test systems. Besides a general observation scale, the focus of the other behavioral tests was on assessment of movements, startle response and anxiety-related behavior as these features are among the most prominent effects of Δ⁹-THC (Chaperon and Thiebot, 1999) and might be of value in the therapeutic fields of recent interest such as movement-related disorders and anxiety disorders (Brotchie et al. 2003, Witkin et al. 2005, Madsen et al. 2006). Behavior related to emotional state was assessed using the human threat test and the startle response. Δ⁹-THC effects on movements were assessed with an observation scale measuring the abnormal involuntary movements (AIMS) and a behavioral test system measuring locomotor activity. The hand-eye coordination task assessed coordinated motor performance.

Materials and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus)*, aged 2-6 years with initial body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments confirming to the European guidelines for Laboratory Animal Care.

Study design

In each animal measurements were performed before and after Δ⁹-THC administration. Δ⁹-THC was orally administered in doses of 2 (n=6), 4 (n=6) and 8 (n=8) mg/kg. Each animal was subjected to each test in a semi-crossover design. Doses and time points of measurements chosen were based on the commonly used oral dose of Δ⁹-THC in non-human primates and the pharmacokinetics of oral Δ⁹-THC in humans (Perlin et al. 1985, Aigner 1988, Grotenhermen 2003). Consequently,

30 minutes after administration, the presence of symptoms was observed and rated with the observation scales and the human threat test was carried out, followed by the hand-eye coordination task at 40 minutes post-dose. At 55 minutes post-dose, locomotor activity was assessed, followed by the startle response at 80 minutes post-dose. Startle response data were based on six animals after 8 mg/kg Δ^9-THC due to a technical failure. Ninety minutes post-dose, the observation of symptoms and the human threat test were repeated to follow behavioral changes over time. Male and female animals were distributed evenly over the groups. Ethanol in the amounts used to dissolve and to administer Δ^9-THC had no intrinsic behavioral effects (established in separate experiments, data not shown).

Drugs

Δ^9-THC given in doses of 2 and 4 mg/kg was dissolved in 28 mg/ml ethanol (Sigma Chemical co., St. Louis, USA) and 8 mg/kg Δ^9-THC was dissolved in 50 mg/ml ethanol (IBL, Leiden, The Netherlands). Δ^9-THC was administered by gavage simultaneously with 1 ml/kg freshly prepared syrup (Karvan Cevitam with water (1:1)).

Behavioral assessment

Observation of signs and symptoms: For the observation of signs and symptoms two rating scales were used. 1) A general clinical scoring list in which the condition of the animal was rated. The following symptoms were registered: inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility; rigidity and presence of tremors. The degrees of severity were coded from 0 (normal) to 4 (severe). 2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976, Di Monte et al. 2000). The AIMS is widely used clinically for qualification of involuntary movements, occurring in PD (Katzenschlager et al. 2004). These scales have successfully been applied to monkey research in our institute for more than 10 years. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity, and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occurred due to stimulation by the observer were rated one step lower than those observed spontaneously.

Auditory startle response: The auditory startle reflex is a motor response following an intense sound stimulus. The apparatus to measure startle response in marmoset monkeys has been described earlier and has been validated by Philippens et al. (2000). The animals were placed in a transparent plastic tube on a pressure transducer in an illuminated sound attenuated box. Twenty startle stimuli (20 ms, 120 dB, white noise) were delivered in random order (inter stimulus interval: 14 ± 4 seconds). For the duration of 200 ms, directly after the stimulus presentation, the force exerted by the animal was registered. The startle reflex was represented by the amplitude of the response.

Human threat test: The human threat test is a non-human primate putative model of anxiety. Marmosets will exhibit fear-related behavior in the presence of a human observer in front of the cage (Carey et al. 1992, Van Vliet et al. 2005). The most pronounced behavior would be retreating to the back of the cage and showing characteristic postures. The behavior was assessed in the home cage (40 x 60 x 60 cm) with a hanging basket in the back of the cage, a wooden board (20 x 10 cm, 30 cm above cage floor) on the left side in the back and on the other side a perch, at the same height, positioned from the back to the front of the cage. To assess the behavior, the observer stood approximately 30-100 cm from the cage front and made eye contact with the marmoset throughout a 2-minute test period. During this period the movements, behavior and position of the marmoset in the cage were recorded by video registration.

A range of parameters was obtained according to Carey et al. (1992), based on Stevenson and Poole (1976): 1) The number of characteristic postures exhibited: tail posture (tail raise to present the genital region), scent marking (the anal and genital area is pressed against the substrate to be marked with the excretion of the glands), arched pilo-erection (arched back posture with full body pilo-erection), slit stare (stare with the eyes half closed in combination with tufts flattened and exposure of the teeth), rearing (upright position with flexed paws), twisting (head and torso movement from side to side), 2) The time spent in the front of the cage, 3) The number of position changes in the cage, 4) The number of movements from the back of the cage to the front, 5) The number of jumps from the left side of the cage to the right side or vice versa.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Hand-eye coordination task: An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed one minute to grasp the non-moving reward. Each type

of trial was presented 14 times per session. At the beginning of each trial a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

Statistics

The results are presented as mean ± SEM and parametric statistical analysis was applied. The study can be considered as a semi-within design, since some animals received multiple doses, however not all doses. An overall repeated measures (RM) analysis was applied where the dose-group (the animals receiving a certain dose of a certain treatment) was the between-factor and the treatments, control and Δ^9-THC, were included as the within-factor. For example, animals receiving one of the doses of Δ^9-THC were compared control vs. drug. If $p<0.05$, a paired t-test was applied to test each dose-group for a difference between the treatment groups. For example, animals receiving 2 mg/kg Δ^9-THC were compared control vs. drug. For this analysis, the significance level of $p<0.05$ was used.

Results

Observation of signs and symptoms

In Table 1 the mean score of the affected animals and the number of affected animals at each observational parameter are presented. The behavior was within normal ranges after 2 mg/kg Δ^9-THC as the severity of the symptoms was normal in more than half of the animals. There was only a minimal change in trunk movement after 90 minutes observed in 5 out of 6 animals.

After 4 and 8 mg/kg Δ^9-THC more minimal to mild changes in behavior were observed. Thirty minutes after 4 mg/kg increased movements of trunk and lower extremities and increased severity were found in at least half of the animals. At 90 minutes grooming and apathy were observed to be worse than in the earlier observations. After 8 mg/kg at 90 minutes immobility and increased apathy, trunk movements and severity were observed in at least half of the animals.

Additional observations, not covered by the observation scales, at 30 and 90 minutes after 4 and 8 mg/kg Δ^9-THC included impairment of balance during jumps and increased bradykinesia in most animals and diminished vocalization, and wet eyes.

Behavioral tests

Δ^9-THC affected the amplitude of the startle response (Fig. 1) and the 'number of body postures' of the human threat test (Fig. 2a). Dose-specifically, the startle

response was significantly reduced after 4 mg/kg Δ⁹-THC ($p<0.05$) and the body postures were decreased 30 minutes after 2 mg/kg Δ⁹-THC ($p<0.05$) and 90 minutes after all doses ($p<0.05$).

The movement-related tests showed contradictory results as no change was found in activity in the bungalow test (Fig. 3), but in the three activity-related parameters of the human threat test were increased, namely the 'number of jumps from side to side', the 'number of movements forward' and 'the number of position changes' (Fig. 2b, c, d; RM, jumps: $p<0.001$, forward and position: $p<0.05$). More specifically, the 'jumps from side to side' were increased 30 and 90 minutes after 2 and 4 mg/kg Δ⁹-THC (paired t-test, $p<0.05$).

The performance on the hand-eye coordination task was not affected after any dose at both time points (data not shown).

Discussion

This study was aimed at the assessment of the dose range of orally administered Δ⁹-THC for behavioral effects in marmoset monkeys. Overall, 4 and 8 mg/kg Δ⁹-THC slightly induced apathy, increased trunk and lower extremity movements,

Table 1 Clinical score and AIMS. Overview of the mean scores of the affected animals on the items of the clinical score and AIMS 30 and 90 minutes after administration of 2, 4 and 8 mg/kg Δ⁹-THC. The most interesting changes are in bold.

		Δ⁹-THC					
		2 mg/kg		4 mg/kg		8 mg/kg	
		30 min.	90 min.	30 min.	90 min.	30 min.	90 min.
Clinical score							
Grooming		1 (2/6)	1 (3/6)	1.5 (2/6)	**1.4 (5/6)**	1.5 (2/8)	1.7 (3/8)
Apathy		0	1 (1/6)	1 (2/6)	**1.3 (4/6)**	1.3 (3/8)	**2 (4/8)**
Rigidity		1 (1/6)	0	2 (1/6)	1.5 (2/6)	1.5 (2/8)	**2 (4/8)**
Immobility		0	0	0	0	0	2 (1/8)
Tremors		0	0	0	0	0	0
Total affected animals		*2/6*	*4/6*	*3/6*	*5/6*	*4/8*	*6/8*
AIMS test							
Facial, oral mov.	Facial expression	0	0	0	1.3 (3/6)	1 (1/8)	1 (1/8)
	Lips, peri-oral area	1 (1/6)	1 (1/6)	1 (2/6)	1 (2/6)	0	1 (1/8)
	Jaw	0	0	1 (1/6)	0	0	0
	Tongue	0	0	0	0	0	0
Extremity mov.	Upper	0	0	1 (1/6)	0	1 (1/8)	0
	Lower	1 (2/6)	1 (2/6)	**1.2 (5/6)**	**1.4 (5/6)**	1 (1/8)	1 (3/8)
Trunk mov.	Neck, shoulder, hips	1 (2/6)	1 (5/6)	**1.3 (4/6)**	1 (6/6)	1.3 (3/8)	**1.3 (6/8)**
Global judgment	Severity	1 (1/6)	1 (2/6)	**1.3 (4/6)**	1 (5/6)	1 (1/8)	**1.1 (7/8)**
	Incapacitation	0	0	1 (2/6)	1 (3/6)	0	1.3 (3/8)
Total affected animals		*2/6*	*5/6*	*6/6*	*6/6*	*6/8*	*7/8*

Score of symptoms 0: normal; 1: minimal, but extreme normal; 2: mild; 3: moderate; 4: severe (# of affected animals/ total group size). Total affected animals: # of animals with ≥ 1 symptom/ total group size. Mov. indicates movements.

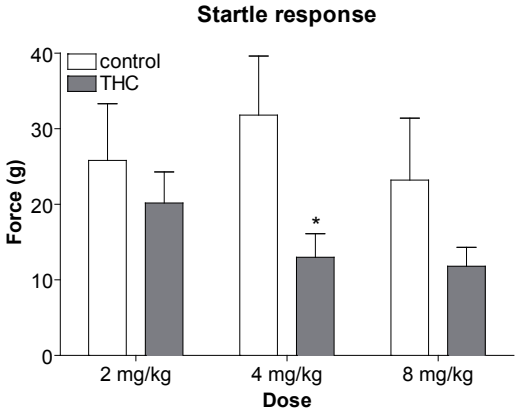


Fig. 1 Startle response. Bars show mean (+ SEM) of the amplitude of control (before administration) and after 2, 4 and 8 mg/kg Δ⁹-THC. *p<0.05 vs. control.

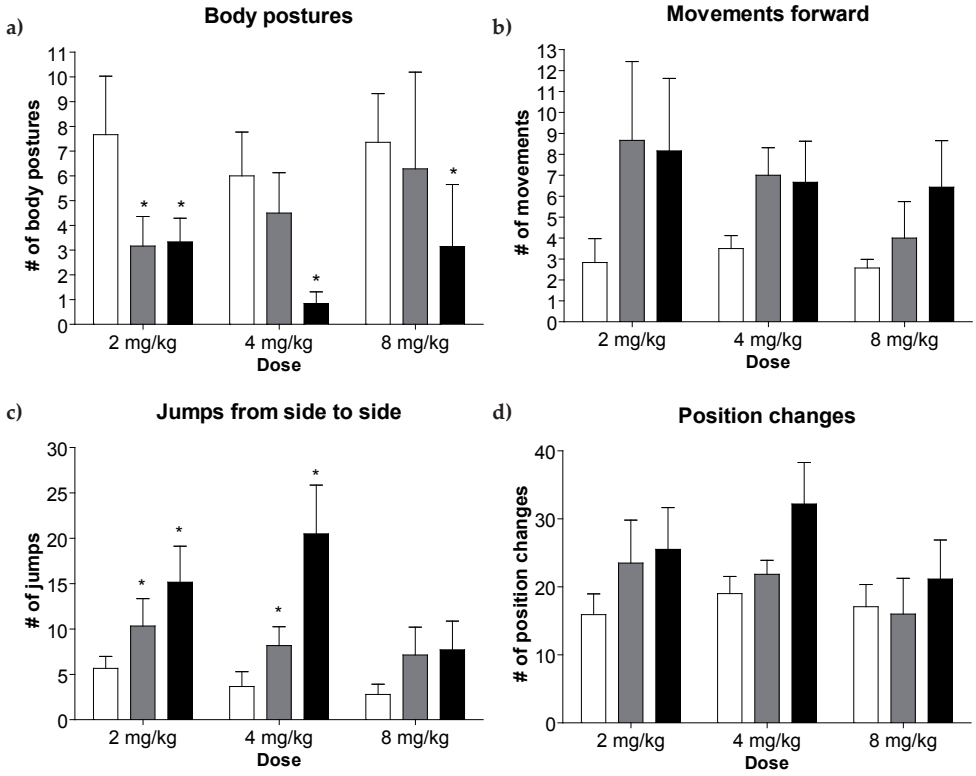


Fig. 2 Human treat test. Bars show mean (+ SEM) of 2, 4 and 8 mg/kg of Δ⁹-THC. White bar: control (before administration); grey bar: 30 minutes after Δ⁹-THC; black bar: 90 minutes after Δ⁹-THC. *p<0.05 vs. control.

bradykinesia and affected balance during jumps as measured with the observational scales. Startle response and anxiety-related behavior were reduced after all doses. Activity was increased test-dependently after 2 and 4 mg/kg Δ⁹-THC. No effects on hand-eye coordination were found.

In this study Δ⁹-THC was orally administered, because therapeutic use of Δ⁹-THC preferably should have a longer lasting effect, which cannot be achieved by inhalation of Δ⁹-THC. Inhalation, as in smoking is the most used method of Δ⁹-THC intake in humans. With the latter route, Δ⁹-THC has been detected in human plasma after 3-10 minutes and after 1 hour has the plasma concentration is minimal. After oral intake, the level of Δ⁹-THC in human plasma is maximal at 60-120 minutes and has an elimination half-life of 25 hours (Grotenhermen 2003). In monkeys the maximum concentration in blood plasma occurs 10 times later after oral administration than after intramuscular injections (Perlin et al. 1985). A disadvantage of oral use of Δ⁹-THC is the lower bioavailability due to degradation by the acid of the stomach, the gut and the first-pass effect (Grotenhermen 2003).

In the present study, the different effects of Δ⁹-THC on the activity seem to be test-dependent. The observations and the human threat test both include parameters for the assessment of activity and were both home cage observations. The difference between these two tests is the interaction with the observer. During normal observation the observer is present in the room without purposely drawing attention, whereas during the human threat test the human observer incites a response by being near the cage and making eye contact. This difference in interaction resulted in an increased activity during the human threat test after 2 and 4 mg/kg Δ⁹-THC, while a higher rating of immobility in some animals after 8 mg/kg Δ⁹-THC during normal observation was found. In the computerized Bungalow test exploratory and locomotor activity were assessed in another environment without human interaction

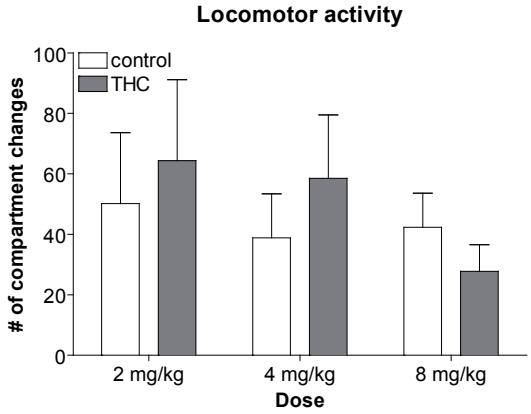


Fig. 3 Locomotor and exploratory activity. Bars show mean (+ SEM) number of compartment changes of control values (before administration) and after 2, 4 and 8 mg/kg Δ⁹-THC.

and were not significantly affected after the three doses of Δ^9-THC.

Intramuscular administration of Δ^9-THC to cynomolgus monkeys did not result in a decrease of locomotor activity, although increased bradykinesia was observed with increasing doses (Meschler et al. 2001). These results are in agreement with our findings in the Bungalow test and observation scales. In contrast, there is a decrease in activity after levonantradol, a cannabinoid agonist, and decreased activity or even catalepsy in rodents after Δ^9-THC (Chaperon and Thiebot 1999, Meschler et al. 2001). The lack of decreased activity is may be due to the Δ^9-THC doses used in both monkey studies, this study and of Meschler et al. (2001), they were probably too low for the induction of this effect. Therefore, higher doses of Δ^9-THC are required to observe immobility after Δ^9-THC in non-human primates.

In this study the quality of movements was also assessed by observation of the animals. The observed bradykinesia, apathy and ataxia are in accordance with observations in other non-human primate studies after treatment with cannabinoid receptor agonists (Young et al. 1981, Beadsley et al. 1987, Meschler et al. 2001).

After cannabinoid use, humans and animals often experience emotional effects (Navarro et al. 1993, Chaperon and Thiebot 1999). Therefore, in this marmoset study the effects of Δ^9-THC on anxiety-related behavior were investigated with the human threat. Furthermore, the startle response, which can be affected by a different emotional state, was measured.

The startle response was reduced after 4 mg/kg Δ^9-THC and a trend towards a reduction was found after 8 mg/kg Δ^9-THC. This corresponds to other rare reports about acoustic startle response and cannabinoids: A decreased startle response was found after the cannabinoid mimetic levonantradol (Geyer 1981) and after the cannabinoid agonist CP 55,940 in rats (Mansbach et al. 1996).

The human threat test showed that the anxiety-related parameter, the 'number of body postures', was reduced after all Δ^9-THC doses. Reduction of body postures is interpreted as a reduction of anxiety, as shown with the anxiolytic diazepam (Carey et al. 1992, Van Vliet et al. 2005). The human threat test parameter 'time spent in front' has also been reported to be sensitive for anxiolytics, but due to variation in baseline anxiety of marmosets this is not always demonstrated (Van Vliet et al. 2005). Because of this high variation within the test population this parameter was excluded in this study.

Effects of Δ^9-THC or other cannabinoid CB_1 receptor agonists on anxiety are rarely reported in non-human primates (Miczek 1978, Chaperon and Thiebot 1999). Studies of cannabinoids on anxiety in rodents show a dose and test-dependent biphasic profile (Chaperon and Thiebot 1999, Witkin et al. 2005). Anxiolytic effects were found after low doses of cannabinoids in various tests in different species (Onaivi et al. 1990, Valjent et al. 2002).

The human threat test measures anxiety and activity parameters. It could be argued that the increase in activity could influence the body postures. Carey et al. (1992) showed that the activity and anxiety measured in the human threat test cannot be directly related to each other because inverse relations are also observed.

Therefore, they conclude that the change in activity during the human threat test can be interpreted as a measure of the ability of anxiolytic and anxiogenic agents to alter anxiety in the marmoset. This also supports the suggestion that the difference in activity measured in the human threat test and the Bungalow test is due to a differential effect of the environment as discussed earlier.

This report is one of the few studies investigating the movement and emotion-related behavioral effects of oral Δ⁹-THC in the marmoset monkey in a range of behavioral observation and test systems. Direct comparison with other studies was hampered due to the differences in administration route, species and dose. Despite this, most of the results are in agreement with findings after other cannabinoid agonists in humans and animals. Still, the inability of Δ⁹-THC to clearly reduce the mobility or the lack of catalepsy observed in this study emphasize the differences between non-human primates and rodents. Therefore, this study underlines the necessity for further knowledge about the behavioral effects of Δ⁹-THC in non-human primates.

Part II

Symptomatic effects

Chapter 5

Therapeutic effects of Δ⁹-tetrahydrocannabinol and modafinil in a marmoset Parkinson model

Sanneke van Vliet, Raymond Vanwersch, Marjan Jongsma, Berend Olivier, Ingrid Philippens

Manuscript submitted

Abstract

Current therapies for Parkinson's disease (PD) like levodopa and dopamine (DA) agonists have declined efficacy after long-term use. Therefore, research towards supplementary or alternative medication is needed. The implementation in PD can be expedited by application of compounds already used in the clinic. In this study, the therapeutic effects of the psychoactive compounds Δ⁹-tetrahydrocannabinol (Δ⁹-THC) and modafinil are tested in the 1-methyl-1,2,3,6-tetrahydropyridine (MPTP)-marmoset model for PD.

The anti-parkinson effects of Δ⁹-THC (4 mg/kg) and modafinil (100 mg/kg) in parkinsonian marmosets were assessed with two behavioral rating scales covering parkinsonian symptoms and involuntary movements and two test systems assessing the locomotor activity and hand-eye coordination.

Δ⁹-THC improved activity and hand-eye coordination, but induced compound-related side-effects. Modafinil improved activity and observed parkinsonian symptoms but not hand-eye coordination. It can be concluded that both compounds have therapeutic values and could supplement existing therapies for PD.

Introduction

PD is a progressive neurodegenerative disorder characterized by akinesia, bradykinesia, rigidity, resting tremor and postural instability. At a neuronal level it is characterized by deterioration of DAergic neurons in the substantia nigra pars compacta (SNpc). The decrease in DAergic tone results in a disturbed balance in the neural circuitry of the basal ganglia (Wichmann and DeLong 1998).

Current PD therapy includes levodopa and DA receptor agonists. These compounds are still the most effective, although long-term medication does not prevent complications such as motor fluctuations and dyskinesia (Lang and Lozano 1998b). As the side-effects are related to direct intervention in the DA system, compounds which are more indirectly affecting the DA system would be good alternatives or supplements to the current DA replacement therapies. Research towards compounds, which are already used by humans for other purposes, can expedite the implementation in PD. Two psychoactive compounds, modafinil and Δ^9-THC do fulfill these criteria and have gained more interest in therapeutic application in PD.

Modafinil is a vigilance-enhancing compound, and used is as treatment for excessive daytime sleepiness (EDS) in narcolepsy (Bastuji and Jouvet 1988, Green and Stillman 1998), but is also suggested to be effective against EDS in PD (Adler et al. 2003, Happe et al. 2001, Nieves and Lang 2002). The mechanisms by which modafinil produces sustained waking are still not clarified. Modafinil is suggested to indirectly increase noradrenaline release via indirect interference with DA release (Wisor and Eriksson 2005). This effect of modafinil on DA could also be of interest in the treatment of motoric complications in PD. The increase locomotor activity in animals after modafinil treatment substantiates this potential (Simon et al. 1995, Ward et al. 2004, chapter 2). Modafinil is also suggested to modulate GABA and glutamate release in some nuclei of the basal ganglia (Ferraro et al. 1997, 1998). Via this pathway modafinil could also establish alleviation of PD-symptoms.

The other compound with therapeutic potential for PD is Δ^9-THC, a cannabinoid derived from the leaves and flower tops of marijuana *(Cannabis sativa)*. Δ^9-THC is used by patients for among others its analgesic properties and ability to reduce spasticity in multiple sclerosis (Croxford 2003). There are also reports, however inconsistent, about PD patients benefiting from cannabinoid use, although more research is needed towards the most effective cannabinoid (Sieradzan et al. 2001, Venderova et al. 2004). Stimulation of cannabinoid CB_1 receptors, among others localized in the basal ganglia, can modulate release of neurotransmitters via interaction with DA receptors and via retrograde signaling the GABA and glutamate release (Brotchie 2003). The cannabinoid system is involved in the PD pathology as an increased number of cannabinoid CB_1 receptors in the basal ganglia of parkinsonian humans, non-human primates and rats is found compared to the distribution in healthy brains (Silverdale et al. 2001, Lastres-Becker et al. 2001). The changed number of receptors is thought to be a compensating mechanism against the degeneration of DAergic innervation (Brotchie 2003). Therefore, intervention via

cannabinoid CB_1 receptors to modulate a disturbed balance is often suggested as a possible therapy against the PD symptoms (Brotchie 2003). In this study Δ⁹-THC, a partial agonist of the cannabinoid CB_1 receptor (Howlett et al. 1999), is tested for its anti-parkinsonian properties. This major constituent of marijuana is an often used cannabinoid in the clinic (Croxford 2003).

To evaluate the potency of modafinil and Δ⁹-THC to alleviate PD symptoms, the effects of these compounds were tested in the MPTP marmoset model. In this animal model, the neurotoxin MPTP, which selectively damages DAergic neurons in the SNpc is used to induce a pathology resembling the human form of PD. Resulting in clear and lasting behavioral features (Jenner and Marsden 1986), which reflects many aspects of human PD symptoms (Gerlach et al. 1991). This is illustrated by the application of a clinically used observational scale for abnormal involuntary movements (AIMS) to the marmoset (Di Monte et al. 2000) without adaptation. The comparability with the human symptoms indicates the suitability of the marmoset model for testing effects of anti-parkinsonian therapy.

In this study, extensive behavioral tests were used to asses the effects on motor function in a detailed fashion. Two rating scales, the clinical score and AIMS, assessing parkinsonian features as immobility, rigidity, tremors and involuntary movements were applied. For quantitative measurement, the activity and mobility of the animals were tested in the Bungalow test (Philippens et al. 2000) and the effects on more complex motor behaviors were tested in the hand-eye coordination task (Philippens et al. 2000).

Material and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus),* aged 2-6 years with body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands. The animals were matched for sex within the study design. The ambient temperature was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained. All animal procedures were approved by local laws and are in line with European Community guidelines.

Study design

Marmosets were treated with total 6.0-8.75 mg/kg MPTP s.c. over 10 days till stable moderate parkinsonian symptoms were established. After recovery of the acute MPTP effects, nine parkinsonian animals received a single oral dose of modafinil (100 mg/kg) and nine parkinsonian animals a single oral dose of Δ⁹-THC (4 mg/kg). Seven parkinsonian animals served as control and received the vehicle (10% sugar solution). The doses and time point of the therapeutic measurement of modafinil were based on behavioral dose effect studies of modafinil in naïve marmosets (chapter 2). The dose and time point of the therapeutic measurement of Δ⁹-THC chosen was

based on a commonly used oral dose of Δ^9-THC in non-human primates and the pharmacokinetics of oral Δ^9-THC (Perlin et al. 1985, Aigner 1988, Grotenthermen 2003) and dose effect studies in our laboratory (data not shown).

Modafinil (Modiodal®; d,1-2-[(diphenylmethyl)sulfinyl]acetamide) was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds such as lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in a 10% sugar solution in a dose volume of 1 ml/kg. Δ^9-THC was given in doses of 4 mg/kg (50 mg/ml ethanol (96%), minimal 90% pure, IBL, Leiden University, The Netherlands) and orally administered simultaneously with 1 ml/kg 10% sugar solution. Ethanol was used to dissolve Δ^9-THC and had no intrinsic behavioral effects as established in separate experiments (unpublished data).

Behavioral assessment

Two hours after modafinil or one hour after Δ^9-THC administration the behavioral effects were assessed by observations with two rating scales, the clinical score and AIMS, followed by the hand-eye coordination task and assessment of the activity in the Bungalow test. Two animals of the vehicle group were excluded from the Bungalow test because they were hyperactive before disease induction. Before disease induction, baseline values of all test systems were obtained and the animals were trained on the hand-eye coordination task.

Observation of signs and symptoms: For the observation of signs and symptoms two rating scales were used. 1) A general clinical scoring list in which the condition of the animal was rated. The following symptoms were registered: inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility; rigidity and presence of tremors. The degrees of severity were coded from 0 (normal) to 4 (severe). 2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976, Di Monte et al. 2000). The AIMS is widely used clinically for qualification of involuntary movements, occurring in PD (Katzenschlager et al. 2004). These scales have successfully been applied to monkey research in our institute for more than 10 years. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity, and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occurred due to stimulation by the observer were rated one step lower than those observed spontaneously.

Hand-eye coordination task: An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving reward in the middle of the window, one using a slow horizontally

moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed one minute to grasp the non-moving reward. Each type of trial was presented 14 times per session. At the beginning of each trial a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Statistics

The data of the Bungalow test and the hand-eye coordination task were statistically analyzed using a one-way ANOVA in SPSS (SPSS inc, Chicago, USA). The behavioral observation data (clinical score and AIMS) were statistically analyzed using the Mann-Whitney procedure for two unrelated groups. The difference between behavioral score pre- and post-dose of each treatment was used, since twice a day was scored to exclude variation disturbing these sensitive observation scales. Differences were considered to be statistically significant if $p<0.05$.

Results

Modafinil

Modafinil improved the locomotor activity of the parkinsonian animals measured in the Bungalow test (Fig. 1a, one-way ANOVA, $p<0.05$). The activity was comparable with the activity before disease induction (paired t-test, $p>0.05$). The improved behavior was also confirmed by the rating scales. Both the total clinical score and total AIMS score (Fig. 2a, b, Mann-Whitney test, $p<0.05$) showed reduction in the score, meaning an alleviation of the parkinsonian symptoms. Furthermore, the item of the AIMS indicating incapacitation was significantly reduced, confirming the increase in activity measured in the Bungalow test. The alleviation of the parkinsonian behavior

did not result in improved performances on the hand-eye coordination task (Fig. 1b, one-way ANOVA, p>0.05).

Δ⁹-THC

Δ⁹-THC (4 mg/kg) was also able to improve the locomotor activity of parkinsonian animals in the Bungalow test (Fig. 3a, one-way ANOVA, p<0.05). The activity was

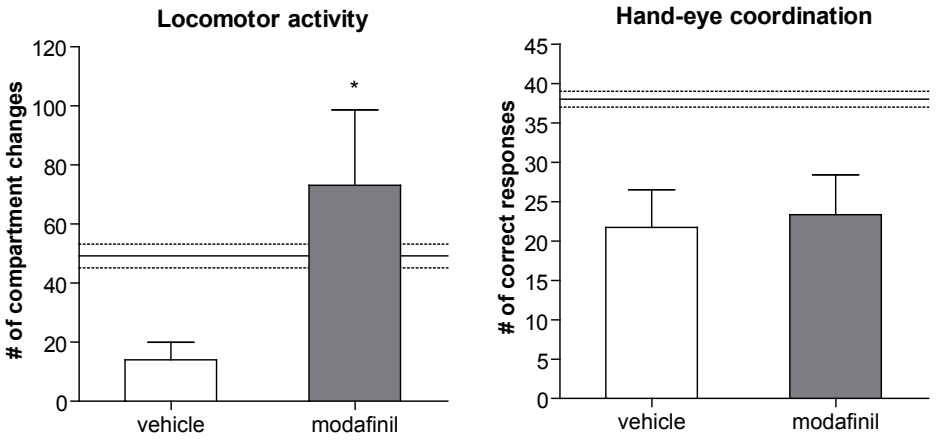


Fig. 1 Behavioral tests. Mean + SEM of number of compartment changes in the Bungalow test and number of correct responses in the hand-eye coordination task of vehicle and modafinil treated parkinsonian animals. Solid and dotted line indicated mean ± SEM of baseline before PD-symptom induction. *p<0.05 vs. vehicle.

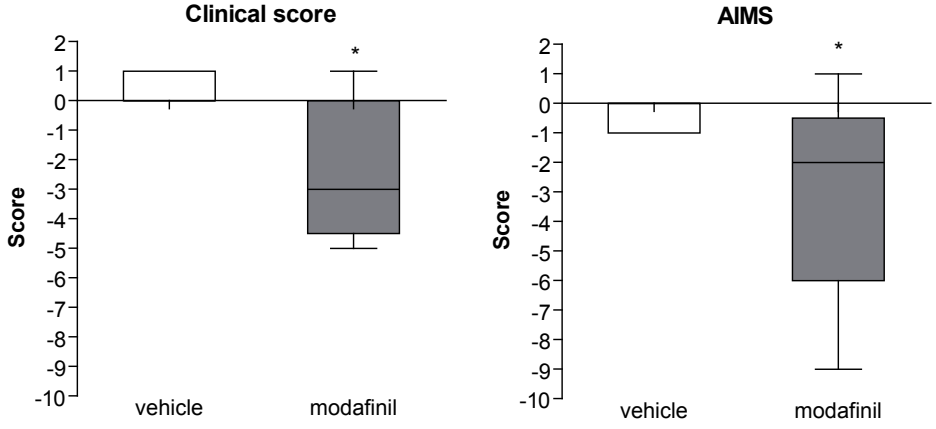


Fig. 2 Behavioral observation scales. Boxplots with mean and SEM of the difference pre- and post-dose in clinical score and AIMS of vehicle and modafinil treated parkinsonian animals. Baseline before PD-symptom induction was 0. Reduction indicates alleviation of the symptoms. *p<0.05 vs. vehicle.

even comparable to the movement level before disease induction (paired t-test, $p>0.05$). The more complex movements were also improved, reflected in an increased performance on the hand-eye coordination task (Fig. 3b, one-way ANOVA, $p<0.05$), although the performance was not comparable with pre-disease level (paired t-test, $p<0.05$). The total scores of clinical score did not change (Fig. 4a, Mann-Whitney, $p>0.05$) and the total AIMS score indicated that the overall rating of the involuntary movements were worsened (Fig. 4b, Mann-Whitney, $p<0.05$).

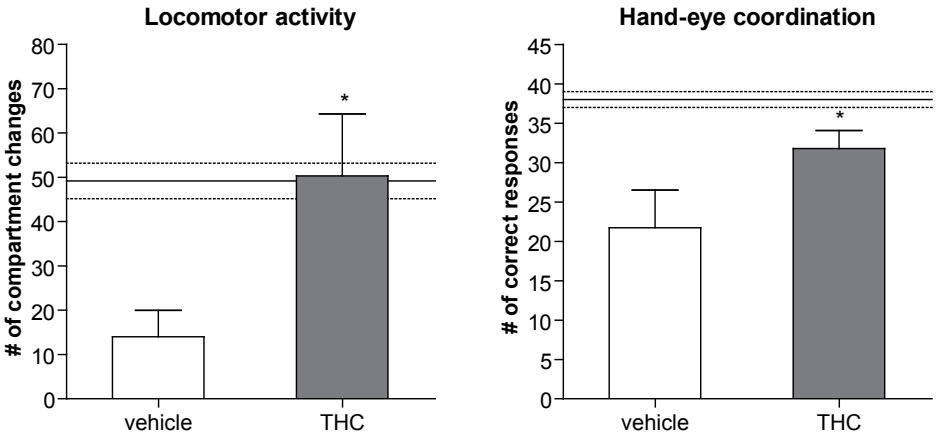


Fig. 3 Behavioral tests. Mean + SEM of number of compartment changes in the Bungalow test and number of correct responses in the hand-eye coordination task of vehicle and Δ⁹-THC treated parkinsonian animals. Solid and dotted line indicated mean ± SEM of baseline before PD-symptom induction. *$p<0.05$ vs. vehicle.

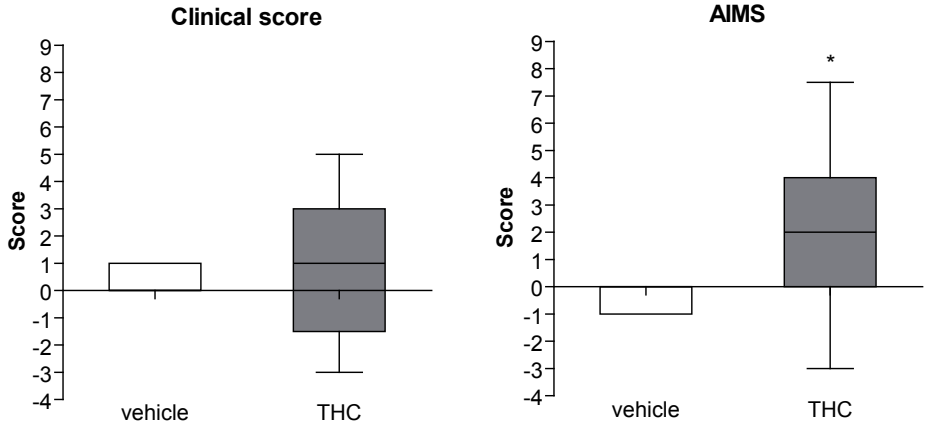


Fig. 4 Behavioral observation scales. Boxplots with mean and SEM of the difference pre- and post-dose in clinical score and AIMS of vehicle and Δ⁹-THC treated parkinsonian animals. Baseline before PD-symptom induction was 0. Reduction indicates alleviation of the symptoms. *$p<0.05$ vs. vehicle.

Discussion

This study showed that two psychoactive drugs with opposite behavioral effects, modafinil, a stimulant and Δ^9-THC, a depressant, are both able to alleviate part of the parkinsonian symptoms in marmoset monkeys. Modafinil and Δ^9-THC were included in this study because of their ability to indirectly interfere with DA release and their effects of GABA and glutamate release. Modulation of these neurotransmitters is thought to counterbalance the disturbed output of the basal ganglia.

Modafinil

Oral modafinil reduced some symptoms of PD. Modafinil was able to increase the locomotor activity without stereotypies, but did not affect the more complex movements needed for the hand-eye coordination task. The effects on locomotor activity are in line with other MPTP-marmoset studies (Jenner et al. 2000). Modafinil is known as a vigilance and arousal-enhancing compound, reflected in naïve non-human primates and rodents as increased locomotor activity (Simon et al. 1995, Ward et al. 2004, chapter 2). MPTP treated non-human primates have a low arousal state and narcolepsy-like daytime naps (Daley et al. 1999). Therefore, the increased locomotor activity found in the modafinil treated parkinsonian animals seems to be generated by the arousal effects of modafinil. This indicates that the low arousal state is a major factor in the inactivity of the parkinsonian animals as illustrated by observations that severely parkinsonian animals were still able to move if they had the urge to reach a goal, i.e. to obtain food. Although the effect on arousal is an effect on non-motor symptoms, the increased arousal state can also affect the motor symptoms via feedback from the wake-promoting areas to the basal ganglia, i.e. the pedunculopontine nucleus (PPN), an area involved in promoting wakefulness, innervates the subthalamic nucleus (STN) and can therefore temper the excitatory output (Wichmann and DeLong 1998).

Despite the effects on gross movements, modafinil was not able to increase the performance on the hand-eye coordination task. Deterioration of the reach and grasp movements in PD is due to loss of control over the execution of complex movements as the basal ganglia increases the speed and efficiency of cortical processing (Gentilucci and Negrotti 1999). The most effective PD medication levodopa was also not able to improve some aspects of skilled movements in human PD patients and parkinsonian rodents (Castiello et al. 2000, Metz et al. 2001). This may indicate that restoration of this executive function of the basal ganglia needs more specialized targeting.

Δ^9-THC

This study indicated that oral Δ^9-THC has beneficial effects on the alleviation of the parkinsonian symptoms. It restored locomotor activity to nearly pre-disease levels and also the hand-eye coordination performance was clearly improved. The same dose of Δ^9-THC given to naïve marmosets at our laboratory did not change the locomotor activity in the Bungalow task and the hand-eye coordination task (data

not published). This indicates that the basal ganglia system after MPTP intoxication becomes more sensitive for the modulatory actions of Δ^9-THC. Which is in line with the finding that the number of striatal cannabinoid CB_1 receptors is increased in parkinsonian marmosets (Lastres-Becker et al. 2001) resulting in enhanced cannabinoid CB_1 receptor signaling.

Our findings of the anti-parkinson effects of Δ^9-THC, a cannabinoid receptor agonist, are in contrast to the beneficial effects of cannabinoid CB_1 receptor antagonists as often suggested in the literature based on *in vivo* rodent data (Romero et al. 2002, Brotchie 2003). In theory, based on findings in a normal functioning circuitry, the anti-parkinsonian and pro-parkinsonian actions of a cannabinoid CB_1 receptor agonist depend on the focus to a specific area of the basal ganglia (reviewed by Brotchie 2003). Stimulation of cannabinoid CB_1 receptors in the striatum could alleviate the symptoms due to reduction of the enhanced glutamate drive to striatal output neurons projecting to the globus pallidus externa (GPe). On the other hand, stimulation of cannabinoid CB_1 receptors in the GPe could worsen the symptoms as the GABAergic drive to the GPe increases due to retrograde signaling and therefore amplify the inhibiting output of the basal ganglia (Gerdeman and Lovinger 2001). Activation of cannabinoid CB_1 receptors in the globus pallidus interna (GPi) has no net effect as it reduces the glutamatergic drive from the STN, but also the GABAergic drive from the striatum (Sanudo-Pena et al. 1998). Experimental rodent data has shown that the cannabinoid CB_1 receptor antagonist rimonabant (SR141716A) enhances the anti-parkinsonian properties of a dopamine D_2 receptor agonist (Di Marzo et al. 2000), whereas an agonist reduces this effect (Maneuf et al. 1997). It is also argued in favor of the use of cannabinoid CB_1 antagonists because cannabinoid CB_1 agonists in naïve rodents also elicit parkinsonian symptoms like catalepsy.

In non-human primates, Δ^9-THC does not induce catalepsy, although other behavioral effects of Δ^9-THC are comparable to rodents (Meschler et al. 2001). Also the cannabinoid CB_1 receptor antagonist SR141716A was not able to alleviate the parkinsonian symptoms in the MPTP-treated primate (Meschler et al. 2001) as proven in rodents (Di Marzo et al. 2000). The species difference is also emphasized by a different distribution of cannabinoid CB_1 receptors through the basal ganglia and differences in DA-CB_1 receptor interaction (Tsou et al. 1998, Ong and Mackie 1999). These differences between rodents and non-human primates argue for a difference in functioning of the endocannabinoid system between species. As almost all anti-parkinsonian effects of cannabinoids as described above are generated in rodents, both the theoretical approach and practical results can be different in non-human primates.

It may be suggested that a cannabinoid CB_1 receptor agonist can produce anti-parkinsonian effects because in a small dose range the cannabinoid CB_1 signaling in the striatum may overrule the effects of cannabinoid CB_1 signaling in the GPe in the non-human primate. This is in line with the expression of more cannabinoid CB_1 receptors in the striatum than in the GP compared to rodents (Tsou et al. 1998, Ong and Mackie 1999).

In contrast to the positive results on motor functioning, no improvements were found on the observation scales. Notable was the worsening of the total AIMS

score. These effects are not necessarily related to an increase of the parkinsonian symptoms as these effects were also observed after the same dose of Δ^9-THC given to naïve marmosets at our laboratory (data not published).

This study is a first attempt in research towards the therapeutic value of modafinil and Δ^9-THC to alleviate the parkinsonian symptoms in the marmoset monkey. Increased focus towards specific symptoms could further define the most efficacious application of the compound within the current range of medication. However, current data show potential for both compounds to be used as a supplement to the current symptomatic therapies. Modafinil could serve as an alternative to levodopa in the early stages of the disease or could be used a concomitant therapy to reduce the side-effects related to reduced arousal. Δ^9-THC could also be applied as concomitant therapy together with levodopa as it could improve the complex movements, which levodopa fails to improve (Castiello et al. 2000, Metz et al. 2001).

Part III

Neuroprotective effects

Chapter 6

Neuroprotective effects of modafinil in a marmoset Parkinson model: behavioral and neurochemical aspects

Sanneke van Vliet, Raymond Vanwersch, Marjan Jongsma, Jan van der Gugten, Berend Olivier, Ingrid Philippens

Behavioral Pharmacology (2006) 17: 453-462

Abstract

The psychostimulant modafinil has neuroprotective properties: it prevents striatal ischemic injury, nigrostriatal pathway deterioration after partial transsection and intoxication with 1-methyl-1,2,3,6-tetrahydropyridine (MPTP). The present study determines the protective effects of modafinil in the marmoset MPTP Parkinson model on behavior and on monoamine levels.

Twelve marmoset monkeys were treated with a total dose of 6 mg/kg MPTP. Simultaneously, six animals received a daily oral dose of modafinil (100 mg/kg) and six animals received vehicle for 27 days. Behavior was observed daily and the locomotor activity, hand-eye coordination, small fast movements, anxiety-related behavior and startle response of the animals were tested twice a week for three weeks. Modafinil largely prevented the MPTP-induced change in observed behavior, locomotor activity, hand-eye coordination and small fast movements, whereas the vehicle could not prevent the devastating effects of MPTP. Dopamine (DA) levels in the striatum of the vehicle + MPTP treated animals were reduced to 5% of control levels, whereas the DA levels of the modafinil + MPTP treated animals were reduced to 41% of control levels.

The present data suggest that modafinil prevents decrease of movement-related behavior and DA levels after MPTP intoxication and can be a potent pharmacological intervention in the treatment of Parkinson's disease (PD).

Introduction

In PD the output of the basal ganglia is irreversibly affected due to degeneration of the neuromelanin-containing DAergic neurons in the substantia nigra pars compacta (SNpc). This results in manifestation of symptoms including akinesia, postural instability, rigidity and resting tremors (Dauer and Przedborski 2003). PD is incurable, since present medications (predominantly with levodopa) do not counteract progression of the disease and long-term medication is associated with declined efficacy and increased side-effects (Clarke 2004). Therefore, a better strategy aims to focus on prevention of the neuronal loss in an attempt to stop or slow down the progression of the disease. One way to achieve neuroprotection is via pharmacological interference aimed at crucial steps in the neuronal cell death process to promote neuronal survival. Although some potential drug candidates were tested in clinical trails there is no proven neuroprotective treatment yet (Clarke 2004).

The actual cause of PD is unknown. There is evidence suggesting that factors like mitochondrial dysfunction, oxidative stress, excitotoxicity and inflammatory processes, either separately or cooperatively, are involved in the neurodegenerative process causing PD (Alexi et al. 2000).

Modafinil is a vigilance-stimulating compound and marketed for treatment of narcolepsy (Bastuji and Jouvet 1988). The mechanism of modafinil is not clear, although it is suggested to increase indirectly wakefulness via α1-noradrenergic neurotransmission (Duteil et al. 1990) but also γ-aminobutyric acid (GABA) release is reduced by it in sleep-related areas and striatum (Ferraro et al. 1996, 1998). Modafinil influences DA release whereas knocking out the DA transporter prevents the stimulative properties of modafinil (Wisor et al. 2001).

Modafinil could also be very promising as a neuroprotective compound. Modafinil in cultured cortical cells prevented glutamate toxicity (Antonelli et al. 1998), it prevented increases in toxic aspartate and glutamate levels after striatal ischemic injury caused by endothelin-1 in rats (Ueki et al. 1993b) and it prevented development of lesions in the hippocampus induced by the neurotoxic nerve gas soman (Lallemant et al. 1997). After partial transsection of the DA pathway (Ueki et al. 1993a) and also in MPTP-induced PD models in mice (Fuxe et al. 1992) and marmosets (Jenner et al. 2000) modafinil protected dopaminergic neurons from degeneration. The latter two suggest that modafinil could be a candidate drug for neuroprotection in PD at behavioral and neuronal level. However, more insight into the effects of modafinil on different DA and non-DA-related symptoms and the relation to neuronal function is needed. Therefore, the present study focuses on putative neuroprotective effects of modafinil in the marmoset MPTP Parkinson model with extensive behavioral tests and biochemical measurements. Earlier studies did not include these parameters.

This MPTP Parkinson model is the most used experimental model for PD (Dauer and Przedborski 2003). The neurotoxic agent MPTP selectively damages neurons in the SNpc by blocking the electron transport chain of the mitochondria

leading to a loss in mitochondrial function resulting in a depletion of ATP and eventually cell death. MPTP is effective in mice and marmosets. However, the mouse MPTP model is not suitable for behavioral studies because parkinsonian symptoms do not develop clearly and disappear within a few days (Schmidt and Ferger 2001). The marmoset model is more suitable for behavioral studies because marmosets show after MPTP treatment clear and lasting behavioral features, which reflect many aspects of human Parkinson symptoms (Jenner and Marsden 1986). Even a clinically used observational scale for involuntary movements (AIMS) can be applied to the marmoset without adaptation (Di Monte et al. 2000).

In the present study, the protective effects of modafinil are tested on behavior using two extensive behavioral observation scales for PD and functional tests measuring locomotor activity, hand-eye coordination, small fast movements and the startle response. The human threat test was applied to measure whether anxiety-related behavior is sensitive for changes induced by MPTP and is possibly changed after a neuroprotective intervention with modafinil.

Another important marker for neuroprotection is the protection of monoaminergic neurotransmission in the brain. In PD and the MPTP model DA levels in the striatum, the main area receiving DAergic output from the SNpc, are most heavily affected due to reduction of the neurons in the SNpc. Metabolites and other monoamines, noradrenaline (NA) and serotonin (5-HT) can also be as markers for neuronal damage and neuroprotection and are studied in brains of vehicle and modafinil treated parkinsonian animals and control brains.

In addition to the work of Jenner et al. (2000), the neuroprotective effects of modafinil against PD-induction on the functional outcome, with an extensive battery of behavioral tests, and on neurotransmitter levels are described in this paper. Results of the measurements with magnetic resonance imaging (MRI) and spectroscopy (MRS) and with immunohistochemistry will be covered in chapter 7.

Materials and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus)*, aged 2-6 years with initial body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

Twelve naïve marmosets were treated in total with 6 mg/kg MPTP s.c. over 9 days (day 1: 2 mg/kg and days 2, 3, 6 and 9: 1 mg/kg). Six of these animals (4 males; 2 females) received a daily oral dose of 100 mg/kg modafinil from experimental day 1 until day 27. The remaining six animals (3 males; 3 females) received a daily oral dose of the vehicle (10% sucrose solution). The dose of modafinil was based on the lowest effective dose in naïve marmosets (chapter 2), which was in accordance with a MPTP study in mice (Fuxe et al. 1992) and a comparable study in marmoset monkeys (Jenner et al. 2000). By using this dose the parallels in the behavioral aspects with the study of Jenner et al. (2000) can be used to increase the comparability and therefore increase the value of contribution to research. The oral modafinil or vehicle treatment was given directly after the s.c. MPTP injections. Of the vehicle group one animal died during the anesthesia procedure before the MRI-scan, therefore the data of this animal is omitted.

Modafinil (Modiodal®, d,1-2-[(diphenylmethyl)sulfinyl]acetamide) was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds: lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in a 10% sucrose solution in a dose volume of 1.5 ml/kg.

The occurrence of parkinsonian symptoms were observed daily before and after administration of the treatment using two rating scales: clinical score and AIMS. On day 13, 17, 20, 24 and 27 the behavioral tests, namely the hand-eye coordination task, locomotor activity, startle response and small fast movements were tested in noninvasive test systems. The human threat test was executed on these days simultaneously with the behavioral observations. Before disease induction, the animals were trained on the hand-eye coordination task and baseline values of all test systems were obtained. The 'after administration' behavioral observations and tests were started two hours after administration. This time span reflects the peak activity of modafinil in marmoset monkeys based on the pharmacokinetic results of modafinil in our institute (Philippens et al. 2006) and the study of Jenner et al. (2000) and the t_{max} of modafinil in humans (Robertson and Hellriegel 2003).

As modafinil is a psychostimulant, these temporary symptomatic effects on motor function can be present besides the neuroprotective effects of modafinil. Therefore, a distinction is made between before and after administration: the behavioral observations were done twice a day and most tests were performed either before the daily administration on day 13, 20 and 27 or two hours after administration on days 17 and 24. Only the small fast movements test was tested in opposite order. In the figures this distinction is indicated with solid and striped bars.

Behavioral assessment

Observation of signs and symptoms: For the observation of signs and symptoms two rating scales were used. 1) A general clinical scoring list in which the condition of the animal was rated. The following symptoms were registered: appetite, inadequacy of

grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility; rigidity and presence of tremors. The degrees of severity were coded from 0 (normal) to 4 (severe). 2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976). The AIMS is widely used clinically for qualification of involuntary movements, occurring in PD (Katzenschlager et al. 2004). These scales have successfully been applied to monkey research in our institute for more than 10 years. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity, and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occurred due to stimulation by the observer were rated one step lower than those observed spontaneously.

Hand-eye coordination task: An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed one minute to grasp the non-moving reward. Each type of trial was presented 14 times per session. At the beginning of each trial a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Small fast movements test: Small fast movements are very hard to detect by observation. Therefore, an automated test system, which makes use of a capacitive transducer, was used. Changes in the transducer capacitance resulted in a signal, which was constructed of different behavioral components. Gross movements to the extremities

were filtered out. Only small fast movements were detected. A transparent plastic tube (diameter: 18 cm and height: 26 cm) was placed in a homogeneous electrical field, created by an electrical potential difference across two vertically placed metal plates. Since the animal, situated in the plastic tube between the plates, was a conducting medium, any change in posture of the animal lead to a change of plate capacitance. Both plates were, via a buffer amplifier, driven at the same potential as the detection plate. These signals were amplified, filtered (5-20 Hz) and fed into the AD converter. Crosses above the noise level were used as an indication for the small fast movements.

Human threat test: The human threat test is a non-human primate putative model of anxiety. Marmosets will exhibit fear-related behavior in the presence of a human observer in front of the cage (Carey et al. 1992, Van Vliet et al. 2005). The most pronounced behavior would be retreating to the back of the cage and showing characteristic postures. The behavior was assessed in the home cage (40 x 60 x 60 cm) with a hanging basket in the back of the cage, a wooden board (20 x 10 cm, 30 cm above cage floor) on the left side in the back and on the other side a perch, at the same height, positioned from the back to the front of the cage. To assess the behavior, the observer stood approximately 30-100 cm from the cage front and made eye contact with the marmoset throughout a 2-minute test period. During this period the movements, behavior and position of the marmoset in the cage were recorded by video registration.

A range of parameters was obtained according to Carey et al. (1992), based on Stevenson and Poole (1976): 1) The number of characteristic postures exhibited: tail posture (tail raise to present the genital region), scent marking (the anal and genital area is pressed against the substrate to be marked with excretion of the glands), arched pilo-erection (arched back posture with full body pilo-erection), slit stare (stare with the eyes half closed in combination with tufts flattened and exposure of the teeth), rearing (upright position with flexed paws), twisting (head and torso movement from side to side), 2) The time spent in the front of the cage, 3) The number of position changes in the cage.

Auditory startle response: The auditory startle reflex is a motor response following an intense sound stimulus. The apparatus to measure startle response in marmoset monkeys has been described earlier and has been validated by Philippens et al. (2000). The animals were placed in a transparent plastic tube on a pressure transducer in an illuminated sound attenuated box. Twenty startle stimuli (20 ms, 120 dB, white noise) were delivered in random order (inter stimulus interval: 14 ± 4 seconds). For the duration of 200 ms, directly after the stimulus presentation, the force exerted by the animal was registered. The startle reflex was represented by the amplitude of the response.

High performance liquid chromatography (HPLC) analysis

For determination of brain monoamine levels, four MPTP-treated animals from the modafinil group and five animals from the vehicle group were used. Furthermore,

six brains of naïve animals were used to establish control values of the monoamines. At day 37, ten days after the last modafinil administration, brains were removed after decapitation of the sedated animals. The striatum of one hemisphere was isolated after termination and was directly frozen in liquid nitrogen. The tissue (100-500 mg) was weighed and homogenized in 10 ml 0.4 M perchloric acid containing 20 ng/ml 3,4-dihydroxybenzylamine hydrobromide (Sigma Chemical co., St. Louis, USA) and 20 ng/ml (±)-isoproterenol hemisulfate salt (Sigma Chemical co., St. Louis, USA) as internal standards. Homogenate was centrifuged at 22,000 g for 30 minutes at 4 °C and 1 ml of supernatant was adjusted to about pH 4.0 with 250 µl 2 M sodium acetate. The homogenate samples were stored at -70 °C for a maximum of 6 weeks. The monoamines NA, DA and 5-HT, and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 5-hydroxyindole-3-acetic acid (5-HIAA) were determined by ion-pair reversed phase liquid chromatography. A 2-50 µl sample was injected on a RP18 LiChrosfer 100 column (125 x 4 mm i.d., 5 µm particle size; Merck, Darmstadt, Germany) connected to a Thermo Separations Products (San Jose, CA, USA) model P100 solvent delivery pump and AS300 autosampler and a Coulochem II Model 5011 electrochemical detector (ESA, Bedford, MA, USA). The mobile phase consisted of a 30 mM citrate/40 mM phosphate buffer, pH 4.0, containing 0.27 mM Na_2EDTA, 1.8 mM heptanesulphonic acid and 5% methanol. The potential of the electrode was set at 420 mV. External standards were determined in each assay run. Calibration plots were linear from 2 to 500 ng/ml for each compound. The lower limit of detection was 2 ng/ml. The intra-assay coefficient of variation amounted to 2%.

Statistics

The results of this study are presented as mean ± SEM and parametric statistical analysis was applied with a significance level of $p<0.05$. The scores of the behavioral observation scales were analyzed with an independent t-test to reveal differences between the two treatment groups. The results of the behavioral tests were analyzed in two ways. First, the difference between the two treatment groups was obtained. Therefore, an overall repeated measure (RM) analysis was applied on the results of day 13, 20 and 27 and of day 17 and 24 (see below). When relevant, an independent t-test was applied. Second, the difference between baseline and test day results of each treatment values was tested with a paired t-test.

Temporary symptomatic effects of modafinil were tested via comparison of observational data before administration (Fig. 1) and after administration (data not shown) with a paired t-test. These effects in the behavioral tests were analyzed with a comparison between pooled data of day 13 and 20 vs. day 17, and pooled data of day 20 and 27 vs. day 24 with independent t-tests. Because of the alternate test schedule, the comparison of the small fast movements was made between the data of day 13 vs. day 17 and day 24 vs. day 27 and pooled data of day 17 and 24 vs. day 20 with independent t-tests.

The difference between the monoamine levels of each treatment was tested with an one-way ANOVA followed by a t-test when relevant.

Results

Behavioral observation

During the first three experimental days, after receiving the first two MPTP injections, all animals developed similar symptoms (Fig. 1). Hereafter, a discrepancy between the groups emerged. The vehicle treated parkinsonian animals showed a mild parkinsonian symptomatology, whereas the modafinil treated parkinsonian animals (from day 3) were not affected by the last MPTP injections and ended with rather weak parkinsonian symptoms. In the modafinil treated parkinsonian group all parameters of the clinical score were present until day 17. After day 17 decreased appetite, rigidity and tremors were most pronounced symptoms, whereas scores of the inadequacy of grooming, apathy and immobility were returned to normal values. In the vehicle treated group all parameters were apparent during the whole experiment.

Hand-eye coordination

On all five test days the performance on the hand-eye coordination task of the modafinil treated parkinsonian animals was clearly better than the performance of the vehicle treated parkinsonian animals (Fig. 2; RM, $p=0.002$ (before administration (BA)) and $p=0.004$ (after administration (AA)); t-test $p<0.05$). Although an improvement of the performance of the vehicle treated parkinsonian animals over time was observed (RM, $p=0.023$ (BA)).

The hand-eye coordination of the modafinil treated parkinsonian animals was comparable to the performance before the disease induction, only at day 13 and 20 the performance was slightly lower (paired t-test, $p<0.05$). The hand-eye coordination of the vehicle treated parkinsonian animals was worse on all test days than at baseline level (paired t-test, $p<0.05$).

Locomotor activity

The modafinil treated parkinsonian animals were more active in the Bungalow test than the vehicle treated parkinsonian animals (Fig. 3; RM, $p=0.012$ (BA), $p=0.004$ (AA)). More specific, the locomotor activity of the modafinil treated group was significantly higher on experimental days 17, 24 and 27 (t-test, $p<0.05$), but also on experimental day 13 a difference between the two treatments was present (t-test, $p=0.09$).

The activity of the modafinil treated parkinsonian animals was comparable with the baseline activity before PD-induction. The activity of the vehicle treated parkinsonian animals was clearly reduced compared to their baseline values on all test days (paired t-test, $p<0.05$, except day 27).

Small fast movements test

The small fast movements were tested to establish whether modafinil was able to restore the lack of these fine motor movements after MPTP. The small fast movements of the vehicle treated parkinsonian animals were less present than before the disease induction (Fig. 2). This was clear after experimental days 13, 17 and 20 (paired t-test, $p<0.05$). The small fast movements of the modafinil treated parkinsonian animals were at the same level as before the disease induction. Therefore, a difference between the experiment groups was also found (RM, $p=0.006$ (AA), $p=0.014$ (BA)).

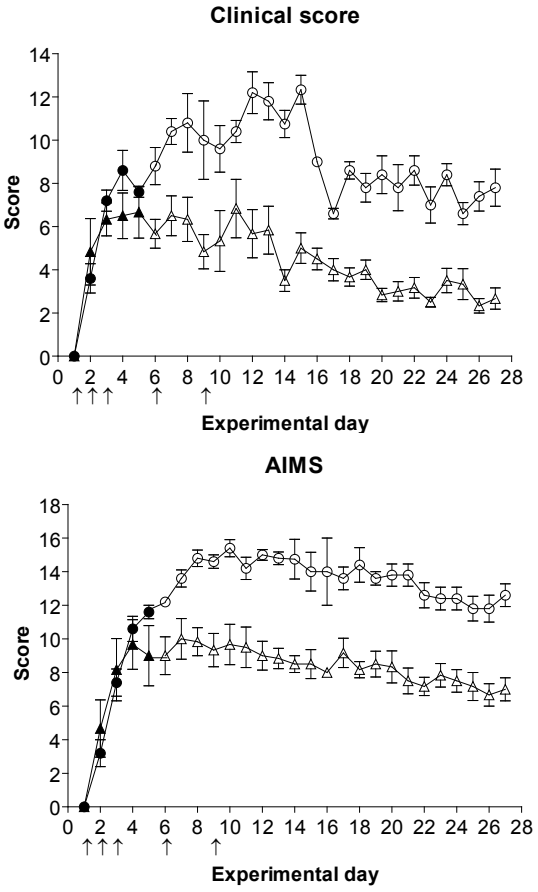
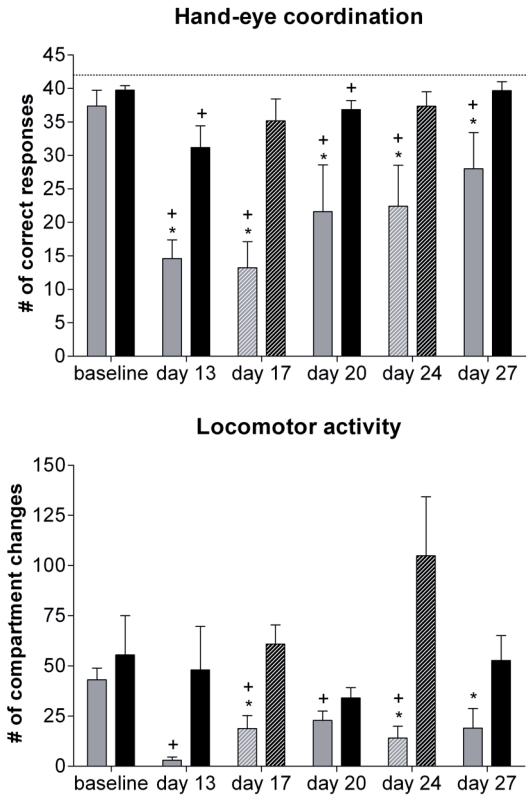


Fig. 1 Behavioral observation scales. Mean (± SEM) of the clinical (a) and AIMS (b) score before daily administration. Circles indicate the vehicle treated parkinsonia animals and triangles modafinil treated parkinsonian animals. On experimental days 14-16 only n=2-4 were scored due to MRI and MRS-scans of part of the animals (chapter 7). Arrows indicate day of MPTP injection. Modafinil or vehicle was given from day 1-27. Open data points indicate significant difference between vehicle and modafinil treated parkinsonian animals (t-test, $p<0.05$).

Human threat test

The two anxiety-related parameters of the human threat test, namely the 'number of body postures' and 'time spent in front', did not differ between the two treatment groups (data not shown; RM, body postures and front, $p>0.05$). No changes were found in both treatment groups compared to baseline.

The activity parameter of the human threat test: 'the number of position changes' showed a significant difference between the vehicle and modafinil treated parkinsonian animals (Fig. 2; RM, $p=0.002$ (BA), $p=0.001$ (AA)). The difference in activity between the two treatment groups was observed on all test days (t-test, $p<0.05$, except day 27, $p=0.08$). The activity of the modafinil treated parkinsonian animals was comparable to baseline values (except day 24 and 27). The activity of the vehicle treated parkinsonian animals was clearly reduced compared to baseline values (paired t-test, $p<0.05$, except day 27).



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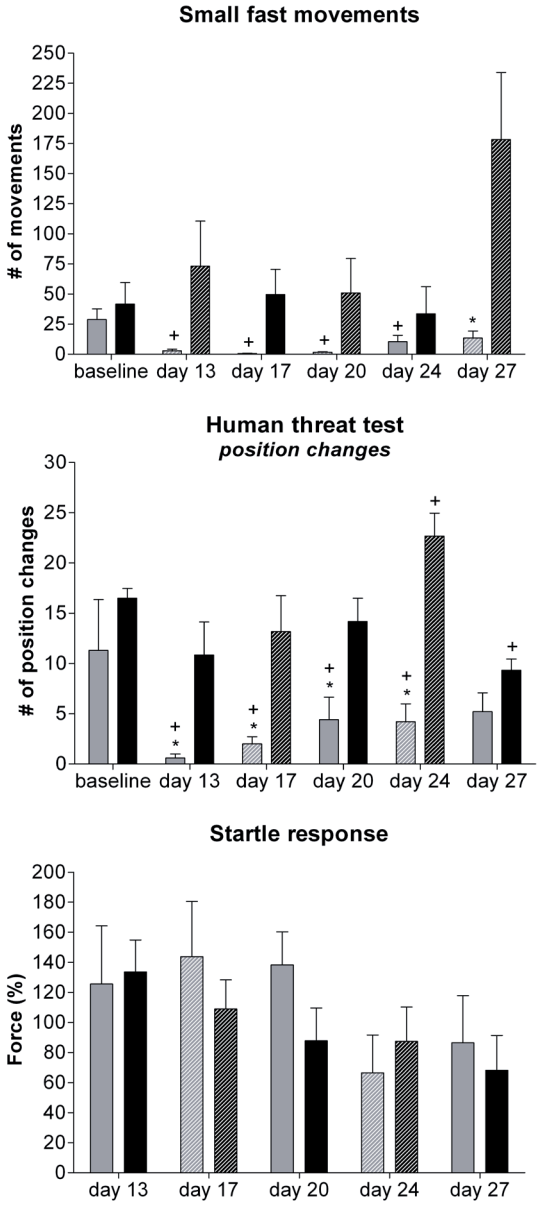

Fig. 2 Behavioral tests. Grey bars show mean (+ SEM) of vehicle treated parkinsonian animals. Black bars show mean (+ SEM) of modafinil treated parkinsonian animals. Solid bars indicate the results before administration. Striped bars indicate results two hours after administration. Dotted line indicates maximum correct responses. *p<0.05 vehicle vs. modafinil treated parkinsonian animals. +p<0.05 before vs. after disease induction.

Startle response

Neither the shape of the curve nor the timing of the startle response of both groups was affected after the induction of parkinsonian symptoms by MPTP (Fig. 2). One animal of the vehicle group was considered an outlier at baseline, because of its extreme startle response and was therefore omitted in the analysis. There was a decrease of the amplitude over time (day 13, 20 and 27, RM, vehicle: p=0.06, modafinil: p=0.03), but this habituation of the startle response was also seen in naïve marmosets.

Difference symptomatic and neuroprotective effects of modafinil

In the protocol a shift in time of observation was included to rule out the temporary symptomatic effects of modafinil on motor function. The animals were observed daily before and after administration and the tests were executed on day 13, 20 and 27 before administration and on days 17 and 24 two hours after administration (the small fast movements test in opposite order). No difference was found between the clinical and AIMS score obtained before and two hours after treatment. Also on most behavioral tests no difference was found between the moments of execution of the tests. Only in the two activity tests (locomotor activity and number of position changes of the human threat test), the activity on day 24 (AA) was higher than the activity of days 20 and 27 (BA, Fig. 2, t-test, $p<0.05$). More small fast movements were present on day 27 (AA) than on day 24 (BA; Fig. 2; t-test, $p<0.05$).

Monoamine levels

DA level in the striatum of modafinil treated parkinsonian animals was reduced to 41% of control DA level, whereas vehicle treated parkinsonian animals showed a reduction of 95% (see Table 1). The DA metabolites, HVA and DOPAC, were reduced in the vehicle treated parkinsonian animals compared to control values. In the modafinil treated parkinsonian animals HVA content was lower than control

Table 1 Monoamine and metabolite levels. Mean ± SEM in μg/g tissue of the striatum of untreated controls or vehicle and modafinil treated parkinsonian animals.

	Control	MPTP + vehicle	MPTP + modafinil
Dopamine	5.47 ± 0.85	0.27 ± 0.08 ***	2.23 ± 0.05 */++
DOPAC	0.73 ± 0.06	0.16 ± 0.06 ***	1.14 ± 0.29 ++
HVA	5.37 ± 0.59	0.34 ± 0.09 ***	2.82 ± 0.48 */++
Dopamine turnover	1.24 ± 0.18	2.10 ± 0.29 *	1.89 ± 0.31+
Noradrenaline	0.49 ± 0.12	0.19 ± 0.04	0.35 ± 0.06
Serotonin	0.36 ± 0.05	0.18 ± 0.04 *	0.15 ± 0.01 *
5-HIAA	1.20 ± 0.11	0.76 ± 0.30	1.27 ± 0.08
Serotonin turnover	3.48 ± 0.40	4.42 ± 1.37	8.34 ± 0.27 ***/+

Dopamine turnover: ((DOPAC +HVA)/ DA); serotonin turnover: (5-HIAA/5-HT); ***p<0.001 vs. control levels, *p<0.05 vs. control levels, ++p<0.01 vs. vehicle treated parkinsonian animals, +p<0.05 vs. vehicle treated parkinsonian animals (ANOVA followed by t-test).

values, but higher than the level of vehicle treated parkinsonian animals. Therefore, a change in the DA turnover, the ratio between degradation and synthesis of DA ((DOPAC + HVA)/DA) is found. DA turnover of the modafinil treated parkinsonian animals was comparable to the turnover of control animals. The vehicle treated parkinsonian animals had a higher DA turnover ratio than the modafinil treated parkinsonian animals and naïve animals. The 5-HT level was reduced in both MPTP treated groups. The 5-HT metabolite, 5-HIAA, was slightly reduced in the vehicle treated group, however the modafinil treated parkinsonian animals were not different from controls. Therefore, an increase in the turnover ratio between 5-HIAA and 5-HT was found in modafinil treated parkinsonian animals and not in vehicle treated parkinsonian animals. The NA levels of the three groups were comparable since MPTP had no effect on these levels.

Discussion

This study shows the protective effects of modafinil against parkinsonian symptoms induced by MPTP in marmoset monkeys on various behavioral aspects and monoamine levels. It also generates information on the sensitivity of behavioral tests for the effects of MPTP, as this is not extensively studied in the marmoset. Clinical and abnormal involuntary movement scores show a clear difference between the modafinil treated parkinsonian animals and vehicle treated parkinsonian animals. Locomotor activity, hand-eye coordination and small fast movements of the modafinil treated parkinsonian animals were comparable to values before disease induction and were clearly better than in vehicle treated parkinsonian animals. DA levels in the striatum showed the same results, although the levels of the modafinil treated group were lower than control values.

As the balance of neurotransmitters in the basal ganglia is disturbed by MPTP, tests measuring movement-related behavior are the most sensitive. The locomotor activity is an often used and well validated parameter in MPTP-marmoset studies (Jenner et al. 2000, Kupsch et al. 2001). In this study two other tests, namely hand-eye coordination task and small fast movements, were included and these proved to be highly sensitive to MPTP-induced deficits. The effect of MPTP on the startle response was also tested. Our results show that neither shape of the curve nor timing of the startle response was changed by MPTP. This is in accordance with the study of Leng et al. (2004) in the MPTP-mouse model. Both studies are in contrast with the delayed startle response found in Parkinson patients and reduced adjustment of the gait during startle stimuli (Vidailhet et al. 1992, Nieuwenhuijzen et al. 2005). Explanation for the different outcome lies in the changed noradrenergic neurotransmission in the Parkinson patients (Braak et al. 2003) in contrast to the unchanged NA system in the marmoset MPTP model (our data, Waters et al. 1987). The central noradrenergic neurotransmission controls the startle response as demonstrated by reduced startle response after 6-hydroxydopamine lesions in the locus coerulus (LC) (Adams and Geyer 1981) and in the Parkin null mouse, which has a clear loss of LC neurons, but

not of the nigrostriatal DA system (von Coelln 2004). Anxiety-related behavior as measured with the human threat test was not changed after the devastating effects of MPTP. It can be concluded that despite deprived movements of vehicle treated animals, anxiety-related behavior as compared to before disease induction was not changed.
In this study vehicle treated parkinsonian animals show an improvement of symptoms over time on clinical score and three tests reflecting movement. This is a general outcome in marmoset monkeys. This is due to compensatory mechanisms to improve DA function like higher DA turnover, reflecting neuronal activity or increase in susceptibility or amount of DA receptors (Bezard and Grossman 1998). Recovery takes place during the first weeks after the MPTP induction and, depending upon the severity of the lesion, eventually resulting in residual parkinsonian symptoms (Rose et al. 1993).

Modafinil may act as a symptom controlling drug by temporary short-living effects and as a neuroprotective drug. Therefore, in this study, behavior was observed twice a day (before and after administration) and an alternation in testing before or after administration is included in the behavioral tests (see materials and methods section). Measurement two hours after administration showed increased activity (Bungalow test and the number of position changes of human threat test) which was not present when measured before administration. This psychostimulating property of modafinil is also apparent in naïve animals (chapter 2). The small fast movements test showed a clear increase after modafinil treatment on the last test day, when tested after administration. When modafinil is given to naïve animals the small fast movements are not changed (chapter 2). As the small fast movements of the modafinil treated parkinsonian animals were comparable to baseline, also after modafinil administration, this extreme behavior on this particular day is probably due to external factors.

The neuroprotective properties of modafinil were already shown at neuronal level in both mice and marmoset MPTP models, whereas tyrosine hydroxylase immunoreactivity, a marker of viable dopaminergic neurons, and DA uptake was higher in protected animals than in vehicle treated parkinsonian animals (Fuxe et al 1992, Jenner et al. 2000). The mechanism of protection of modafinil is not clarified yet. Clear is that modafinil does not act as a MAO-B inhibitor, to obstruct conversion of MPTP into 1-methyl-4-phenylpyridinium (MPP^+), the actual damaging compound, as these are ineffective if administered 5 minutes after MPTP (Sundstrom et al. 1986). Fuxe et al. (1992) showed that the neuroprotective abilities of modafinil against MPTP are independent of the time of administration (15 minutes before until 3 hours after administration). Therefore, modafinil is not a DA uptake blocker, because a blocker cannot prevent damage when administrated 2 hours after MPTP (Sundstrom et al. 1986).

The effects of modafinil on GABA and glutamate release in distinct areas can play a role in the protection of the neurons. Modafinil inhibits GABA release in areas

involved in the direct and indirect pathways of the basal ganglia-thalamus-cortex loop (Ferraro et al. 1997). The liquidation of the inhibitory effects of GABA by modafinil can result in a normalization of the MPTP-induced disturbed balance of the basal ganglia-thalamo-cortical circuitry, especially in the indirect pathway between the striatum and the external globus pallidus (Wichman and DeLong 1998). The stimulation of glutamate release in the ventrolateral and ventromedial thalamus (Ferraro et al. 1997) can result in an increased excitatory output towards the cortex and therefore restore the dysfunctional motor loop (Wichman and DeLong 1998). The improved function of the dysfunctional motor loop is reflected in this study, whereas the motor behavior of the modafinil treated parkinsonian animals is nearly normal.

In this study, two things became apparent during the period of disease induction. First, the first three subsequent MPTP injections resulted in comparable observational scores, both the clinical score and the involuntary movements scale, in both treatment groups. During the following days the scores of modafinil treated parkinsonian animals stayed at the same level, whereas scores of vehicle treated parkinsonian animals still worsened. Second, the last two MPTP injections on days 6 and 9 did not affect the modafinil treated parkinsonian animals. In the marmoset study of Jenner et al. (2000) a comparable picture was shown: The MPTP injections were given on five subsequent days and after this period the difference between the modafinil and vehicle treatments became apparent due to an improvement of symptoms of the modafinil treated group over time, whereas in our study a stable level of motor deficits was reached. An explanation of the delayed protective effects of modafinil can be the reduction of excitotoxicity as discussed in other studies covering the neuroprotective effects of modafinil (see introduction). Modafinil is able to increase the glutamine synthase activity in glial cells resulting in a reduction of glutamate (Touret et al. 1994). The number of glial cells is increased after MPTP in mouse and marmoset (Mackenzie et al. 1997, Kurosaki et al. 2004) and this activation occurs within a time frame that enables these glial cells to participate in the DAergic demise (Teisman et al. 2003). The more glial cells are present due to MPTP, the more glutamate is removed due to modafinil administration and the less excitotoxicity will take place and therefore more cells are protected. In the vehicle treated group the excitotoxicity will continue to result in more damage. The insensitivity of the modafinil treated parkinsonian animals against the last two MPTP injections can on one hand be a result of the above described processes, but on the other hand the result of a change in neurotransmitter balance in the basal ganglia after the repeated modafinil treatment.

Monoamine levels in the striatum of MPTP treated animals were in line with the behavioral observations. DA levels in the modafinil treated parkinsonian animals were lower than control values, although behavior of these animals was nearly normal. The reason of this deviation in the parameters might be that due to compensatory mechanisms more than 60% of the DA neurons have to be lost before manifestation of the parkinsonian symptoms (Dauer and Przedborski 2003). The

higher DA turnover observed in vehicle treated parkinsonian animals has also been reported in other marmoset MPTP studies and parkinsonian patients (Scatton et al 1983, Rose et al. 1989). The observed changes in 5-HT levels of the vehicle treated parkinsonian animals are comparable with chronic and more severe MPTP studies (Perez-Otano et al. 1991, Russ et al. 1991). Remarkable is the changed 5-HT turnover in the modafinil treated parkinsonian animals due to reduced 5-HT levels, but normal metabolite production. As the animals are 10 days off-treatment before decapitation, direct influence of modafinil can be excluded. Presumably, the direct or indirect protective effects or the sustained administration of modafinil could have increased the activity of the remaining serotonergic neurons in the striatum, as modafinil does affect 5-HT levels in the brain (Ferraro et al. 2002).

In conclusion, this study shows that modafinil has protective properties against MPTP damage of the substantia nigra neurons on functional outcome as seen in clinical and abnormal involuntary movement scores and behavioral tests concerning movements and coordination, and on monoamine levels in the striatum. The focus on the functionality of neurons is an extension of earlier studies about neuroprotective effects of modafinil in PD-models. It is as yet unclear what the actual protective mechanism of modafinil is, although it is likely a multifactorial drug effect interfering with acute cellular processes within the first hours after the intoxication, tempering the excitotoxicity and changing the neurotransmitter balance resulting in reduction of the sensitivity of the neurons and restoration of the basal ganglia-thalamo-cortical loop.

Chapter 7

Exploring the neuroprotective effects of modafinil in a marmoset Parkinson model with immunohistochemistry, magnetic resonance imaging and spectroscopy

Sanneke van Vliet, Erwin Blezer, Marjan Jongsma, Raymond Vanwersch, Berend Olivier, Ingrid Philippens

Manuscript submitted

Abstract

Neuroprotective therapeutics stop or slow down the degeneration process in animal models of Parkinson's disease (PD). Neuronal survival in PD animal models is often measured by immunohistochemistry. However, dynamic changes in the pathology of the brain can not be explored with this technique. Application of proton magnetic resonance (MR) imaging (MRI) and spectroscopy (MRS) can cover this lacuna as these techniques are noninvasive and can be repeated over time in the same animal. Therefore, the sensitivity of both techniques to measure changes in PD-pathology was explored in an experiment studying the neuroprotective effects of the vigilance enhancer modafinil in a marmoset PD model.

Eleven marmoset monkeys were treated with the neurotoxin 1-methyl-1,2,3,6-tetrahydropyridine (MPTP). Six of these 11 animals, simultaneously, received a daily oral dose of modafinil (100 mg/kg) and five received vehicle for 27 days. MR experiments were performed at baseline, 1 and 3.5 weeks after the MPTP intoxication period after which brains were analyzed with immunohistochemistry. Tyrosine hydroxylase immunoreactive (TH-IR) staining of dopamine neurons of the substantia nigra pars compacta (SNpc) showed that modafinil was able to partially prevent the MPTP-induced neuronal damage. In MRS, N-acetylaspartate (NAA)/ phosphocreatine (tCR) ratios confirmed the protective effect indicating that this is a sensitive measure to detect neuroprotection in the MPTP marmoset model. Furthermore, the number of TH-IR positive neurons and the NAA/tCR ratio were significantly correlated to behavioral observations indicating that the changes measured in the brain are also reflected in the behavior and vice versa.

Introduction

In PD the output of the basal ganglia is irreversibly affected due to degeneration of neuromelanin-containing dopaminergic (DAergic) neurons in the substantia nigra pars compacta. This results in manifestation of neurological symptoms including akinesia, postural instability, rigidity and resting tremors (Lang and Lozano 1998a). PD is incurable, since present medications (predominantly with levodopa) do not counteract progression. Furthermore, long-term medication is associated with declined efficacy and increased side-effects (Clarke 2004). Therefore, a better strategy would be to focus on preventing neuronal loss in an attempt to stop or slow down the progression of the disease.

In experimental research of PD the 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) intoxicated animal model is widely accepted and mimics for the most part neurodegeneration as observed in human PD brains (Dauer and Przedborski 2003). The metabolite 1-methyl-4-phenylpyridinium (MPP^+) of the neurotoxic agent MPTP selectively damages neurons in the SNpc by blocking the electron transport chain in mitochondria leading to mitochondrial dysfunction resulting in a depletion of ATP and eventually cell death (Dauer and Przedborski 2003). The non-human primate model is preferable over rodents as these animals show clear and lasting behavioral features after MPTP treatment, which reflect many aspects of human parkinsonian symptoms (Jenner and Marsden 1986). Furthermore, similarities with humans in the anatomy of the striatum, distribution of DA cells in the SNpc and striatal DA function are of consideration in the choice of the non-human primate model (Eslamboli 2005).

In order to study neuroprotection in this model a range of markers can be used ranging from various behavioral tests to analysis of catecholamine levels and changes of cellular function (reviewed by Emborg 2004). In PD research, staining of tyrosine hydroxylase, which is the first and rate-limiting enzyme in the synthesis of the catecholamines, is often used as a quick and sensitive method for visualization of the surviving DAergic neurons in the MPTP model (Pearson et al. 1983). However, immunohistochemistry is an invasive and static read-out value. Dynamic changes in the brain due to cell death, recovery and compensation mechanisms can not be explored with this technique. Application of proton magnetic resonance techniques, i.e. MR imaging and MR spectroscopy can cover this lacuna as these techniques are non-invasive and can be repeated over time in the same animal. These MR techniques can also have scientific and ethical benefits as the animal acts as its own control thereby reducing animal usage for research.

MRI and MRS are very versatile techniques to examine structural and physiological processes in living organisms and are widely used in clinical and experimental research (Dijkhuizen and Nicolay 2003). Application of MRI/MRS in PD-related research generated differences between PD patients and healthy subjects, although these findings are not consistent and seem to depend on the form of parkinsonism (Brooks 2000, Seppi 2005). A typical MRI technique used for PD research is T2-weighted imaging (T2W) in which changes in signal intensities

are partly due to altered water content of tissue, mostly caused by the presence of extracellular edema (Dijkhuizen and Nicolay 2003). Earlier examinations with T2W imaging of the effect of MPTP intoxication in animals showed changes in relevant brain areas like the SNpc, caudate nucleus and putamen (Miletich et al. 1994, Zhang et al. 1999, Podell et al. 2003). MRS visualizes signals from carbon-bound protons from various metabolites (Kauppinen and Williams 1994). Different variables like the quantity of the observed metabolite and strength of the magnetic field determine the detection level of a specific metabolite. Consequently, only few metabolites may be examined by MRS. NAA is frequently used as a neuronal marker (Gujar et al. 2005, Castillo et al. 2006) and reductions of NAA levels have been observed in the striatum and SNpc in parkinsonian mice, cats and cynomolgus monkeys (Brownell et al. 1998, Podell et al. 2003, Boska et al. 2005).

To investigate whether MR techniques have added value in neuroprotection research in the MPTP marmoset model, both MRI and MRS were applied to investigate the neuroprotective effects of modafinil. Modafinil is a vigilance-stimulating compound and marketed for treatment of narcolepsy (Bastuji and Jouvet 1988). Modafinil is suggested to stimulate wakefulness indirectly via noradrenergic α_1 neurotransmission, DA-dependent mechanisms or γ-aminobutyric acid (GABA) and glutamate release (Duteil et al. 1990, Ferraro et al. 1996, 1998, Wisor et al. 2001). The neuroprotective characteristics of modafinil are shown after mechanical, ischemic and neurotoxic injuries in both non-DAergic and nigrostriatal DAergic neurons (Antonelli et al. 1998, Ueki et al. 1993b, Lallemant et al. 1997). The protection of DAergic neurons in the SNpc indicate that modafinil is a promising compound in slowing down the degeneration in PD (Fuxe et al. 1992, Ueki et al. 1993a, Jenner et al. 2000, chapter 6). Chapter 6 reported the effects of modafinil using extensive behavioral test systems and catecholamines measurement in the marmoset MPTP model. Treatment which started simultaneously with MPTP intoxication decreased parkinsonian symptoms, improved activity and hand-eye coordination. Furthermore, DA and its metabolites were significantly increased compared to vehicle treatment. In this report, the neuroprotective effects of modafinil are explored by immunohistochemistry and MR techniques. These results are compared to the behavioral observations, to investigate if the effects on pathology of the SNpc are also reflected in the behavior and vice versa.

Materials and methods

Animals

Adult marmoset monkeys *(Callithrix jacchus)* aged 2-6 years with initial body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm. All aspects of animal care are described in Standard Operating Procedures, which are in

agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

Eleven naïve marmosets were treated in total with 6 mg/kg MPTP s.c. (Sigma chemical Co. St. Louis, MO, USA) over 9 days (experimental day 1: 2 mg/kg and experimental days 2, 3, 6 and 9: 1 mg/kg). Six of these animals (4 males and 2 females: modafinil treated parkinsonian group) received a daily oral dose of 100 mg/kg modafinil from experimental day 1 until day 27. The other five animals (3 males and 2 females: vehicle treated parkinsonian group) received a daily oral dose of the vehicle (10% sucrose solution). The treatments were given directly after the MPTP injections. MR experiments were performed before the start of the experiment (baseline) and at 1 (experimental days 14-16) and 3.5 weeks (experimental days 35-36) after the 9-day MPTP treatment period. Brains were removed for immunohistochemistry after decapitation of isoflurane sedated animals the day after the last MR session (experimental day 37).

Drugs

MPTP (Sigma chemical Co. St. Louis, MO, USA) was dissolved in saline and was subcutaneously given in a dose volume of 0.5 ml/kg. Modafinil (Modiodal®, d,1-2-[(diphenylmethyl)sulfinyl]acetamide) was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds: lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in a 10% sucrose solution in a dose volume of 1.5 ml/kg.

Behavioral assessment

Two rating scales were used as a tool to quantify the observed signs and symptoms. 1) A clinical scoring list in which the PD-related symptoms of the animal were rated. The following symptoms are included: appetite; inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility; rigidity and presence of tremors. 2) The abnormal involuntary movement scale (AIMS) is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976, Di Monte et al. 2000). AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity, and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Baseline scores are zero for both tests. All tests were done before the daily administration of modafinil or vehicle. The dynamic results on this marker are extensively reported earlier (chapter 6). In this report only the scores on experimental day 13 and day 27 are used.

MRI/MRS protocol

After sedation with ketamine (30 mg/kg i.m., ASP Pharma, The Netherlands) the animals were prepared for mechanical ventilation by an endotracheal intubation. Animals were immobilized in a specially designed stereotactic holder and placed in an animal cradle, which was inserted into the NMR spectrometer. During the experiments animals were ventilated with isoflurane (1.5-2.0%) in N_2O/O_2 (70/30). Expiratory CO_2 was monitored, and the body temperature was maintained at 37 °C with a heated water pad. An infrared sensor (Nonin Medical Inc, Plymouth, Minnesota, USA) was attached to the animal hind paw for monitoring heart rate and blood oxygen.

High-resolution MRI experiments were performed using a 4.7 T horizontal bore NMR spectrometer (Varian, Palo Alto, California), equipped with a high-performance gradient insert (12 cm inner diameter, maximum gradient strength 220 mT/m). A Helmholtz volume coil (∅ 85 mm) and an inductively coupled surface coil (∅ 35 mm) were used for radio frequency transmission and signal reception, respectively. On a sagittal scout image, 11 contiguous transversal slices of 0.75 mm were defined covering an area in which the SN was centrally located. From these slices, quantitative T2 relaxation maps were obtained by a mono-exponential fit of eight multi-echo images. Repetition time (TR)=2000 ms; Echo time (TE)=17.5 + 7 * 17.5 ms. The T2W images from the second echo-time, i.e. 35 ms, were used for data evaluation (see also later). Field of view 4 x 4 cm, matrix 128 x 128 pixels, zero-filled

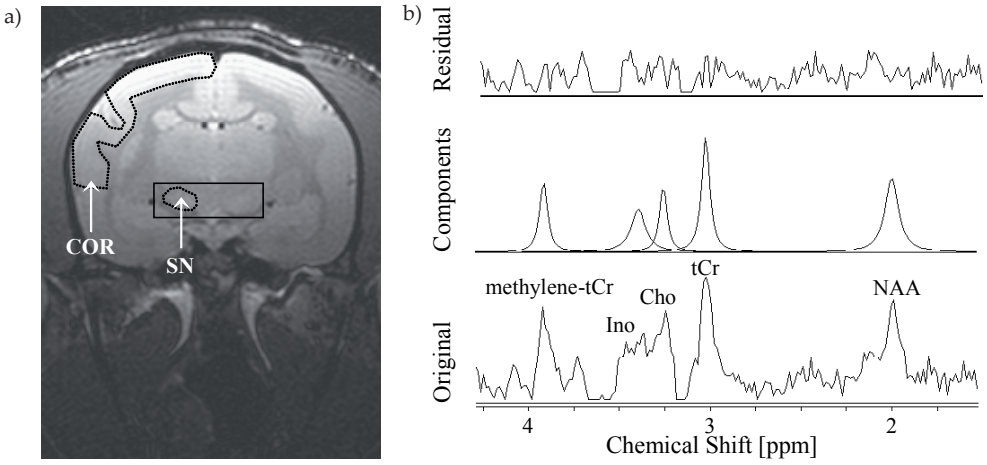

Fig. 1 MRI/MRS analyses. a) example of the outlined ROIs (dashed lines) of the substantia nigra (SN) and reference area (REF) in the cortex. Placement of the VOI for single voxel spectroscopy (squared box). b) Example of data analyses of proton MR spectra using jMRUI. Bottom row: original spectrum. Middle row: fitted individual components using the AMARES algorithm. Top row: residuals signal after subtraction of the individual components from the original data set. NAA: N-acetylaspartate, tCr: total (phospho) creatine, Cho: choline, Ino: inositol.

256 x 256 pixels, two transitions.

Single Voxel MRS was performed using a point resolved spectroscopy sequence (TR=4000 ms; TE=30 and 144 ms, 64 transitions). The voxel of interest (VOI, 3 x 9 x 3 mm=81 mm^3) was positioned to cover the both left and right SN (Fig. 1a). Chemical shift selective pulses and dephasing gradients were used to suppress the water signal.

MRI/MRS data evaluation

The quantitative T2 relaxation maps were analyzed with in-house developed software. In both hemispheres, regions of interest (ROI) were outlined on the T2W images. The ROIs were drawn around the whole substantia nigra (SN) and in a reference area in the cortex as indicated in Fig. 1a (marmoset brain atlas: Stephan et al. 1980). No differentiation could be made between pars reticulata and pars compacta of the substantia nigra therefore the ROI was placed around the whole SN to analyze water content. The ratio between the SN and the reference area were used for data analyses.

Spectra obtained with TE=30 ms were analyzed using the java based magnetic resonance user's interface (jMRUI, version 2.1; EU Project TMR, FMRX-CT97-0160). Free induction decays were pre-processed before fitting. The first two data points of every spectrum were removed. Thereafter, data were apodized with a 5 Hz Gaussian filter. Residual water signal was removed with a Hankel Lanczos Singular Values Decomposition filter. After autophasing, the data set was fitted in the time-domain using the AMARES (Vanhamme et al. 1997) procedure. An a priori knowledge database was constructed with the following 5 peaks, NAA (2.02 ppm; neuronal marker), methyl protons of creatine and phosphorylated creatine, i.e. phosphocreatine (tCr: 3.03 ppm; energy buffer marker), choline containing compounds (Cho: 3.22 ppm; involved in membrane synthesis/degradation), inositol (Ino: 3.54 ppm; involved in intracellular signalling pathways) and the methylene protons of tCr (methylene-tCr: 3.93 ppm). Soft constraints were used during the fitting procedures. The maximal metabolite line width was limited to 25 Hz, peakshifts were restricted to ± 10 Hz of the theoretical peak location. From the obtained fits we calculated the NAA/tCr, Cho/tCr and Ino/tCr ratios assuming that tCr is a suitable *in vivo* concentration reference, since the sum of (phospho) creatine is rather constant in adulthood (Gujar et al. 2005, Castillo et al. 2006). The spectra obtained with TE=144 ms were qualitatively examined for the presence of the inverted doublet peak arising from lactate (Gujar et al. 2005, Castillo et al. 2006). Fig. 1b shows a typical example of how the data were processed using the AMARES algorithm in jMRUI.

Immunohistochemistry

Brains (vehicle treated parkinsonian group: 5; modafinil treated parkinsonian group: 5) were analyzed for the presence of DAergic neurons with TH-IR. Four brains of naïve animals (control group) were used to establish control values. From collected brains, the right hemisphere was fixated in 4% paraformaldehyde at 4 °C and after 48 hours the brains were transferred to 0.5% paraformaldehyde at 4 °C. Brains were

dehydrated in graded ethanol and xylene and subsequently embedded in paraffin. Serial transversal sections of 5 μm were cut on a microtome and collected serially on aminosilane/acetone solution coated slides. Every 4th section was mounted on egg-white coated glass slides for cresylviolet staining which was used as reference for the TH-IR localization.

The sections used for the TH-IR were deparaffinated and rehydrated in xylene and graded ethanol. The citrate buffer method was applied for antigen retrieval. Sections were pre-incubated with 0.3% H_2O_2 in PBS to quench endogenous peroxidase activity. Thereafter, the sections were pre-incubated in PBS with 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100. Incubation in anti-TH antibody (1:80000, Sigma chemical Co. St. Louis, MO, USA) was overnight at room temperature. The secondary antibody (1:2000, Santa Cruz Biotechnology inc., CA, USA) was incubated for 90 minutes followed by 90-minute incubation with Vector ABC (1:800, Vector laboratories inc., Burlingame, Canada). PBS-washes were applied after each pre- and antibody incubation step. Thereafter the sections were pre-incubated for 10 minutes with 0.025% 3'3'-diaminobenzidine containing 0.15% nickel ammonium sulphate (DAB-NI solution) followed by a 10-minute incubation in the DAB-NI solution with 0.00015% H_2O_2 to visualize bound immunocomplexes. After a PBS-wash the sections were dehydrated in alcohol series, cleared up in xylol and cover-slipped with DePeX (BDH Laboratory supplies, England).

TH-IR positive neurons were counted in 4 sections of the SNpc located 4-5.5 mm anterior of the external auditory meati (marmoset brain atlas: Stephan et al. 1980). Within each section, TH-IR neurons were counted manually in three medial to lateral parts of the SNpc using an eye-piece grid of 10 mm x 10 mm at a magnification of 400x using an Olympus light microscope. Qualitative screening for abnormalities was executed throughout the whole SNpc and the caudate nucleus at anterior A5.5 (marmoset brain atlas: Stephan et al. 1980).

Statistics

Results are presented as mean ± SEM. The difference in behavior between treatment groups was analyzed with an independent t-test. MRI/MRS data are presented as changes relative to the baseline time-point. The results of the MRI/MRS data were analyzed with an overall repeated measure analysis. When relevant, a paired t-test was applied to test for differences between baseline and measurements after MPTP intoxication and an independent t-test was applied to test for difference between the two treatment groups.

The MRS data contained two missing values. These missing values are replaced by the mean of the group. Immunochemical data were evaluated with a one-way ANOVA followed by a post-hoc multiple comparison procedure (Bonferroni corrected t-test). Correlations between neurological scores and time matching MRI and MRS data (independent of treatment procedure) or neurological scores and histological outcome (last behavioral observation and TH-IR positive counts) were evaluated by Pearson correlation and linear regression analysis. There was a small time discrepancy between the neurological scores and the last MR session and

immunohistochemistry. Despite this inconsistency, we believe that these correlations can be calculated as the behavioral score of MPTP marmosets does not change much in time.

Results

Neurological function

MPTP intoxication resulted in mild parkinsonian symptoms in the vehicle treated parkinsonian animals. Modafinil protected the animals against the MPTP intoxication as the neurological symptoms were significantly reduced (t-test, $p<0.05$). The effects of modafinil on diverse aspects of parkinsonian symptoms are extensively described in chapter 6. In Table 1 an overview is given of the behavioral scores on relevant time-points.

MRI and MRS

Fig. 2 shows the relative changes of the T2 relaxation times of the SN of the vehicle and modafinil treated parkinsonian groups. A tendency in decrease of relative relaxation times measured in the vehicle treated parkinsonian animals was found 3.5 weeks after termination of the MPTP intoxication (paired t-test, 3.5 weeks $p=0.141$) which was absent in the modafinil treated parkinsonian animals.

Fig. 3 shows the relative changes of the NAA/tCr, Cho/tCr and Ino/tCr ratios compared to the baseline measures. NAA/tCr ratios of the vehicle treated parkinsonian animals significantly reduced 1 week after MPTP intoxication (paired t-test, $p=0.05$) and a tendency to decrease 3.5 weeks after MPTP intoxication (paired t-test, $p=0.123$). The NAA/tCR ratio of the modafinil treated parkinsonian animals was significantly increased 3.5 weeks after MPTP intoxication compared to baseline values (paired t-test, $p<0.05$). This resulted in a difference between the vehicle and modafinil treated parkinsonian animals 3.5 weeks after MPTP intoxication (t-test, $p<0.05$). None of the other ratios changed significantly during the experiment. Furthermore, no lactate peaks were observed throughout the experiment in any of the animals.

Table 1 Neurological changes. Clinical score and AIMS of the vehicle and modafinil treated parkinsonian animals. Data are presented as mean ± SEM. Baseline score is 0. **$p<0.01$ vs. vehicle treated parkinsonian animals.

	Clinical score		AIMS	
	Day 13	Day 27	Day 13	Day 27
MPTP + vehicle	11.8 ± 0.9	9.8 ± 1.5	15.0 ± 0.4	12.6 ± 0.7
MPTP + modafinil	4.3 ± 1.5 **	2.8 ± 0.5 **	8.3 ± 1.2**	6.3 ± 0.8**

Immunohistochemistry

MPTP intoxication resulted in a reduction of 75.8 ± 5.3% of TH-IR positive neurons (vs. control, one-way ANOVA $p<0.001$, Bonferroni t-test $p<0.001$) in the vehicle treated parkinsonian group. The reduction of TH-IR positive neurons in the modafinil treated parkinsonian group was 39.1 ± 6.7% of control values and was significantly different from the vehicle treated parkinsonian group (vs. control and vs. MPTP + vehicle, one-way ANOVA $p<0.001$, Bonferroni t-test $p<0.05$).

Qualitative histological examination of the remaining neurons (Fig. 4) also showed clear differences among groups. The surviving neurons of the vehicle treated parkinsonian animals were small without a clear nucleus (Fig. 4c). Some of the surviving neurons of the modafinil treated parkinsonian group had a darkly stained nucleus with inclusions and an increased size (Fig. 4e). Although the number of TH-IR positive fibers and varicosities, typical of a certain fibre type of the striatum were not quantified, a clear reduction in varicosities of the fibres was noted in the vehicle treated parkinsonian animals (Fig. 4d). The modafinil treated parkinsonian animals showed an intermediate density of the varicosities (Fig. 4f).

Correlations

A significant negative correlation between the NAA/tCr ratios and the behavioral scores was found. This indicates that a decrease of the NAA/tCR ratio is correlated to state of behavior (clinical score $r=-0.60$, AIMS $r=-0.64$, $p<0.05$). No significant correlations were found between any of the other MRS ratios or T2 relaxation times and behavioral observations. Behavioral scores were also significantly negative correlated to the TH-IR positive counts. This indicates that a decrease of the TH-IR

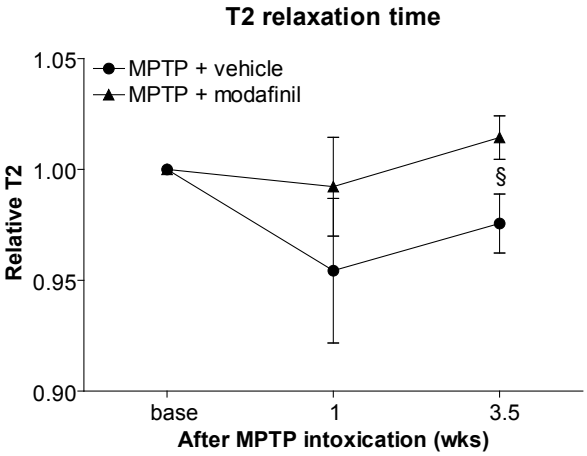

Fig. 1 T2 relaxation times. Relative change of the T2 relaxation times at 1 week and 3.5 weeks (mean ± SEM) after MPTP intoxication as compared to baseline values in the vehicle and modafinil treated parkinsonian groups. §$p<0.05$ between treatments.

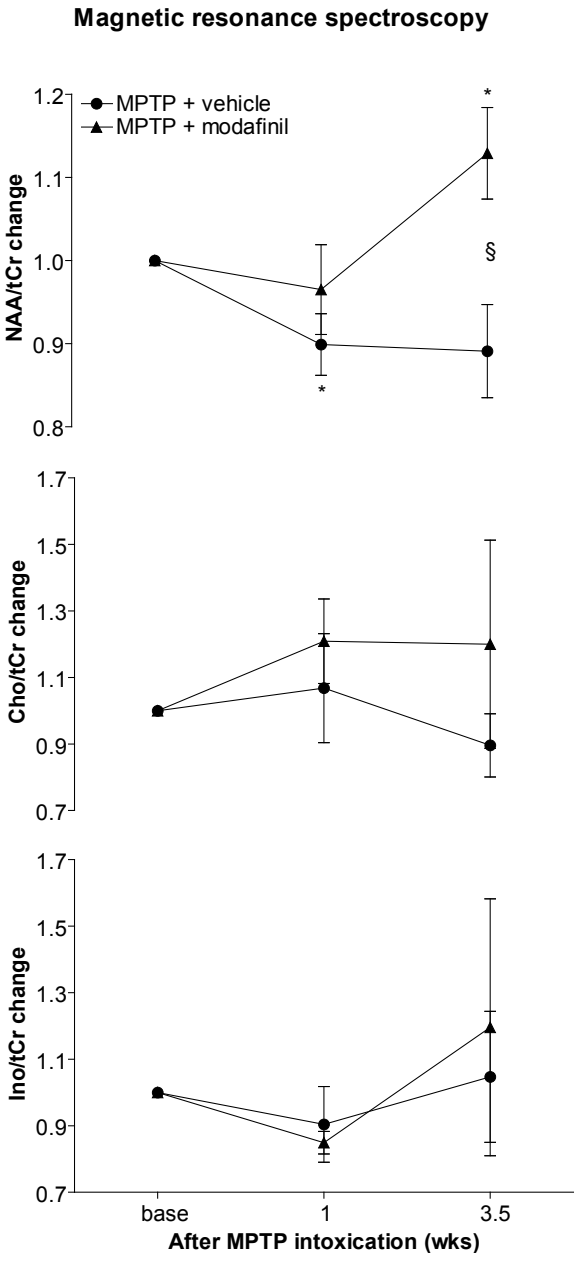

Fig. 3 Magnetic resonance spectroscopy. Relative changes of the NAA/tCR, Cho/tCR and Ino/tCR ratios (mean ± SEM) at 1 week and 3.5 weeks after MPTP intoxication as compared to baseline values in the vehicle and modafinil treated parkinsonian groups. *p<0.05 vs. baseline, §p<0.05 between treatments. NAA: N-acetylaspartate, tCr: total (phospho) creatine, Cho: choline, Ino: inositol.

positive neurons is correlated to increase of affected behavior (clinical score r=-0.88, AIMS r=-0.86, $p<0.05$).

Discussion

This study focused on the neuroprotective effects of modafinil in the marmoset MPTP model as well as on the added value of MR experiments besides other regularly used markers, like immunohistochemistry, in neuroprotection research. The TH-IR staining as well as the NAA/tCR ratio of the MRS signified the neuroprotective ability of modafinil to prevent neuronal death. Furthermore, correlations of these markers with parkinsonian behavior showed that the pathology is reflected in the behavior and vice versa and are both valuable markers to establish neuronal death and survival in the MPTP marmoset model.

MPTP induction results in a clear loss of DAergic neurons in the SNpc as also indicated by the immunohistochemical data of this study. This is the most striking pathological feature of the MPTP model as these neurons are more susceptible to MPTP than mesolimbic DAergic neurons or noradrenergic neurons (Burns et al. 1983, Gerlach and Riederer 1996). Survival of DAergic neurons is most often investigated with staining of TH (Waters et al. 1987). Important for the interpretation of these data is the possible discrepancy between the TH-IR positive neurons and surviving DA-neurons as MPTP is suggested to induce reduction of TH-IR activity without cell loss (Tatton et al. 1990). However, a similar reduction in TH-IR neurons and DAergic neurons was found shortly after MPTP induction and months thereafter (Jackson-Lewis et al. 1995, Kupsch et al. 1996).

Another way of measuring neuronal damage could be the use of MRI/MRS. The advantage is that this non invasive technique can be applied repeatedly during a prolonged time with the opportunity to follow the progress of the disease in time. In this study MPTP intoxication resulted in a tendency towards reduction in T2 relaxation times in the SN. This contrasts earlier reports where increases of T2W signal intensities (which is normally a result of an increased T2 relaxation time) were found in the SNpc, caudate nucleus, putamen and globus pallidus, which are DAergic projection receiving areas (Miletich et al. 1994, Zhang et al. 1999, Podell et al 2003). In general an increase in T2 relaxation time is associated with the presence of extracellular edema (Dijkhuizen and Nicolay 2003). Probably this was not apparent in this study due to a relatively low MPTP dosage (total 6 mg/kg) and a relatively long time interval of the first MR experiment after the MPTP intoxication period. Our first MR experiment was done 1 week after cessation of MPTP intoxication in contrast to most studies in which measurements were done within several hours or days.

In contrast to the lack of reduction of T2 relaxation times in the other MPTP experiments, T2 relaxation time decrease of SNpc has been reported in PD patients (Kosta et al. 2006). The reduction is caused by PD-related iron deposition. Iron creates magnetic field inhomogeneties which dephase water protons nearby resulting in a

shortening of the T2 relaxation time (Kosta et al. 2006). Interestingly, iron deposition has been reported in the SN in MPTP models (Temlett et al. 1996, Mochizuki et al. 1994). Although the exact function of the increased iron levels is unknown, it is

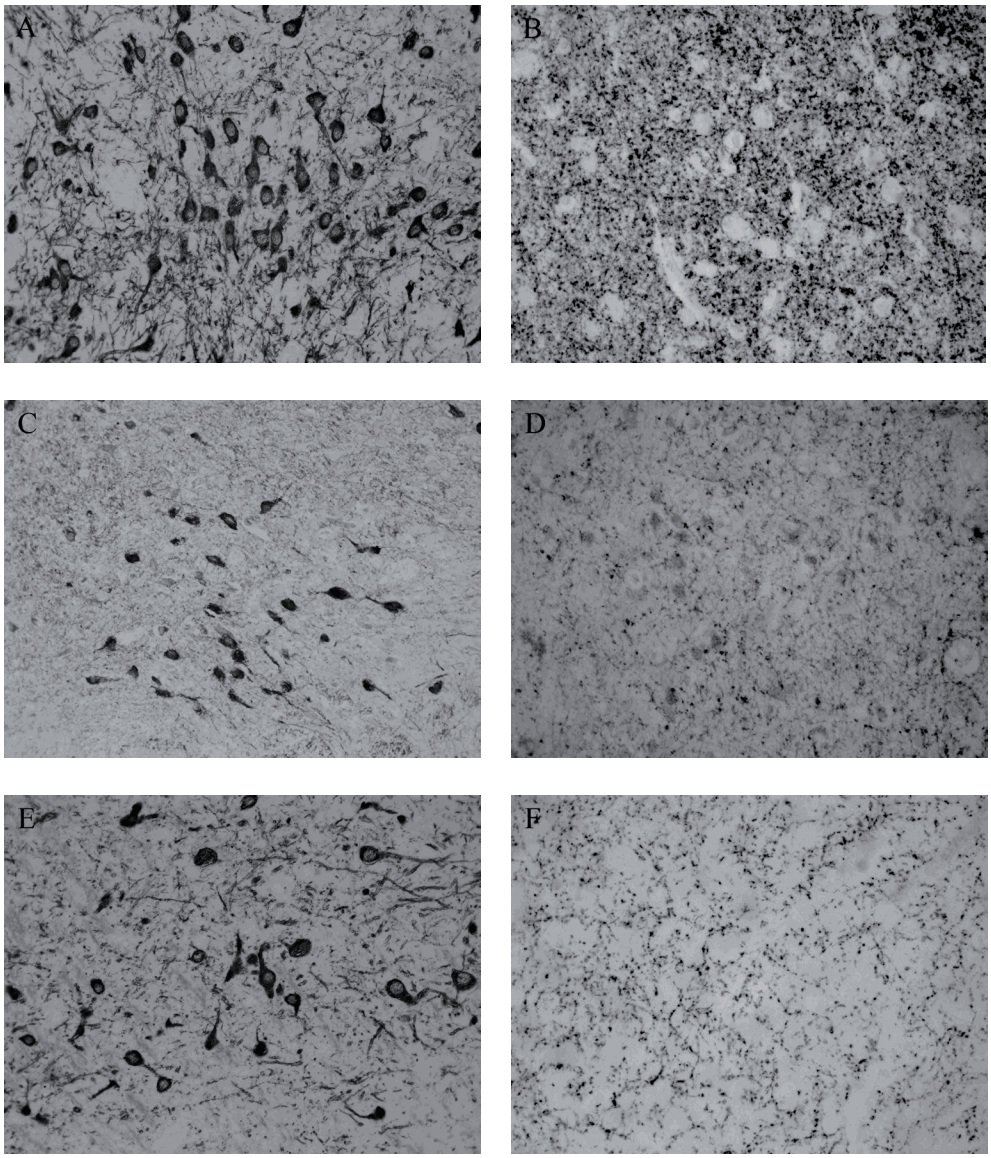

Fig. 4 Tyrosine hydroxylase immunoreactivity. Images of dopaminergic neurons in the substantia nigra pars compacta (A,C, E, magnification 200x) and varicosities in the caudate nucleus (B, D, F, magnification 400x) of a control animal (A, B), vehicle treated (C, D) and modafinil treated parkinsonian animal (E, F).

suggested that it contributes to oxidative stress in PD and MPTP models (Yantiri et al. 1999).

NAA/tCr ratios decreased both 1 week and 3.5 weeks (not significant) after MPTP intoxication. The decrease in the NAA/tCr ratios is in concordance with other MPTP studies. Mice also show a clear decrease in the absolute NAA concentration in the SN both 2 and 6 days after MPTP intoxication (Boska et al. 2005). Cats show a decrease in NAA/tCr ratios in the striatum 12 hours after MPTP intoxication (Podell et al. 2003). Furthermore, chronic MPTP intoxicated cynomolgus monkeys show a persistent reduction in NAA/tCr ratio in the caudate and putamen (Brownell et al. 1998).

In this study the presence of lactate was not detected in the SN after MPTP intoxication. Although there are no reports about presence of lactate in the SN in MPTP models, lactate has been observed in other areas. In the striatum a decrease in lactate was found in mice directly after MPTP intoxication (Koga et al. 2006) and in cynomolgus monkeys after chronic MPTP intoxication (Brownell et al. 1998).

Modafinil treatment during MPTP intoxication resulted in increased survival of DAergic neurons in the SNpc as measured with TH-IR. The protective effects of modafinil in this study are in line with the earlier reported effects on behavior and neurochemistry (chapter 6) and other positive findings in mice and marmoset PD models (Fuxe et al. 1992, Ueki et al. 1993a, Jenner et al. 2000).

Neuroprotective actions of modafinil were also reflected in the MR data. T2 relaxation times did not change after modafinil treatment compared to baseline indicating that modafinil was able to prevent iron disposition. Modafinil treatment also prevented the decrease in NAA/tCr ratio. Surprisingly, these animals showed a clear increase in NAA/tCr ratio 3.5 weeks after modafinil treatment. In general the concentration of NAA depends on the balance between synthesis and degradation pathways, cellular density and presence or absence of edema. To our knowledge it is not known that modafinil increases the density of neurons. Furthermore, it is unlikely that the water content in the area of interest is decreased as we did not observe an increase in T2 relaxation of water in this area. Perhaps a more chronic effect of modafinil results in an altered biosynthesis of NAA. Increase in NAA is not an unknown phenomenon and has for example been observed in Canavan's disease, a pediatric leukoencephalopathic disorder (Matalon et al. 1989). Another explanation may be that modafinil stimulates the production of mast cells and oligodendrocytes in which NAA has been detected *in vitro* (Urenjak et al. 1992, Burlina et al. 1997). Finally, the increase in NAA/tCr ratio could be a result of a decreased tCr concentration. Decrease in the tCr concentration has been reported in the chronic phase of many pathologies although this is always associated with a total tissue damage which we did not observe (Zimmerman and Wang 1997). Furthermore, a decrease in tCr is unlikely as both Cho/tCr and Ino/tCr ratios of modafinil treated animals did not change throughout the experiment.

Nevertheless, it was shown that modafinil is able to prevent the neurodegeneration induced by MPTP as reflected in the behavioral and pathological markers. Therefore, these markers are of value in neuroprotection research, especially

since correlations were found between these markers.

A decrease of TH-IR was negatively correlated to increase of affected behavior. Interestingly, the neurological symptoms of modafinil treated parkinsonian group were reduced almost to normal levels at the end of the experiment, but this was not reflected in the number of TH-IR neurons which differed significantly from normal, non-MPTP intoxicated, animals. In PD patients it has been shown that DAergic neuronal death has to exceed a critical threshold of 50-60% reduction before parkinsonian signs occur (Bernheimer et al. 1973, Riederer et al. 1976). In this study, the reduction of 40% of the neurons in the modafinil treatment group is below this threshold which may explain the lack of change in behavior. Presumably, this discrepancy between cell death and behavior is due to the compensatory mechanisms in the DAergic system (Bezard et al. 2001).

The NAA/tCR ratio is able to predict the disease state of the animal as a decrease in NAA/tCr ratio is associated with increase of affected behavior after MPTP intoxication. This correlation suggests that NAA/tCr ratio measurement is a good tool to study the effects of neuroprotective drugs on the metabolic state of a neuron.

In this study, the relation between the MR-parameters and histology could not be calculated due to the low number of corresponding data points. However a modest correlation between NAA levels and TH-IR neurons (r=0.6) was found in a mouse MPTP model (Boska et al. 2005) indicating that both of these parameters predict neuronal damage.

In conclusion, this study shows improved survival of MPTP intoxicated DAergic neurons in the SNpc after modafinil treatment. This is in line with earlier reported positive effects at the behavioral and neurochemical level (chapter 6) and emphasizes that modafinil is a potential neuroprotective drug for treatment of PD. Furthermore, it can be concluded that MRS (NAA/tCR ratio) is a valuable tool for neuroprotective research in the MPTP intoxicated marmoset as correlations indicate a clear relationship between behavioral deficits and measurements of brain damage. All data together indicate that the combination of measurement of functionality and brain processes over time result in a more complete picture of the actions of a neuroprotective compound.

Chapter 8

Neuroprotective actions of modafinil: Generation via gene expression?

Sanneke van Vliet, Hans van Noort, Carla Persoon-Deen, Berend Olivier, Ingrid Philippens

Abstract

Modafinil is able to prevent nigrostriatal damage in animal models for Parkinson's disease (PD). The pathways via which modafinil exerts its neuroprotective actions are unknown. There are suggestions about direct interference with cell death processes, but there are also indications of indirect actions. To explore the possibility that the neuroprotective actions of modafinil are associated with indirect effects on local neural gene expression two experiments were conducted.

First, the indirect neuroprotective actions were tested by giving modafinil 12 hour before treatment with the neurotoxin 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) in mice. The behavioral and immunohistochemical outcome was compared to the outcome of modafinil given simultaneously with disease induction. Early administration of modafinil did not protect the neurons against MPTP intoxication, in contrast to simultaneous administration of modafinil and MPTP.

Second, in an *in vitro* experiment the effects of modafinil on gene expression of human astrocytes were investigated with microarrays to define possible modafinil-triggered changes in the cytokine and chemokine profile of astrocytes. This experiment indicated that modafinil had no broad direct effects on this gene expression profile.

Together, these data indicate that the neuroprotective actions of modafinil are most likely not exerted via indirect actions.

Introduction

Modafinil, a wake-promoting compound, is a potential neuroprotective compound in the treatment of PD. Modafinil prevents degeneration of dopaminergic neurons in the substantia nigra after partial transsection of the nigrostriatal DA pathway and after the neurotoxin MPTP (Fuxe et al. 1992, Ueki et al. 1993a, Jenner et al. 2000, chapter 6). Furthermore, modafinil prevents glutamate toxicity in cultured cortical cells (Antonelli et al. 1998), increases in toxic aspartate and glutamate levels after striatal ischemic injury caused by endothelin-1 in rats (Ueki et al. 1993b) and development of lesions in the hippocampus induced by the neurotoxic nerve gas soman (Lallemant et al. 1997).

The exact mechanisms behind the neuroprotective actions of modafinil are unknown. However, the effects of modafinil on the diversity of lesion models indicate a mechanism independent of neuron type and localization in the brain. Suggestions have been made about direct interference with processes that affect cell death like energy metabolism, synthesis and release of neurotrophic factors, recovery of calcium homeostasis and improvement in metabolic activity (Fuxe et al. 1992, Ueki et al. 1993a, b, Lallement et al. 1997, Antonelli et al. 1998, Jenner et al. 2000), although no direct evidence for this exist. Furthermore, modafinil induces higher levels of glutamine synthase-encoding mRNA in rat brain, which could result in an increase of enzymatic breakdown of glutamate and a reduction in excitotoxicity (Touret et al. 1994). Moreover, modafinil can, in fact, exert direct effects on gene expression levels in the brain.

This observation resulted in the hypothesis that modafinil might stimulate gene expression of enzymes and other proteins, which are involved in cell survival processes and exerts its neuroprotective actions via these proteins. To explore this hypothesis two experiments were conducted. First, an *in vivo* experiment, in which the indirect neuroprotective effects of modafinil against MPTP intoxication were tested after elimination of modafinil from the body. Second, an *in vitro* experiment in which the direct effects of modafinil on gene expression of human astrocytes was investigated.

The first experiment intended to fill the niche about the timing of modafinil administration in relation to prevention of neurodegeneration after disease induction. Fuxe et al. (1992) reported earlier that modafinil given 15 minutes before or simultaneously with the neurotoxin MPTP, was neuroprotective, whereas modafinil given 3 hours after MPTP was not. The neuroprotective effects of modafinil given hours earlier have not yet been documented. In the present study, therefore, the effects of modafinil given 12 hours before MPTP in mice have been compared to the effects of modafinil given directly after MPTP. Modafinil-induced effects were evaluated using behavioral and immunohistochemical parameters.

The interval between modafinil administration and disease induction with MPTP was chosen such that no or only a small amount of modafinil was expected to be present at the time of MPTP induction, and that this interval was long enough to allow for gene expression effects to become apparent. The interval chosen was 12

hours, based on the pharmacokinetic measurements after 100 mg/kg modafinil in mice, showing an elimination half life of 1-3 hours (Maochon et al. 1996).

Neuroprotection research in the MPTP marmoset model has shown that changes in motor behavior and staining of the DA neurons with tyrosine hydroxylase immunoreactivity (TH-IR) are sensitive parameters for detection of neuronal damage (chapter 6, 7). However, parkinsonian-like behavior in a MPTP-intoxicated mouse recovers within a few days (Schmidt and Ferger 2001). Despite this, open field and rotarod are often reported as sensitive measurements of motoric skills of mice for a few days after MPTP intoxication (Sedelis et al. 2001).

By using an *in vitro* approach we examined the direct effects of modafinil on human astrocytes in cell cultures that could be relevant to explain the neuroprotective effects of modafinil *in vivo*. As the most abundant cell type in the central nervous system (CNS), astrocytes play a central role in the maintenance of the blood-brain barrier, the control of inflammatory processes and neuronal signaling, and a role during repair and regeneration of the CNS after trauma. Key mediators in these astrocyte functions include a large variety of cytokines, chemokines and growth factors that are produced and secreted when homeostasis is disturbed. To examine the impact of modafinil on the expression profile of these mediators, adult human astrocytes in cell culture were exposed to modafinil and gene expression levels were evaluated using cDNA arrays tailored to analyze the cytokine/chemokine/growth factor profile (Meeuwsen et al. 2003). This experiment was performed twice, using astrocytes isolated from two different post-mortem human brains.

The combination of the results of both experiments was expected to yield clues for any indirect or delayed effect of modafinil on MPTP-induced disease that would point to a neuroprotective effect of modafinil being in part the result of changes in local gene expression.

Materials and methods

In vivo experiment

Animals

Ten weeks old male C57BL/6 mice (Harlan Netherlands b.v., Horst, The Netherlands) were used in this study. Ambient temperature in the housing room was regulated at 20 ± 2 °C and relative humidity was always around 50%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm with free access to food pellets and water. Four animals were housed in one cage, one of each treatment group. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

The four experimental groups are displayed in Table 1. The behavior of all groups was tested in open field and rotarod tests at 6, 24, 48 and 72 hours post-intoxication. After the last behavioral test the brains of isoflurane-sedated animals were obtained for immunohistochemical analysis. The dose of modafinil, MPTP and vehicles were based on the MPTP mice study of Fuxe et al. (1992). Modafinil (d,1-2-[(diphenyl methyl)sulfinyl]acetamide) was used in grinded tablet form (Laboratoire L. Lafon, France). One tablet contains 100 mg modafinil and filling compounds: lactose, cornstarch, magnesiummonosilicate $2H_2O$, sodiumcroscarmellose, polyvidon, talc and magnesium stearate. Before usage the grinded tablets were freshly homogenized in a 0.5% arabic gum (Sigma chemical Co. St. Louis, MO, USA) solution and given in the dose of 100 mg/kg i.p. MPTP (Sigma chemical Co. St. Louis, MO, USA) was dissolved in saline and given in the dose of 40 mg/kg s.c.

Behavior

Rotarod: The rotarod test, which requires animals to balance and walk on a rotating cylinder, is a widely used test to measure coordinated motor skills (Kelly et al. 1998). In this experiment animals were trained to walk on the rotarod (TSE systems GmbH, Bad Homburg, Germany) at a speed of 12 rotations per minute (rpm) for 180 seconds. Two tests were performed. First, animals were tested twice at the training speed for 120 seconds and the longest stay was used in the data analyses. This test was followed by the acceleration test, in which the animals were placed on a 15 rpm turning rod. Five seconds after placement the rod accelerated to a speed of 55 rpm within 175 seconds. The maximum speed of the rod before the fall was used in the data analyses. If the animal fell directly off the rod a speed of 0 was registered. There was a 120 seconds rest period between the two trials of the 12 rpm test and between tests. Baseline values of tests were obtained one day before disease induction.

Open field: The level of activity and exploratory behavior is a measurement in the parkinsonian behavior of MPTP mice. The open field is a commonly used behavioral test system to asses this type of behavior (Sedelis et al. 2001). The apparatus consists of a 100 x 100 cm white square field with high walls placed in a room with a light intensity of 96 lux. The animals could freely explore the open field for 10 minutes.

Table 1 Treatment schedule of the *in vivo* experiment.

Treatment group	N	Treatment		
		t=-12 h	t=0	t=10 min.
Control	6	0.5% arabic gum	saline	0.5% arabic gum
MPTP + vehicle	6	0.5% arabic gum	MPTP	0.5% arabic gum
MPTP + modafinil	8	0.5% arabic gum	MPTP	100 mg/kg modafinil
Modafinil 12 h before MPTP	6	100 mg/kg modafinil	MPTP	0.5% arabic gum

A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the distance moved in pixels. Before start of the experiment all animals were placed in the open field twice to habituate to this environment and to exclude novelty effects. The baseline values were obtained one day before disease induction.

Immunohistochemistry

Brains were analyzed for the presence of DAergic neurons with TH-IR. The right hemisphere was fixated in 4% paraformaldehyde at 4 °C and after 48 hours the brains were transferred to 0.5% paraformaldehyde at 4 °C. After dehydration in graded ethanol and xylene, brains were embedded in paraffin. Serial sagittal sections of 5 μm were cut on a microtome and collected serially on AAS (aminosilane/acetone solution) coated slides. The sections were deparaffinated and rehydrated in xylene and graded ethanol. The citrate buffer method was applied for antigen retrieval. The sections were pre-incubated with 0.3% H_2O_2 in PBS to quench endogenous peroxidase oxidase. Thereafter the sections were pre-incubated in PBS with 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100. Incubation in anti-TH serum (rabbit anti-TH, 1:1000, Chemicon) was overnight at room temperature. The secondary antiserum (goat anti-rabbit 1:600, Santa Cruz Biotechnology inc., CA, USA) was incubated for 90 minutes followed by a 90-minute incubation with Vector ABC (1:800, Vector laboratories inc., Burlingame, Canada). PBS-washes were applied after each pre-incubation and antibody incubation step. Thereafter the sections were pre-incubated for 10 minutes with 0.025% 3'3'-diaminobenzidine containing 0.15% nickel ammonium sulphate (DAB-NI solution) followed by a 10-minute incubation in the DAB-NI solution with 0.00015% H_2O_2 to visualize bound immunocomplexes. After a PBS-wash the sections were dehydrated in alcohol series, cleared up in xylol and cover-slipped with DePeX (BDH Laboratory supplies, England).

For the identification of the treatment effects the number of immunoreactive neurons was counted with a 10 x 10 mm grid at a magnification of 400x on a Olympus microscope. The TH-IR neurons were counted in 4 sections of the substantia nigra pars compacta (SNpc). Within each section, TH-IR neurons were also counted in three parts of the SNpc.

Statistics

The data were analyzed using Repeated Measures ANOVA in SPSS (SPSS Inc, Chicago, USA). When relevant, an one-way ANOVA with a post-hoc test (LSD) was applied to test for differences between the treatment groups. Differences were considered to be statistically significant if $p<0.05$. Immunochemical data were evaluated with a one-way ANOVA followed by a post-hoc multiple comparison procedure (Bonferroni corrected t-test).

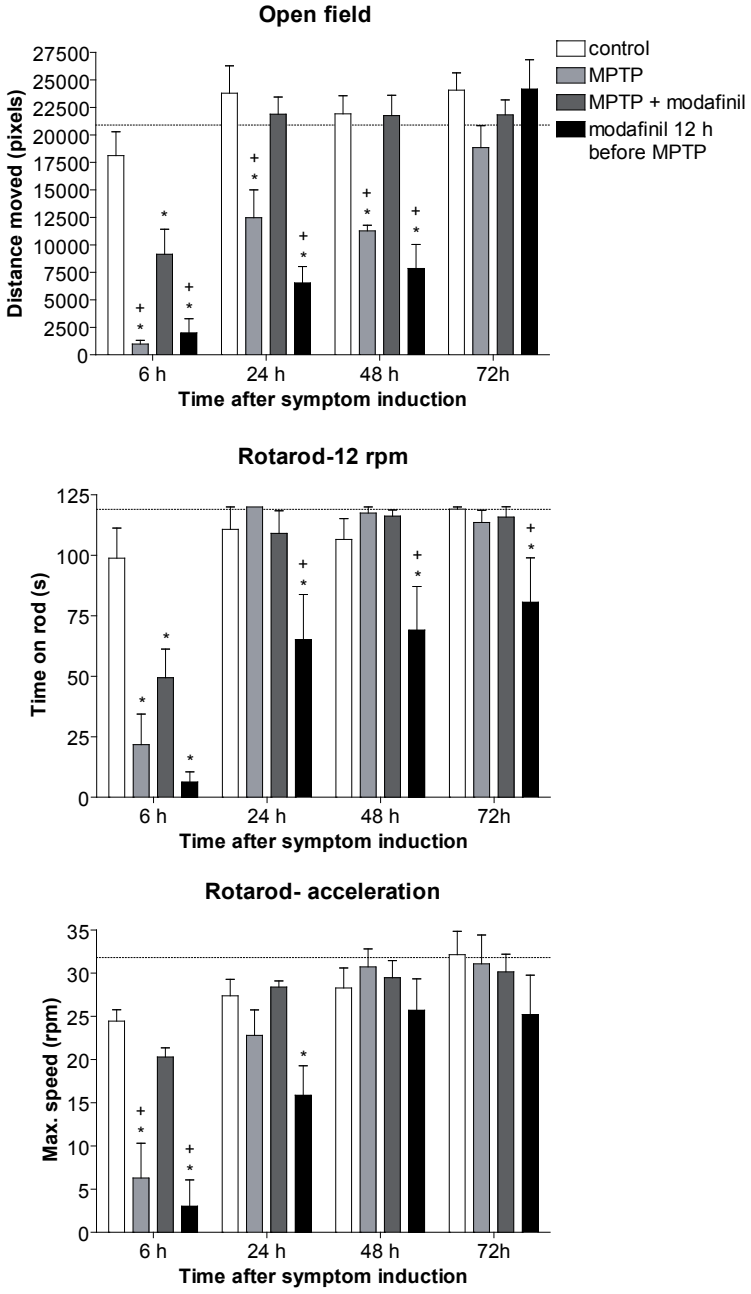

Fig. 1 Behavioral tests. Mean + SEM of performance on the open field test and rotarod measured at 6, 24, 48 and 72 hours after MPTP intoxication. *p<0.05 vs. control values. +p<0.05 vs. MPTP + modafinil. Dotted line indicates baseline measures.

In vitro experiment

Gene profiling of human astrocytes in the presence or absence of modafinil was performed by hybrid selection of ^{32}P-labeled cDNA on Clontech Atlas® arrays as previously described (Meeuwsen et al. 2003). Briefly, adult human astrocytes were isolated of post-mortem white matter tissue samples from two donors that were free from any clinical or pathological signs of neurodegeneration. After isolation, astrocytes were cultured in poly L-lysine coated 96 well-flat-bottom plates at 1 x 10^4 cells per well in 100 µl DMEM/HAM-F10 medium containing 10% FCS and antibiotic supplements. Confluent cultures of astrocytes were supplied with HPLC-purified modafinil to a final concentration of 100 µM, or left untreated as a control. After 48 hours, RNA was isolated from the cells and reverse transcribed into cDNA in the presence of [^{32}P]-ATP. Next, the radiolabeled cDNA was hybridized onto a cDNA array containing specific probes in duplicate for 268 human genes encoding various cytokines, chemokines, growth factors and their receptors. Hybridization signals for each of these genes were calculated as the mean of the duplicate measurements, corrected for background intensity and quantified using software provided by the manufacturer. Relative expression was calculated by dividing these hybridization signals by the mean signal for all nine house-keeping genes that are present on each array as an internal reference standard. The resulting relative signal was multiplied by 1,000. Thus, a relative level of expression of 100 for example implies a hybridization signal at 10% the intensity of the average house-keeping gene.

Results

In vivo experiment

Behavior

The activity and the motor skills of the MPTP + vehicle treated mice were reduced after MPTP treatment compared to control levels (LSD t-test, $p<0.05$) at 6 hour in the rotarod test and at 6, 24 and 48 hour in the open field test (Fig. 1). MPTP + modafinil treatment resulted in improved performance in the open field test and the acceleration rotarod test compared to the MPTP + vehicle treatment (LSD t-test, $p<0.05$), whereas a tendency was found on the 12 rpm rotarod test (LSD t-test, $p=0.086$). This improvement was not seen in the 'modafinil 12 hours before MPTP' group (LSD t-test, $p>0.05$). Remarkably, the performance on the rotarod in this group is reduced for a longer time span than in the vehicle + MPTP treated mice. The reduction in the 12 rpm challenge was sustained during all measurements and the reduction in the acceleration rotarod test remained reduced 24 hours longer than the MPTP + vehicle treated mice.

Immunohistochemistry

MPTP intoxication in the vehicle group resulted in a reduction of 71.7 ± 11.1% of control TH-IR neurons (one-way ANOVA, $p<0.05$ vs. control). The reduction of neurons in the MPTP + modafinil treatment group was only 41.8 ± 26.9% of control values which was significantly different from the MPTP + vehicle treated group (one-way ANOVA, $p<0.05$ vs. MPTP + vehicle). Modafinil treatment prior to MPTP did not result in a comparable reduction. The reduction of neurons was 70.9 ± 16.5% of control, significantly lower than simultaneous modafinil treatment values (one-way ANOVA, $p<0.05$ vs. control, vs. MPTP + modafinil) and thus comparable to the MPTP + vehicle treatment group.

In vitro experiment

Modafinil revealed a very limited direct effect on expression levels of astrocyte genes within the 48-hour monitoring time. Only in a few cases (Table 2) a consistent and marked change was observed in gene expression levels with most changes involving down-regulation. The most striking change observed in both experiments was a modafinil-induced 5-fold suppression of insulin-like growth factor binding protein 5 (IGFBP-5). In addition, a mild 2-fold down-regulation of brain-derived

Table 2 Effects of modafinil on the gene expression profile of human astrocytes.

Gene transcripts down-regulated by modafinil			
Gene product	*Reference signal*	*Treated signal*	*Ratio*
BDNF	628/436	354/212	0.56/0.49
IGFBP5	160/293	22/58	0.14/0.20
EDF	102/155	30/126	0.29/0.81
ERBB2	184/268	89/226	0.48/0.84
HGF receptor	141/265	41/189	0.29/0.71
Follistatin-related protein	140/94	9/48	0.06/0.51
PAF-receptor	149/149	130/56	0.87/0.38
Interleukin-5	100/177	40/147	0.40/0.83
Interleukin-15	131/183	55/140	0.42/0.76
Interleukin-12 receptor	107/130	53/94	0.49/0.72
Gene transcripts up-regulated by modafinil			
Gene product	*Reference signal*	*Treated signal*	*Ratio*
Frizzle-related FrzB	60/208	180/265	3.02/1.28
B-cell growth factor	66/24	126/141	1.91/5.91
EPO receptor	87/36	99/100	1.14/2.78
G-CSF receptor	17/94	102/122	5.95/1.29

Data are given for two independent experiments using different astrocyte isolates. For further details, see materials and methods. Selection criteria for down- or up-regulation include a strongest expression signal > 90 in both experiments, and a change factor > 2 in at least one experiment.

neurotrophic factor (BDNF). was observed. Other changes affected genes that were expressed in astrocytes at rather low levels, and are therefore less likely to be of biological significance.

Discussion

The experiments reported here aimed to explore the possibility that the neuroprotective mechanism of modafinil is associated with effects on local gene expression. Evidence was generated via an *in vivo* experiment assessing the indirect neuroprotective actions of modafinil and an *in vitro* experiment assessing the direct effects of modafinil on astrocyte gene expression.

The results of the *in vivo* experiment showed that modafinil administered 12 hours before MPTP intoxication was not able to prevent neuronal damage induced by MPTP while concurrent administration of modafinil with MPTP did result in a higher survival of the neurons in the SNpc. The latter group was included to obtain a proof of principle as this effect has already previously been reported by Fuxe et al. (1992). This difference in efficacy was also clearly revealed by the open field test. However, the behavioral improvement in the modafinil + MPTP group at 6 hours was not completely devoid of symptomatic effects of modafinil itself. Modafinil given to naïve mice also exerted an increase in locomotor activity as well as improvement on the rotarod test, which is a known effect of modafinil in intact animals (Simon et al. 1995, chapter 2). The behavioral improvement after 24 hours is only a result of the neuroprotective effects of modafinil.

It cannot be excluded that the 12-hour interval chosen between modafinil administration and disease induction could still allow for any unnoticed effect of modafinil on transcript levels. Since it is unknown which proteins could possibly mediate an indirect effect of modafinil, relevant protein expression could either have already disappeared, or not yet be produced at sufficient levels. More direct analyses of gene expression profiles over several points in time will be required to fully address this issue.

The experiments in which direct effects of modafinil were examined on the expression profile of human astrocytes with regard to inflammatory and trophic mediators indicate an absence of broad effects at that level. This appears consistent with the observation that the neuroprotective effects of modafinil *in vivo* are apparent only shortly after administration, but not when modafinil is given 12 hours before. Previous studies have indicated that cultured astrocytes tend to respond to different molecular signals by accumulating responsive transcripts over periods of days rather than hours (Meeuwsen et al. 2003). Resulting proteins would thus be expected to be produced over a similarly long period. We consider it likely that this time frame would also apply to any comprehensive effect that would be relevant to modafinil's actions. While modafinil at 100 μM clearly did not trigger any marked overall change in expression levels of the vast majority of the 268 cytokine, chemokine and

growth factor genes examined, a few changes did occur. It is of interest to note that modafinil appears to strongly and selectively down-regulate production of IGFBP-5 by astrocytes. IGFBPs regulate the functions of insulin-like growth factors (IGFs) that play central roles in CNS plasticity and regeneration during recovery from trauma. IGFBP-5 in particular has been demonstrated to play a stimulatory role in fibrosis in both the lung and skin (Yasuoka et al. 2006a, b). Assuming a similar function of IGFBP-5 in the CNS, a reduction of IGFBP-5 release by astrocytes could conceivably inhibit gliosis in response to MPTP-induced damage. Thus, modafinil would increase the opportunities for functional recovery rather than the formation of dysfunctional gliotic scars following trauma. Additional studies will be required to confirm and further clarify this potential effect of modafinil.

Taken together, the data obtained in the two experiments performed in this study both point to a lack of a substantial indirect and lasting neuroprotective effect of modafinil. To fully rule out indirect effects, additional studies will be required to address the present experimental limitations such as the timing interval in the *in vivo* experiment, and the selective focus on astrocytes. Despite this, our data give a clear indication that the neuroprotective effects of modafinil do not last for 12 hours and that they are probably not mediated via modulation of trophic support or inflammatory responses by astrocytes.

Chapter 9

Neuroprotective effects of Δ⁹-tetrahydrocannabinol in a marmoset Parkinson model

Sanneke van Vliet, Raymond Vanwersch, Marjan Jongsma, Jan van der Gugten, Berend Olivier, Ingrid Philippens

Manuscript submitted

Abstract

The present medication in Parkinson's disease (PD) is unable to stop or slow down the progression of the disease. Therefore, pharmacological intervention at crucial steps in the neuronal cell death processes would be a better strategy. Cannabinoids are potent neuroprotective compounds in models of oxidative stress and excitotoxicity and offer potential protection in models of PD.

Therefore, the present study determines the neuroprotective effects of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) in the marmoset 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model on behavior and pathology. Twelve marmoset monkeys were treated with a total cumulative dose of 6 mg/kg MPTP in 9 days. Seven of these animals received simultaneously, a daily oral dose of Δ⁹-THC (4 mg/kg) and five animals received simultaneously vehicle for 27 days. The parkinsonian symptoms were observed daily and locomotor activity and hand-eye coordination were tested once a week during the experimental period. Post-mortem, dopamine (DA) levels in the striatum were analyzed and tyrosine hydroxylase immunohistochemistry (TH-IR) was applied to determine viable DAergic neurons in the substantia nigra. Δ⁹-THC has no protective effects on any parameter. These negative results might be related to the severity of the cell death induction by MPTP in relation to the low dose of Δ⁹-THC used in this Parkinson model.

Introduction

In PD the output of the basal ganglia is irreversibly affected by degeneration of the neuromelanin-containing DAergic neurons in the substantia nigra pars compacta (SNpc). This results in symptoms including akinesia, postural instability, rigidity and resting tremors (Fearnley and Lees 1991).

PD is incurable, and present medications (predominantly levodopa) do not counteract progression of the disease, and long-term medication is associated with declined efficacy and increased side-effects (Clarke 2004). Therefore, a better strategy aims to focus on prevention of the neuronal loss in an attempt to stop or slow down the progression of the disease. One way to achieve neuroprotection is via pharmacological interference aimed at crucial steps in the neuronal cell death process to promote neuronal survival. Although some potential drug candidates were tested in clinical trials there is no proven neuroprotective treatment yet (Clarke 2004).

The actual cause of PD is unknown. There is evidence suggesting that factors like mitochondrial dysfunction, oxidative stress, excitotoxicity and inflammatory processes, either separately or cooperatively, are involved in the underlying neurodegenerative process (Alexi et al. 2000).

Cannabinoids appeared neuroprotective in cerebral ischemia (Nagayama et al. 1999), brain trauma (Panikashvili et al. 2001), multiple sclerosis (Lyman et al. 1989) and nerve gas-induced seizures (Filbert et al. 1999), but also *in vivo* and *in vitro* models of oxidative stress and excitotoxicity (reviewed by Grundy et al. 2001). Most of these protective effects appear mediated by activation of the cannabinoid CB_1 receptor (Parmentier-Batteur et al. 2002), although the contribution of other mechanisms (i.e., anti-oxidant and/or anti-inflammatory properties of cannabinoids) have also been reported (Grundy et al. 2001).

Δ^9-tetrahydrocannabinol (Δ^9-THC), the main component of marijuana, induces psychoactive properties via agonistic actions on cannabinoid CB_1 receptors. Δ^9-THC has neuroprotective properties in cellular and animal models of oxidative stress, ouabain -a sodium pump blocker- induced excitotoxicity or glutamate-induced excitotoxicity (Hampson et al. 1998, Van der Stelt et al. 2001a, El-Remessey et al. 2003, Chen et al. 2005 a, b). Δ^9-THC administration resulted in delay of motor deterioration in an animal model of amyotrophic lateral sclerosis (Raman et al. 2004) and Δ^9-THC partially protected against 3,4-methylenedioxy-N-methylamphetamine (MDMA)-induced serotonin depletion (Morley et al. 2004). These findings indicate Δ^9-THC to be a potent anti-oxidant and anti-excitotoxitant and suggest its use as a neuroprotective drug in PD. Recently, the group of Lastres-Becker (2005) showed that Δ^9-THC protects against unilateral infusion of 6-hydroxydopamine (6-OHDA)-induced neurodegeneration of the SNpc neurons.

To extend the investigation towards application of Δ^9-THC in the protection of the SN neurons in PD, we tested the neuroprotective effects of Δ^9-THC in the MPTP marmoset model for PD. MPTP, a neurotoxic agent, selectively damages DAergic neurons in the SNpc by blocking the electron transport chain of the

mitochondria leading to a loss in mitochondrial function resulting in a depletion of ATP and eventually cell death (Dauer and Przedborski 2003). In experimental PD research the MPTP animal model is widely accepted and findings are confirmed in human PD brains (Dauer and Przedborski 2003). The non-human primate model is preferable as these animals show clear and lasting behavioral features after MPTP treatment, which reflect many aspects of human Parkinson symptoms (Jenner and Marsden 1986). Even a clinically used observational scale for involuntary movements (AIMS) can be applied to the marmoset without adaptation (Di Monte et al. 2000). Furthermore, similarities to humans with regard to the anatomy of the striatum, distribution of DA neurons in the SNpc and striatal DA function are supportive of the choice of the non-human primate model (Eslamboli 2005).

In the present study, the protective effects of Δ⁹-THC are measured with various markers including motor functions by behavioral tests and pathological measurements at the brain level. These markers are sensitive to reflect neurodegeneration and neuroprotection in the MPTP marmoset model (chapter 6). The parkinsonian symptoms are assessed by using two extensive behavioral observation scales for PD and functional tests measuring locomotor activity and hand-eye coordination (Wolthuis et al. 1994, Philippens et al. 2000). The survival of the DAergic neurons in the SNpc is investigated with TH-IR. Staining of TH, the first and rate-limiting enzyme in the synthesis of the catecholamines, is often used as a quick and sensitive measure for localization of surviving DAergic neurons in the MPTP-model (Pearson et al. 1983). The functionality of the surviving neurons was assessed by measurement of the level of DA neurotransmission in the striatum.

Materials and methods

Animals

Adult male and female marmoset monkeys *(Callithrix jacchus),* aged 2-6 years with initial body weights between 350-550 g were obtained from the Biomedical Primate Research Centre (BPRC), The Netherlands and Harlan, United Kingdom. The ambient temperature in the housing room was regulated at 25 ± 2 °C and the relative humidity was always >60%. A 12-hour light-dark cycle was maintained, lights on from 7 am to 7 pm. All aspects of animal care are described in Standard Operating Procedures, which are in agreement with current guidelines of the European Community. The independent TNO committee on Animal Care and Use approved all protocols for the animal experiments.

Study design

Twelve naïve marmosets were treated, cumulatively, with 6 mg/kg MPTP s.c. over 9 days (2 mg/kg at day 1 and 1 mg/kg at days 2, 3, 6 and 9). Seven of these animals (4 males; 3 females) additionally received a daily oral dose of 4 mg/kg Δ⁹-THC from experimental day 1 until day 27. The remaining five animals (3 males; 2 females) additionally received a daily oral dose of the vehicle (10% sucrose solution in water).

Δ^9-THC or vehicle treatment was given directly after the MPTP injections.

The dose of Δ^9-THC chosen was based on a commonly used oral dose of Δ^9-THC in non-human primates, the pharmacokinetics of oral Δ^9-THC (Perlin et al. 1985, Aigner 1988, Grotenthermen 2003) and behavioral studies at our laboratory (chapter 4). Δ^9-THC was dissolved in 50 mg/ml ethanol (IBL, Leiden, The Netherlands) and orally administered simultaneously with 1 ml/kg 10% sucrose solution.

Before disease induction, animals were trained on the hand-eye coordination task and baseline values of all test systems were obtained. The occurrence of parkinsonian symptoms was observed daily before administration of the treatment using two rating scales: clinical score and AIMS. At day 13, 20 and 27, before daily administration of the treatment, the hand-eye coordination task and locomotor activity were tested in non-invasive test systems. At day 37, ten days after the last Δ^9-THC administration, brains were removed after decapitation under isoflurane anesthesia. One hemisphere was used for immunohistochemistry and the other for neurochemical measurements.

Behavioral assessment

Observation of signs and symptoms: For the observation of signs and symptoms two rating scales were used. 1) A general clinical scoring list in which the condition of the animal is rated. The following symptoms were registered: appetite, inadequacy of grooming by inspection of the fur; apathy by testing the responsiveness of the animal to its surroundings; immobility; rigidity and presence of tremors. The rates of severity were coded from 0 (normal) to 4 (severe). 2) The AIMS is a 9-item rating scale, designed to record in detail the occurrence of involuntary movements (Guy 1976). The AIMS is widely used clinically for qualification of involuntary movements, occurring in PD (Katzenschlager et al. 2004). These scales have successfully been applied for more than 10 years in monkey research in our institute. The AIMS includes facial, mouth (lips, peri-oral area, jaw and tongue), extremity and trunk movements. The global judgment of the severity and the incapacitation due to the abnormal movements were also scored. All items were rated from 0 (normal) to 4 (severe). Movements that occur upon stimulation by the observer were rated one step lower than those observed spontaneously.

Spontaneous exploratory behavior (Bungalow test): The levels of spontaneous activity and exploratory behavior can play an important role in practically all measurements of animal behavior. A device called the 'Bungalow test' automatically and quantitatively assesses these variables and has been extensively described and validated (Wolthuis et al. 1994, Philippens et al. 2000). The apparatus consists of four horizontally placed non-transparent boxes (23 x 23 x 23 cm) all interconnected by 6 PVC tubes (inner diameter 9.5 cm). Each animal was placed in the same compartment at the start of each session. There was one animal per session. The animals could freely move and change from one compartment to another during the 20-minute session. A video tracking system (Ethovision, Noldus, Wageningen, The Netherlands) registered the locomotor activity of the animal, expressed as the number of compartment changes during the session.

Hand-eye coordination task: An automated robot-guided apparatus with positive reinforcement as a motivating stimulus (small pieces of marshmallow) has been used to asses the hand-eye coordination (Philippens et al. 2000). The marmoset is placed in front of a window in the test panel (8 x 5 cm). A robot arm presents a reward behind the window. With this system three types of trials were performed: one using a non-moving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving reward (0.08 m/s). The animal was allowed one minute to grasp the non-moving reward. Each type of trial was presented 14 times per session. At the beginning of each trial a sound signal was presented, intended to alert the animal. A pressure detector in the robot arm and infrared detectors in the window registered hits and attempts and speed of performance. A 'hit' was registered when the animal successfully retrieved the reward from the robot arm. The percentage of correct hits was used as a criterion to judge the performance of the animal. Before the start of the study, all animals were trained to successfully grasp a minimum of 80% of the presented rewards.

High performance liquid chromatography (HPLC) analysis

For determination of brain DA levels, five brains of the Δ⁹-THC treated parkinsonian group and five from the vehicle treated parkinsonian group were used. Furthermore, six brains of naïve animals were used to establish control values of the DA. At day 37, ten days after the last Δ⁹-THC administration, brains were removed after decapitation of the sedated animals. The striatum of one hemisphere was isolated after termination and was directly frozen in liquid nitrogen. The tissue (100-500 mg) was weighed and homogenized in 10 ml 0.4 M perchloric acid containing 20 ng/ml 3,4-dihydroxybenzylamine hydrobromide (Sigma Chemical co., St. Louis, USA) and 20 ng/ml (±)-isoproterenol hemisulfate salt (Sigma Chemical co., St. Louis, USA) as internal standards. Homogenate was centrifuged at 22,000 g for 30 min. at 4 °C and 1 ml of supernatant was adjusted to about pH 4.0 with 250 µl 2 M sodium acetate. The homogenate samples were stored at -70 °C for a maximum of 6 weeks. DA and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA) were determined by ion-pair reversed phase liquid chromatography. A 2-50 µl sample was injected on a RP18 LiChrosfer 100 column (125 x 4 mm i.d., 5 µm particle size; Merck, Darmstadt, Germany) connected to a Thermo Separations Products (San Jose, CA, USA) model P100 solvent delivery pump and AS300 autosampler and a Coulochem II Model 5011 electrochemical detector (ESA, Bedford, MA, USA). The mobile phase consisted of a 30 mM citrate/40 mM phosphate buffer, pH 4.0, containing 0.27 mM Na_2EDTA, 1.8 mM heptanesulphonic acid and 5% methanol. The potential of the electrode was set at 420 mV. External standards were determined in each assay run. Calibration plots were linear from 2 to 500 ng/ml for each compound. The lower limit of detection was 2 ng/ml. The intra-assay coefficient of variation amounted to 2%.

Immunohistochemistry

Brains (vehicle treated parkinsonian group: n=5; Δ⁹-THC treated-parkinsonian group:

n=5) were analyzed for the presence of DAergic neurons with TH-IR. Four brains of naïve animals (control group) were used to establish control values. From collected brains, the right hemisphere was fixated in 4% paraformaldehyde at 4 °C and after 48 hours the brains were transferred to 0.5% paraformaldehyde at 4 °C. Brains were dehydrated in graded ethanol and xylene and subsequently embedded in paraffin. Serial transversal sections of 5 μm were cut on a microtome and collected serially on aminosilane/acetone solution coated slides. Every 4th section was mounted on egg-white coated glass slides for cresylviolet staining which was used as a reference for the TH-IR localization.

The sections used for the TH-IR were deparaffinated and rehydrated in xylene and graded ethanol. The citrate buffer method was applied for antigen retrieval. Sections were pre-incubated with 0.3 % H_2O_2 in PBS to quench endogenous peroxidase activity. Thereafter the sections were pre-incubated in PBS with 0.1% bovine serum albumin (BSA) and 0.3% Triton X-100. Incubation in anti-TH serum (1:80000, Sigma chemical Co. St. Louis, MO, USA) was overnight at room temperature. The secondary antibody (1:2000, Santa Cruz Biotechnology inc., CA, USA) was incubated for 90 minutes followed by a 90-minute incubation with Vector ABC (1:800, Vector laboratories inc., Burlingame, Canada). PBS-washes were applied after each pre- and antibody incubation step. Thereafter the sections were pre-incubated for 10 minutes with 0.025% 3'3'-diaminobenzidine containing 0.15% nickel ammonium sulphate (DAB-NI solution) followed by a 10-minute incubation in the DAB-NI solution with 0.00015% H_2O_2 to visualize bound immunocomplexes. After a PBS-wash the sections were dehydrated in alcohol series, cleared up in xylol and cover-slipped with DePeX (BDH Laboratory supplies, England).

TH-IR positive neurons were counted in 4 sections of the SNpc A4-5.5 mm anterior of the external auditory meati (marmoset brain atlas: Stephan et al. 1980). Within each section, TH-IR neurons were counted manually in three medial to lateral parts of the SNpc using an eye-piece grid of 10 mm x 10 mm at a magnification of 400x on a Olympus light microscope.

Statistics

The results of this study are presented as mean ± SEM and parametric statistical analysis was applied with a significance level of $p<0.05$. The scores of the behavioral observation scales were analyzed with independent t-tests to reveal differences between the two treatment groups. The results of the behavioral tests were analyzed in two ways. First, the difference between the two treatment groups was obtained. An overall repeated measure analysis was applied on the results of day 13, 20 and 27. When relevant, an independent t-test was applied. Second, the difference between baseline and test day results of each treatment was tested with a paired t-test.

The difference between the DA levels of each treatment was tested with a one-way ANOVA followed by a t-test when relevant. Immunochemical data were evaluated with a one-way ANOVA followed by a post-hoc multiple comparison procedure (Bonferroni corrected t-test).

Results

Behavior

The behavior rated with the observation scales showed that Δ⁹-THC could not prevent the development of parkinsonian symptoms during MPTP intoxication as both groups had comparable observational scores (Fig. 1a, b). During the first three MPTP injections the Δ⁹-THC treated parkinsonian animals were even worse than the

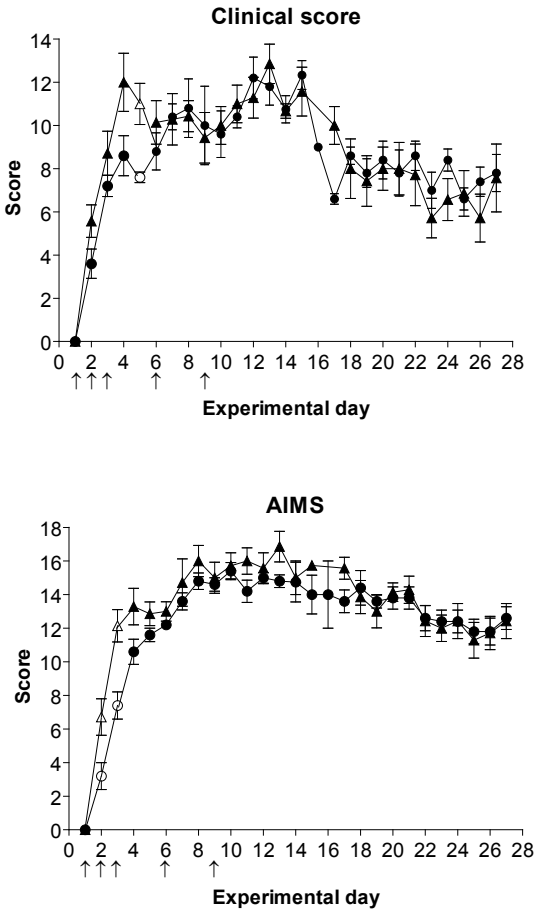

Fig. 1 Behavioral observation scales. Mean (± SEM) of the clinical (a) and AIMS (b) score before daily administration. Circles indicate the vehicle treated parkinsonian animals and triangles Δ⁹-THC treated parkinsonian animals. On experimental days 14-16 only n=0-4 were scored due to MRI and MRS-scans of part of the animals (chapter 7). Arrows indicate day of MPTP injection. Δ⁹-THC or vehicle was given from day 1-27. Open data points indicate significant difference between vehicle and Δ⁹-THC treated parkinsonian animals (t-test, $p<0.05$).

vehicle treated parkinsonian animals. Significant differences were found with the AIMS at day 2 and 3 and with the clinical score at day 5 (t-test, $p<0.05$). A tendency to a difference was found with the AIMS at day 3 and with the clinical score at day 2 and 4 (t-test, $p<0.1$).

Similar to the findings during observations, the hand-eye coordination task performance and locomotor activity (Fig. 2), tested during day 13, 20 and 27, were lower than before disease induction in both treatment groups (paired t-test, $p<0.05$). Moreover, no differences between these two groups were found in the test systems.

Biochemical analysis

The levels of DA and its metabolites, HVA and DOPAC, were clearly reduced in the MPTP + vehicle and MPTP + Δ⁹-THC groups (t-test, $p<0.001$, Table 1). No differences were found between both groups.

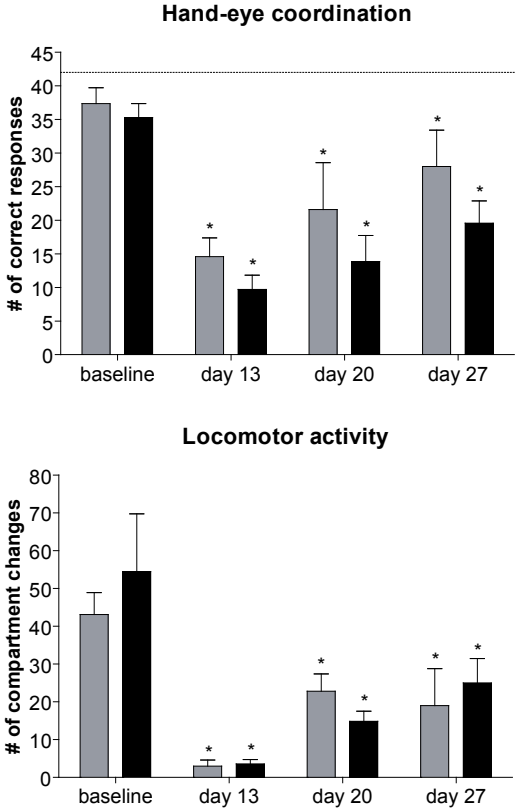

Fig. 2 Behavioral tests. Grey bars show mean (+ SEM) of vehicle treated parkinsonian animals. Black bars show mean (+ SEM) of modafinil treated parkinsonian animals. Dotted line indicates maximum correct responses. *$p<0.05$ vehicle vs. Δ⁹-THC treated parkinsonian animals.

Immunohistochemistry

At the cellular level the inability of Δ⁹-THC to protect against the MPTP intoxication was also clear (Fig. 4). Both MPTP groups had a similar number of remaining DAergic neurons. In the MPTP + vehicle group a reduction of 75.8 ± 5.3% of TH-IR neurons was found (one-way ANOVA, $p<0.001$ vs. control) and in the Δ⁹-THC treated MPTP group a reduction of 70.7 ± 4.2% of number of TH-IR neurons was found (one-way ANOVA, $p<0.001$ vs. control).

Discussion

This study is one of the first investigating the neuroprotective effects of Δ⁹-THC in a monkey model of PD. Recently, the group of Lastres-Becker (2005) reported positive results with Δ⁹-THC, a cannabinoid CB_1 receptor agonist, in the unilateral 6-OHDA rat model. In that study 3 mg/kg Δ⁹-THC i.p. was given 16 hours after the 6-OHDA infusion and continued daily for 2 weeks. In contrast to the findings in the 6-OHDA model, orally given Δ⁹-THC in a dose of 4 mg/kg was not able to protect the DAergic neurons in the SNpc against MPTP-induced neurodegeneration in the marmoset monkey. The parkinsonian symptoms were not reduced after Δ⁹-THC treatment, damage of the DAergic neurons was not prevented and the functionality of the surviving neurons was comparable to vehicle treatment.

The MPTP model has proven to be useful for studying neuroprotective effects. The behavioral markers used for parkinsonian symptoms, DA levels and neuronal survival, are sensitive to neurodegeneration by MPTP and neuroprotection in the marmoset monkey (chapter 6). Therefore, the cause of the discrepancy between the results of the 6-OHDA rat study and this study can be due to the differences between the experimental designs, which will be discussed in the following sections.

The application of two different neurotoxins could be an explanation for the discrepancy. However, the mechanisms of 6-OHDA and MPTP intoxication imply comparable cellular modifications susceptible to induce cell death of DAergic cells

Table 1 Monoamine and metabolite levels. Mean ± SEM in μg/g tissue of the striatum of untreated controls or vehicle and Δ⁹-THC treated parkinsonian animals.

	Control	MPTP + vehicle	MPTP + Δ⁹-THC
Dopamine	5.47 ± 0.85	0.27 ± 0.08 ***	0.47 ± 0.17**
DOPAC	0.73 ± 0.06	0.16 ± 0.06 ***	0.28 ± 0.02 ***
HVA	5.37 ± 0.59	0.34 ± 0.09 ***	0.66 ± 0.15***
Dopamine turnover	1.24 ± 0.18	2.10 ± 0.29 *	3.50 ± 1.28

Dopamine turnover: ((DOPAC +HVA)/ DA); *** $p<0.001$ vs. control levels, ** $p<0.01$ vs. control levels, *$p<0.05$ vs. control levels (ANOVA followed by t-test).

(Blum et al. 2001). Both methods result in mitochondrial deficits and oxidative stress, although the induction of oxidative stress is slightly different as 6-OHDA generates reactive oxygen species (ROS) via deamination by monoamine-oxidase-B or auto-

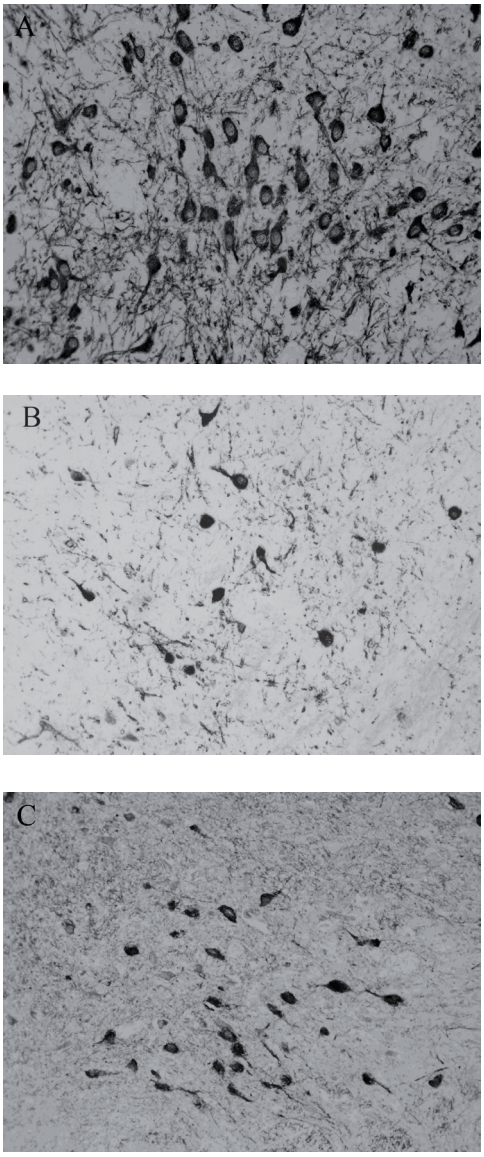

Fig. 4 Tyrosine hydroxylase immunoreactivity. Images of the dopaminergic neurons in the substantia nigra pars compacta (magnification 200x) of a control animal (A), Δ⁹-THC treated (B) and vehicle treated (C) parkinsonian animal.

oxidation and 1-methyl-4-phenylpyridinium (MPP^+), the effective metabolite of MPTP, becomes a radical after reaction with xanthine oxidase (Blum et al. 2001). ROS and radicals can both be deactivated with anti-oxidants (Alexi et al. 2000). This implicates that cell death processes induced via MPTP or 6-OHDA would be comparably susceptible for neuroprotective intervention with cannabinoids.

Despite the comparable cellular modification, the time frame of cell death differs between the models. Degeneration starts 12 hours after a single MPTP dose and continues till day 4 post-treatment (Jackson-Lewis et al. 1995), whereas after a single dose of 6-OHDA degeneration starts 12 hours post-injection and ends 7-10 days later (Jeon et al. 1995).

Furthermore, in the present study MPTP was given with repeated injections, which is a normal procedure in the MPTP non-human primate model. Therefore, the severity of DA depletion in this study is higher than in the 6-OHDA study of Lastres-Becker et al. (2005) (95% vs. 46% reduction). The severity level of cell death might be of primary importance in the success of neuroprotective compounds to prevent cell death. Therefore, the more severe induction scheme used in the present study might need a different strategy in the application of the neuroprotective compound, e.g. different doses.

The dose of the compound is essential for the success of a neuroprotective compound. Oral administration of 4 mg/kg Δ⁹-THC used in this study was a pharmacological effective dose as this dose evoked behavioral responses in naïve marmoset monkeys (chapter 4). This dose did not differ much with the dose of 3 mg/kg used in the 6-OHDA model, although the method of administration can affect the dose available for the neuroprotection processes. In this study, Δ⁹-THC was orally administered as the plasma concentration of Δ⁹-THC is more stable over time in contrast to inhalation and could therefore generate a longer lasting neuroprotective effect. After oral intake, the level of Δ⁹-THC in human plasma is maximal 60-120 minutes post-dosing and has an elimination half-life of 25 hours (Grotenthermen 2003). The low clearance level after oral intake may benefit the neuroprotective effects of Δ⁹-THC as the degeneration process takes a couple of days (Jackson-Lewis et al. 1995). A disadvantage of oral administration is the low bioavailability (50% lower than after inhalation or rectal administration) due to degradation by the acid of the stomach, the gut and the first-pass effect (Grotenthermen 2003). I.p. injections bypass these effects and the total bioavailability is expected to be higher as well as the peak concentration. Therefore an assumption may be that in the present MPTP experiment the Δ⁹-THC concentration in the brain to exert neuroprotective actions was clearly lower than after i.p. injections in the 6-OHDA experiment, although the higher metabolic rate in rodents might result in a higher clearance level.

The concentration of cannabinoids in the brain during neuroprotection is of importance as for effective protection via anti-oxidant properties a higher concentration is needed than via receptor mediated protection (Grundy 2001). The anti-oxidant properties of cannabinoids responsible for the protection of DAergic neurons are further consolidated with a recent demonstration in the 6-OHDA model. It revealed that neuroprotection was established via cannabinoids

which acted independently of the cannabinoid CB_1 receptor and proved failure of neuroprotection by synthetic cannabinoid CB_1 agonists (Garcia-Arencibia et al. 2007). Δ^9-THC has, besides its agonistic actions on the cannabinoid CB_1 receptors also anti-oxidant properties as shown in oxidative stress models (Hampson et al. 1998). If the protection of the DAergic neurons is indeed not mediated via cannabinoid CB_1 receptors, a too low Δ^9-THC dose may contribute to the explanation of failure in preventing DAergic neuronal death after MPTP. The dose used in this study should be high enough to stimulate the cannabinoid CB_1 receptor. This dose was based on previous reports in which behavioral and anti-parkinson effects of Δ^9-THC were studied (chapter 4 and 5). This emphasizes the finding of Garcia-Arencibia et al. (2007) that the neuroprotective effect of Δ^9-THC is not regulated by direct action on the cannabinoid CB_1 receptor.

In conclusion, in this study oral Δ^9-THC in a dose of 4 mg/kg did not prevent severe DAergic cell death after repeated MPTP injections. These results are in contrast to the findings of Δ^9-THC in the 6-OHDA model (Lastres-Becker et al. 2005). The underlying explanation could be a combination of a more severe damage model and a rather low dose for anti-oxidant protection. A higher dose of Δ^9-THC and administration before disease induction to create a steady state level of Δ^9-THC is needed to examine whether the neuroprotective potential of Δ^9-THC is mediated via its anti-oxidant properties or to refute the existence of neuroprotection by Δ^9-THC in a PD model close to man.

Chapter 10

General discussion

The focus of this thesis is on neuroprotection in Parkinson's disease (PD). For this purpose compounds with putative neuroprotective potential, modafinil and Δ^9-tetrahydrocannabinol (Δ^9-THC) were tested in an animal model for PD, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model. Neuroprotective outcome was established with behavioral assessment for functional aspects and pathological measures reflecting cell survival and function. Furthermore, the putative mechanism behind the neuroprotective actions of modafinil was explored.

The neuroprotection studies were preceded by experiments to explore the behavioral responses on different doses of the compounds. Determination of the therapeutic characteristics of the compounds in intact animals supplemented the neuroprotection data.

Modafinil

Behavioral effects

Studies towards behavioral effects of modafinil preceded the therapeutic and neuroprotective studies. Reasons were to study the side-effects of modafinil in the behavioral read-out systems used in the MPTP marmoset studies as well as to establish the dose of modafinil to be applied in these studies. Modafinil had no clear side-effects, except on locomotor activity which increased (chapter 2). Furthermore, it was shown that repeated administration of modafinil (100 mg/kg) did not induce tolerance (chapter 3). Based on these studies 100 mg/kg was used as dose for further exploration of its therapeutic and neuroprotective effects.

The increased locomotor activity induced by modafinil seems to be independent of the state of the animal as healthy animals, sleep deprived animals, parkinsonian animals or animals during the neuroprotection experiment all showed increased activity after modafinil administration (chapter 2, 3, 5 and 6). Increased activity is a common characteristic of modafinil in mice, rats and marmosets (Simon et al. 1995, Jenner et al. 2000, Ward et al. 2004). This is in contrast to humans where modafinil only increases arousal in a sleep deprived state (Samuels et al. 2006, Ballon and Feifel 2006). However, related side-effects of modafinil such as nervousness and insomnia are reported (Robertson and Hellriegel 2003).

Therapeutic effects

Modafinil improved most of the motor-related symptoms in the parkinsonian marmoset (chapter 5). This indicates that modafinil could be applied in the clinical treatment of motor symptoms of PD-patients. The potential of modafinil is emphasized by the properties that it shares with levodopa. The superior therapeutic effect of levodopa is attributed to its indirect effects on DA receptors. This indirect modulation might also activate adrenoceptors, dopamine transporters (DAT) and trace amines (Mercuri and Bernardi 2005). Modafinil also has modulatory effects on the DAergic system without direct actions on dopamine D_1 and D_2 receptors. In line with levodopa, modafinil might therefore have a more widespread effect than

DA receptor agonists, which are less effective than levodopa in the treatment of PD (Lang and Lozano et al. 1998b).

There is no direct evidence of clinical translation of the therapeutic effects of modafinil in PD. However, indirect evidence could be derived from clinical studies towards treatment of excessive daytime sleepiness of PD patients with modafinil. Unfortunately, these clinical studies reveal only improvement on the Epworth sleepiness scale, but not on the Unified Parkinson's disease rating scale (UPDRS) (Adler et al. 2000, Högl et al. 2002, Ondo et al. 2005), which is a standard rating scale for the cardinal aspects of PD. However, the positive effects of modafinil on the motor symptoms may go unnoticed as only the total UPDRS score was reported and the focus of the study was towards effects on sleep. A more specific study in PD patients must be conducted to confirm the clinical importance of the findings in the marmoset.

Neuroprotection

Neuroprotective effects in MPTP model In chapters 6, 7 and 8 it is demonstrated that modafinil is able to partially prevent damage of the neurons in the SNpc caused by MPTP as proven with immunohistochemistry and magnetic resonance (MR) imaging. This probably resulted in improved DA release in the striatum and improved motor functions measured with behavioral tests. These experiments confirm earlier neuroprotective actions of modafinil described in various *in vitro* and *in vivo* models of ischemic, neurotoxic and mechanical injury in different brain structures (Fuxe et al. 1992, Ueki et al. 1993a, b, Lallement et al. 1997, Antonelli et al. 1998, Jenner et al. 2000).

In most experimental PD models neuronal damage is induced by external factors creating the opportunity for a neuroprotective compound to block the disease induction instead of protecting the processes that result from the disease induction. Therefore, the possibility of modafinil interfering with the MPTP transport towards the mitochondria has to be excluded.

The process of DAergic neuronal death after MPTP administration includes several steps. The first step is conversion of MPTP into MPP^+ by the enzyme monoamine (MAO)-B. Modafinil is expected to have no inhibitory actions on the function of MAO-B, since five minutes after MPTP administration the conversion to MPP^+ is complete (Sundstrom and Jonsson, 1986), whereas no substantial increase of modafinil in blood plasma is observed in that period (Philippens et al. 2006).

The next major step in the pathway of MPTP is the influx of MPP^+ into DAergic neurons via the DAT (Schulz 1988, Bezard et al. 1999). At this point modafinil could interfere with the influx and therefore interfere with disease induction as modafinil's actions are suggested to be related to direct or indirect modulation of the DAT. This is based on evidence including the lack of behavioral response in a DAT knockout mouse and modafinil's low affinity for the DAT (Mignot et al. 1994, Wisor et al. 2001, Madras et al. 2006). This indicates that modafinil could interfere with the influx of MPP^+ via the DAT and can therefore prevent the disease induction to some degree. However, there are many arguments indicating that modafinil is not blocking the

influx of MPP^+.

First, the neuroprotective capacities of modafinil are independent of brain structure and type of neuronal damage indicating that modafinil does not need to block DAT to induce neuroprotection (Fuxe et al. 1992, Ueki et al. 1993a, b, Lallement et al. 1997, Antonelli et al. 1998, Jenner et al. 2000). Furthermore, modafinil is neuroprotective 3 hours after MPTP administration, which is not the case for high affinity DAT inhibitors (Fuxe et al. 1992). Additionally, the blockade of DAT by modafinil was refuted in a comparable marmoset experiment with simultaneous administration of MPTP and modafinil (Jenner et al. 2000). In that experiment the protective actions of different doses of modafinil appeared in a dose-dependent manner after cessation of the MPTP intoxication (Jenner et al. 2000). This indicates that modafinil did not interfere with the disease induction. If so, a dose-dependent effect of modafinil would have been apparent during the 5-day MPTP intoxication period.

Finally, the minimal overlap in the pharmacokinetics of MPP^+ and modafinil reduces the possibility of modafinil's interference with MPP^+ influx. MPP^+ is stored in monoamine vesicles within 30-60 minutes after MPTP administration, while orally administered modafinil has a peak concentration in marmoset blood plasma at 4 hours after intake (Schulz 1988, Philippens et al. 2006).

In summary, the protective effects of lower doses as well as the protective effects in other DAergic and non-DAergic neuronal degeneration models indicate that modafinil is a potent neuroprotective compound against PD. However, DAT blockade might still play a limited role in the neuroprotective actions of high doses of modafinil in the MPTP model, since, despite modafinil's low affinity for DAT, high doses of modafinil may affect DAT.

Mechanism of neuroprotection The neuroprotective mechanisms of modafinil are unknown. It could be via modulation of neurotransmitters or it could be via relatively unexplored mechanisms connected with interference with cell death processes.

The first group of theories is based on the capacity of modafinil to modulate neurotransmitter levels in specific brain areas. These theories are mostly suggested in relation to models inducing nigrostriatal damage, e.g. MPTP and partial hemisection of the pathway (Fuxe et al. 1992, Ueki et al. 1993a, Jenner et al. 2000). The disturbed balance of basal ganglia in the PD brain, in particular the glutamatergic overactivity in the subthalamic nucleus, induces excitotoxicity. Modafinil could normalize the disturbed balance by affecting glutamate and γ-aminobutyric acid (GABA) release in specific areas of the basal ganglia (Ferraro et al. 1997, 1998) and therefore reduce the excitotoxicity. However, this theory focuses more on a symptomatic aspect, namely improvement of output of the basal ganglia rather than protection of the neurons of the substantia nigra pars compacta (SNpc). Moreover, to establish protection there is friction between cause and effect: the protection of the neurons is suggested to be generated by normalization of the disturbed basal ganglia system, whereas on the other hand, the cause of the disturbance of the basal ganglia is the degeneration of the DA neurons.

Another theory based on neurotransmitter modulation is that reduction of

GABA release by modafinil could reduce the activity of DAergic neurons in the SNpc, which could therefore save energy and increase their chance of survival (Fuxe et al. 1992). However, this is in contrast to other findings demonstrating that increased GABA levels are neuroprotective in stroke (Sternau et al. 1989).

The second group of theories addresses the interference of modafinil with cellular processes. These theories are based on the assumption that modafinil may have other mechanisms of action besides neurotransmitter modulation, such as improvement of energy metabolism, synthesis and release of neurotrophic factors, recovery of calcium homeostasis, improvement in metabolic activity, radical scavenging or stimulation of repair processes as axonal sprouting from the surviving cell bodies (Fuxe et al. 1992, Ueki et al. 1993a, b, Lallement et al. 1997, Antonelli et al. 1998, Jenner et al. 2000). Another possible mechanism of action is interference via glial cells, i.e. stimulation of the enzymatic breakdown of glutamate resulting in reduction of excitotoxicity or stimulation of the immune response (Touret et al. 1994). However, direct evidence of modafinil inducing these processes does not exist. Therefore, chapter 8 explored the neuroprotective effects of modafinil generated via gene expression and suggested a lack of substantial indirect neuroprotective effect of modafinil. The study revealed that the neuroprotective effects of modafinil do not last for 12 hours and that they are probably not mediated via modulation of trophic support or inflammatory responses by astrocytes. This strengthens the suggestions that modafinil directly interferes with the primary cell death processes in the neuron.

Δ⁹-THC

Behavioral effects

Chapter 4 describes the effects of varying concentrations of oral Δ⁹-THC on movement and emotion-related responses in the naïve marmoset monkey. The main goal was to identify basal behavioral effects of Δ⁹-THC preceding the studies towards the therapeutic and neuroprotective effects of Δ⁹-THC in the MPTP model. Furthermore, behavioral data of Δ⁹-THC in non-human primates are sparse and species differences in the cannabinoid system are suggested (Meschler et al. 2001). This is illustrated by the different effects on sedation in monkeys and rats after combined therapy of dopamine (DA) and cannabinoid ligands (Meschler et al. 2000a, b). Δ⁹-THC resulted in apathy, involuntary movements, bradykinesia, affected balance, reduced startle response and anxiety-related behavior and increased activity at lower doses (chapter 4). As discussed in chapter 4, the findings were generally comparable with findings in rodents and humans. Except the often reported cataplexy in rodents (Chaperon and Thiebot 1999).

Based on these behavioral observations a dose of 4 mg/kg Δ⁹-THC was chosen for the therapeutic and neuroprotective experiments (chapter 5 and 9). This dose evoked Δ⁹-THC-related behavioral responses, which were of acceptable severity to allow for repeated administration.

Therapeutic effects

Δ^9-THC administered to parkinsonian marmosets alleviated some parkinsonian symptoms as locomotor activity and the hand-eye coordination task performance were improved after a single dose of Δ^9-THC. The improvement resulted in behavior comparable to near pre-disease level. The same dose of Δ^9-THC in naïve animals slightly, but not significantly, increased the locomotor activity and no effect was found on the hand-eye coordination task. This may indicate that the anti-parkinsonian effects of Δ^9-THC depend on disease-related changes. One of these changes is upregulation of cannabinoid CB_1 receptors in the striatum (Lastres-Becker et al. 2001). Activation of these receptors with Δ^9-THC would reduce the disease-related striatal glutamatergic hyperactivity or stimulate the co-localized dopamine D_1 and D_2 receptors, both resulting in abolishment of the increased inhibitory output of the basal ganglia (Brotchie 2003).

The results described in chapter 5 are as far as we know the first that indicate anti-parkinsonian properties of a cannabinoid CB_1 receptor agonist. This is contradictory to the findings and suggestions made from rodent studies. As discussed in chapter 5, rodent studies suggested that cannabinoid CB_1 receptor antagonists would have more anti-parkinsonian properties as they inhibit the extensive GABA release of the globus pallidus interna (GPi) and substantia nigra pars reticulata (SNpr) (Di Marzo et al. 2000, Gonzalez et al. 2006). In contrast, the positive findings of the cannabinoid receptor antagonists were not observed in the parkinsonian non-human primate (Meschler et al. 2001), which emphasizes the differences in the cannabinoid system between rodents and non-human primates. The higher number of cannabinoid receptors in the primate striatum compared to rodents (Ong and Mackie 1999) together with upregulation of the striatal cannabinoid CB_1 receptors after MPTP induction (Lastres-Becker et al. 2001), may result in domination of the alleviating actions of the striatum on the parkinsonian symptoms over the inhibitory actions of the GPi and SNpr after cannabinoid CB_1 receptor stimulation. This hypothesis is only based on the behavioral outcome after a single pharmacological intervention. Further research with pharmacological interventions with different doses of Δ^9-THC or use of selective cannabinoid CB_1 receptor agonists in MPTP-treated non-human primates as well as studies towards the cannabinoid receptor distribution in different species are needed to confirm the hypothesis and to extend knowledge about the cannabinoid system in PD.

Neuroprotection

Cannabinoids, and more specific Δ^9-THC, have potential to protect neurons as positive results are reported in various models of oxidative stress and excitotoxicity as well as in more clinically oriented models for multiple sclerosis and brain trauma (reviewed by Grundy et al. 2001). Despite this potential neuroprotective effect, Δ^9-THC given simultaneously with MPTP in the marmoset monkey was not able to protect the neurons against the induced cell death processes (chapter 9). The results are in contrast to findings in which Δ^9-THC protected neurons against the intoxication with 6-hydroxydopamine in rodents (Lastres-Becker et al. 2005). In

the discussion of chapter 9, it was suggested that the failure of Δ^9-THC to control cell death processes induced by MPTP was a combination of a more severe disease induction and a rather low dose needed for anti-oxidative protection. Nevertheless, the negative results indicate that both doses used as well as types of PD animal model are important parameters in studies towards the neuroprotective effects of Δ^9-THC. Furthermore, it might indicate that Δ^9-THC is not neuroprotective in a PD model closer to man. However, additional research by variations in experimental setup in different PD models has to ascertain the potential of Δ^9-THC and cannabinoids in the neuroprotective treatment of PD patients.

During the neuroprotection study with Δ^9-THC, the behavior was also tested after daily Δ^9-THC administration. As the protection of the neurons by Δ^9-THC failed, the outcome could reveal therapeutic effects of Δ^9-THC. Despite this, no changes in behavior were found (data not included in this thesis), probably due to the experimental setup. In the therapeutic experiment (chapter 5) the animals were parkinsonian for some weeks before they received a single dose of Δ^9-THC. This is in contrast to the animals in the neuroprotection experiment (chapter 9) that received daily Δ^9-THC simultaneous with the disease induction for 13 days before the first behavioral test. Repeated Δ^9-THC administration is known to induce tolerance (Iversen 2003). Therefore, it seems that the reduced sensitivity of the cannabinoid receptors for the agonistic actions of Δ^9-THC underlie the lack of therapeutic effect in the neuroprotection study.

Δ^9-THC in the clinic

The diverse outcome of therapy and neuroprotection studies emphasize the importance of dosing of Δ^9-THC. Therefore, the lack of control in dosing Δ^9-THC, when smoking marijuana, may be the underlying reason leading to inconsistent results in PD patients (Muller-Vahl et al. 1999, Venderova et al. 2000). Dosing via capsules could make the intake more controllable and excludes the side-effects of smoking. Disadvantages of oral intake are delayed effects and varying uptake capacity due to differences in digestion rate and first pass effect. For comparison psychoactive effects of inhaled Δ^9-THC are present after 15-30 minutes and last 2-3 hours, whereas after oral ingestion, the psychoactive effects are present after 30-90 minutes and last about 4-12 hours (Grotenthermen 2003). Sublingual sprays, which are subject to research, could decrease the delay and evade the side-effects of smoking.

Therapeutic application of Δ^9-THC is also hindered by side-effects like euphoria and depression, due to the wide distribution of cannabinoid CB_1 receptors throughout the brain. This is also illustrated by the increased apathy and reduction of facial movements after Δ^9-THC administration to both naïve and parkinsonian marmosets (chapter 4 en 5). Furthermore, Δ^9-THC use is also associated with rapid development of tolerance and neurotoxicity, although the latter effects are inconsistent (Iversen 2003).

Therefore, the way to success of clinical application of cannabinoids is to take the administration route, the side-effects and the tolerance effects into account.

Neuroprotection in the clinic

The neuroprotection studies in this thesis proved the presence of a neuroprotective profile of modafinil and reported about the neuroprotective potential of cannabinoids. If those compounds would be clinically applied against PD, would it be feasible to use these compounds on a daily basis from the early diagnosis to the end of life?

An ideal neuroprotective compound will need to be potent, have a long-lasting effect and should be devoid of uncontrollable side-effects or risk of major toxicity (Drukarch and Van Muiswinkel 2001). Modafinil matches this profile as daily use is considered safe without tolerance effects based on daily use in narcolepsy patients (Bastuji and Jouvet 1988). Moreover, modafinil elicits no uncontrollable side-effects in humans (Robertson and Hellriegel 2003). Cannabinoids do not yet fit this profile due to psychoactive effects, tolerance and suggested neurotoxicity of cannabinoids. However, there are indications that the protective actions of cannabinoids are independent of the cannabinoid CB_1 receptors, which are underlying most of the side-effects of cannabinoids (Garcia-Arencibia et al. 2007). On the other hand, the side-effect profile of modafinil or cannabinoids would be less prominent in the clinic as much lower doses can be used. The reason for this is that the degeneration process in PD patients is much slower than the degeneration processed developed after subacute induction of neuronal death in the experimental models and therefore lower doses are needed to counteract the degeneration.

The prospect of clinical application of modafinil and cannabinoids in the near future is uncertain independent of their side-effect profile. The reason for this is that clinical neuroprotection is much more difficult to establish, despite the success of neuroprotective experimental studies in cellular lines and animal models.

Neuroprotection trials have substantial problems e.g. symptomatic effects of medication may be mistaken for neuroprotection, misdiagnosis of patients (parkinsonism rather than idiopathic PD), inclusion of patients in different phases of the disease, low sensitivity of outcome markers and the need for large sample sizes to demonstrate small changes (Clarke 2004). Therefore, optimization of the design of a clinical trial would also benefit the success of neuroprotective compounds in the clinic.

Furthermore, the discrepancy between experimental outcomes and clinical use may be a result of the shortcomings of PD experimental models to completely reproduce the complex clinical PD pathogenesis. The slow progression of the disease may result in a different balance between the many pathways involved in cell death and survival compared to the existing animal models. Research towards the development of more ideal PD models may therefore contribute to translating preclinical effects to a clinical level. However, research in the current animal models are of great value as they revealed cell death processes in PD and have indicated the potential neuroprotective compounds for clinical use.

In the application of neuroprotective treatments the moment of intervention seems crucial and could define the efficacy of a neuroprotective compound. In

neurodegenerative diseases this is more difficult than during acute neuronal damage. Cerebral ischemia can still be effectively treated when the neuroprotective compound id administered within hours after the insult. In contrast, in PD the first motoric symptoms appear when 50-60% of the DAergic neurons in the SNpc have disappeared over a time frame of 6 years. To be effective, the neuroprotective treatment in PD has to start as early as possible. Reliable biomarkers, which can identify patients early in the disease process or ideally, before onset of PD can aid in early diagnosis. Early biomarkers being studied are rapid eye movement sleep disorder, olfactory dysfunction, constipation and nigrostriatal DAergic deficits on positron emission tomography scans or other scans (Michell et al. 2004).

Another point of consideration in the application of neuroprotective treatment is the maintenance of daily medication use. This needs dedication of the (potential) patient as preventive treatment has no short-term noticeable effects. Development of slow-release medication would therefore be beneficial as it is more practical for the patient and result in continuous neuroprotective control over the degeneration processes.

Despite the above described hurdles in clinical application of neuroprotective treatment, neuroprotection is still the best perspective in the treatment of PD patients as long as the cause of PD remains undiscovered. Experimental techniques like glial derived neurotrophic factor gene therapy and stem cell therapy do also have good prospects but scientific, safety and ethical considerations limit their application and, more importantly, their neurorestorative properties can not stop or prevent the underlying degeneration.

Conclusion

This thesis explored the neuroprotective capacities of modafinil and Δ⁹-THC in the marmoset MPTP model. Modafinil showed to be a potent neuroprotective compound in the marmoset PD model. The compounds used in this thesis were chosen because of their potential to act via multiple mechanisms on the cell death processes. Based on exploring experiments on the neuroprotective mechanism of modafinil it can be concluded that the actions are within a short time span, probably directly acting on the cell death processes.

Furthermore, modafinil showed to also have positive actions on the PD symptoms indicating that clinical application for neuroprotection is not frustrated by pro-parkinsonian actions. Therefore, testing for clinical efficacy of its therapeutic and neuroprotective actions in PD would be a logical and relatively easy second step as modafinil is already a marketed drug. Furthermore, more knowledge about modafinil's mechanisms of action would benefit its application and further development of modafinil-like compounds.

In the present study, Δ⁹-THC did not show neuroprotective capacities in the marmoset MPTP model. Variation in the experimental setup and application of other cannabinoids has to confirm or refute this observation and the possibility of

neuroprotective treatment of PD by cannabinoids. On the other hand, Δ^9-THC seems to be useful in symptomatic therapy as it alleviated the motor disturbances with the occurrence of some cannabinoid-related side-effects. This confirms the studies about the beneficial effects of marijuana in PD patients. Increasingly, more knowledge of the tolerance and toxic effects of cannabinoids on the brain is needed for safe application in the clinic.

PD is in time seriously deteriorating quality of life of its sufferers due to the progressive nature of this disease. With the findings of modafinil and Δ^9-THC presented in this thesis there a contribution is made towards a treatment to stop or slow down this progression.

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Nederlandse samenvatting

Ziekte van Parkinson

De ziekte van Parkinson is een progressieve neurodegeneratieve ziekte, die vooral gekenmerkt wordt door verstoring van de motoriek. De drie belangrijkste symptomen zijn tremoren in rust, stijfheid van de ledematen en de afwezigheid van onbewuste bewegingen. Andere vaak voorkomende kenmerken zijn traagheid en verkleining van bewegingen en problemen met het starten van de bewegingen. Niet motorisch gerelateerde verschijnselen zoals dementie en depressie komen ook vaker voor bij patiënten met de ziekte van Parkinson. De gemiddelde leeftijd waarop de ziekte wordt gediagnosticeerd is 55 en het aantal gevallen neemt toe naar mate de leeftijd stijgt. Het geschatte aantal patiënten in Nederland is 60.000.

Pathologisch wordt de ziekte vooral gekenmerkt door het afsterven van neuronen in een klein gebied in de hersenstam, de substantia nigra. Deze neuronen geven dopamine af aan de basale ganglia. De basale ganglia zijn onderling verbonden hersengebieden die verantwoordelijk zijn voor de planning en uitvoering van bewegingen. In een gezonde situatie worden de bewegingen geïnhibeerd door de basale ganglia. Bij de juiste input (dopamine) wordt dit tijdelijk opgeheven waardoor er een beweging uitgevoerd kan worden. Door de verminderde dopaminerge input bij de ziekte van Parkinson, wordt de inhibitie van de basale ganglia versterkt. Dit heeft grote invloed op de uitvoering van bewegingen en resulteert in de voor de ziekte van Parkinson kenmerkende verslechterde motoriek.

Naast het afsterven van de neuronen zijn er, bij patiënten met de ziekte, eiwitophopingen in de neuronen gevonden en ook andere neurotransmittersystemen zijn, hetzij in mindere mate, aangedaan.

Bij de meeste patiënten met de ziekte van Parkinson is de oorzaak onbekend. Vijf procent van de patiënten heeft een genetische vorm van de ziekte van Parkinson. Tevens is bekend dat sommige toxische stoffen waaronder bepaalde bestrijdingsmiddelen Parkinson-achtige verschijnselen kunnen veroorzaken. Men denkt daarom dat een combinatie van genetische gevoeligheid en omgevingsfactoren resulteren in het ontstaan van de ziekte.

Er is een breed spectrum aan medicijnen ontwikkeld die de motorische symptomen kunnen verminderen. Een veelgebruikt geneesmiddel is levodopa. Deze stof wordt in de hersenen omgezet in dopamine, zodat de dopamine tekorten aangevuld kunnen worden. Dopamine agonisten worden ook veel gebruikt. Dit zijn stoffen die hetzelfde effect kunnen genereren als dopamine door aan de dopamine receptoren binden.

Het grootste nadeel van de huidige medicatie, naast het feit dat deze medicatie de progressie van de ziekte niet stopt, is dat langdurig gebruik resulteert in ontwikkeling van verstorende bijwerkingen.

Naast het verbeteren van de huidige therapeutische medicatie wordt er onderzoek

gedaan naar methoden die de degeneratie tegen kunnen gaan. Ontwikkeling en onderzoek naar neuroprotectieve stoffen is veelbelovend. Bij neuroprotectie is het doel de cel te beschermen door middel van ingrijpen in de schadelijke processen die leiden tot celdood. Er is nog geen neuroprotectieve stof gevonden die effectief is bij Parkinson patiënten. Verder onderzoek is dus nodig. Een ideaal neuroprotectief werkende stof zou de complexe celdood processen moeten kunnen aanpakken via meerdere werkingsmechanismen. Uit de literatuur blijkt dat de stoffen modafinil en Δ^9-tetrahydrocannabinol (Δ^9-THC) dit potentieel hebben. Een bijkomend voordeel is dat deze stoffen in de kliniek bekend zijn dus bij bewezen effectiviteit gemakkelijk toegepast kunnen worden. Onderzoek naar de neuroprotectieve eigenschappen, maar ook de therapeutische effecten van deze stoffen bij de ziekte van Parkinson is dan ook het hoofdonderwerp van dit proefschrift.

Modafinil

Modafinil is een alertheidbevorderende stof. Het wordt onder andere voorgeschreven bij narcolepsie patiënten. Hoewel het precieze werkingsmechanisme van modafinil niet bekend is, wordt het wel geassocieerd met toename in activiteit van neurotransmitters als noradrenaline, glutamaat en hypocretine. Verder zijn afname in activiteit van de neurotransmitter GABA, en effecten op het dopaminerge systeem bekend. Op basis van dit brede spectrum aan effecten zou modafinil mogelijk ook de Parkinson effecten kunnen reduceren. Naast de therapeutische effecten is modafinil ook neuroprotectief in verschillende *in vivo* en *in vitro* neuronale schade modellen, waaronder specifieke schade in de verbinding tussen de substantia nigra en het striatum die leidt tot Parkinson symptomen.

Δ^9-THC

Δ^9-THC is een van de werkzame stoffen uit de bladeren en bloemknoppen van marihuana. Δ^9-THC is vooral bekend van het opwekken van euforische gevoelens en veranderingen in cognitie via het cannabinoid systeem. Het cannabinoid systeem bestaat uit twee receptoren, de cannabinoid CB_1 en CB_2 receptor waarvan in de hersenen de eerstgenoemde het meeste voorkomt. Stimulatie van die receptoren met onder andere Δ^9-THC leidt tot modulerende effecten op de afgifte van neurotransmitters als glutamaat, GABA en dopamine. Van cannabinoiden in het algemeen, maar Δ^9-THC specifiek is aangetoond dat ze therapeutische effecten kunnen hebben. Ze reduceren bijvoorbeeld spasticiteit bij multipele sclerose en zijn als eetlustopwekker toepasbaar bij chemotherapie of bij HIV patiënten. Cannabinoiden hebben ook neuroprotectieve eigenschappen zoals bewezen in diverse modellen van neuronale schade. Een anti-oxidatieve werking of effecten via receptorstimulatie zijn onder andere gesuggereerd als neuroprotectief mechanisme. De positieve resultaten en het brede werkingsmechanisme tonen het neuroprotectieve potentieel van Δ^9-THC tegen de ziekte van Parkinson.

Diermodel

Het onderzoek in dit proefschrift is experimenteel van aard en kan niet getest worden in patiënten met de ziekte van Parkinson. Als alternatief is er gebruik gemaakt van twee veelgebruikte diermodellen voor de ziekte van Parkinson, het niet-humane primaat (marmoset) en het muis MPTP model. Toediening van het neurotoxine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) resulteert in degeneratie van de dopaminerge neuronen in de substantia nigra. Deze methode is veel gebruikt in experimenteel onderzoek naar de ziekte van Parkinson. Een beperking is wel dat de ziekte acuut wordt geïnduceerd en dat er geen sprake is van progressieve celdood.
Het marmoset model wordt vooral gebruikt wanneer er gekeken wordt naar gedragsmatige effecten van een behandeling voor parkinsonisme. De marmosetten laten namelijk, na toediening van MPTP, Parkinson-achtige symptomen zien, die bijna identiek zijn aan symptomen van de patiënten. Hierdoor kan in een marmoset, een behandelingseffect specifiek gemeten worden. Muizen laten echter kortstondige gedragsmatige effecten zien na toediening van het neurotoxische MPTP, hoewel er wel celdood plaatsvindt. Dit model kan dus goed gebruikt worden wanneer er vragen over het werkingsmechanisme van celdood zijn.

Inhoud proefschrift

Dit proefschrift bestaat uit drie delen. Het eerste deel bestaat uit onderzoek naar effecten van modafinil en Δ⁹-THC bij gezonde marmosetten. Het tweede deel beschrijft het onderzoek naar de symptomatische effecten van modafinil en Δ⁹-THC bij marmosetten met Parkinson symptomen Het derde deel beschrijft onderzoek naar de neuroprotectieve eigenschappen van modafinil en Δ⁹-THC en onderzoek naar het neuroprotectieve werkingsmechanisme van modafinil.

Effecten van modafinil en Δ⁹-THC op gedrag

In *hoofdstuk 2* is er onderzocht wat de gedragseffecten van modafinil zijn in een gezonde marmoset. Twee uur na orale toediening van modafinil in doseringen 50, 100, 150 en 225 mg/kg zijn de dieren getest in diverse gedragstesten. Een toename van locomotor activiteit was gevonden in doseringen hoger dan 100 mg/kg in de Bungalow test en op de activiteitsparameters van de human threat test. Modafinil toediening leidde ook tot reductie van angst gerelateerde gedragingen gemeten met de human threat test. Modafinil veroorzaakte geen veranderingen in de gedragingen gescoord met behulp van observatielijsten en had ook geen effect op de startle reflex en de hand-oog coördinatie. Er is geconcludeerd dat naast psychostimulatie, modafinil geen andere bijwerkingen veroorzaakt binnen de gemeten parameters. De angstverlagende effecten van modafinil kunnen mogelijk een nieuwe therapeutische toepassing van modafinil vormen.

Het onderzoek naar de gedragseffecten van modafinil wordt in *hoofdstuk 3*

uitgebreid met een studie naar de mogelijkheid of modafinil de negatieve gevolgen van slaapdeprivatie zoals vermoeidheid, verminderde alertheid en verslechterde taakuitvoering kan tegengaan. De effecten zijn vergeleken met de effecten van de alertheidbevorderende stof, cafeïne. De dieren zijn na een normale actieve dag, gedurende een nacht wakker gehouden en met de alertheidverhogende stoffen of een vehicle behandeld. Drie maal gedurende de slaapdeprivatie nacht zijn de dieren getest in de Bungalow test om de mate van vermoeidheid te testen en in de hand-oog coördinatie taak om de effecten op taakuitvoering te meten.

De resultaten toonden dat beide stoffen in staat waren om de verslechterde taakuitvoering als gevolg van de slaapdeprivatie in zekere mate tegen te gaan. Modafinil hield de activiteit op een niveau die vergelijkbaar was zonder slaapdeprivatie, maar kon de taakuitvoering niet de gehele nacht op niveau houden. Cafeïne was echter in staat om de taakuitvoering op een niveau te houden van voor slaap deprivatie, maar de activiteit verslechterde wel gedurende de slaapdeprivatie nacht. Chronisch gebruik van de stoffen verslechterde de effecten niet. Er is dan ook geconcludeerd dat modafinil en cafeïne beide in staat zijn om in zekere mate de vermoeidheid en verslechterde taakuitvoering als gevolg van slaapdeprivatie tegen te gaan.

In *hoofdstuk 4* zijn de gedragseffecten van orale toediening van Δ⁹-THC in de marmoset onderzocht. De dieren zijn 30 en 90 minuten na toediening van Δ⁹-THC in de doseringen van 2, 4 en 8 mg/kg getest op activiteit gerelateerd gedrag, taakuitvoering en emotioneel gerelateerd gedrag. 4 en 8 mg/kg Δ⁹-THC veroorzaakten apathie, onvrijwillige bewegingen van de romp en onderste ledematen, traagheid in beweging en verslechtering van de balans bij het springen. Δ⁹-THC verlaagde tevens de emotionele respons zoals aangetoond met een verlaging van de startle reflex en vermindering van angst gerelateerd gedrag tijdens de human threat test. Δ⁹-THC reduceerde na de doseringen 2 en 4 mg/kg de activiteit, maar dit is afhankelijk van de toegepaste test, de Bungalow test of parameters van de human threat test. De conclusie is dan ook dat orale Δ⁹-THC in de marmoset resulteert in dosis afhankelijke effecten op de activiteit, startle respons en angst gerelateerd gedrag.

Parkinson symptoom bestrijdende effecten van modafinil en Δ⁹-THC

In *hoofdstuk 5* is onderzocht of modafinil en Δ⁹-THC in staat zijn de Parkinson symptomen te reduceren in een MPTP behandelde marmoset. Reden is dat beide stoffen mogelijk een bijdrage kunnen leveren in het huidige medicatie spectrum omdat beide stoffen indirect de dopamine release kunnen beïnvloeden. In het onderzoek zijn modafinil (100 mg/kg), Δ⁹-THC (4 mg/kg) of een vehicle toegediend bij dieren met Parkinson symptomen. Vervolgens zijn de dieren geobserveerd met behulp van scoringsschalen voor Parkinson symptomen en het effect op beweging is gekwantificeerd met een locomotor activiteit test en de hand-oog coördinatie test. Δ⁹-THC toediening resulteerde in verbeterde activiteit en hand-oog coördinatie, maar ook Δ⁹-THC gerelateerde bijeffecten zijn geobserveerd. Modafinil resulteerde in verbeterde activiteit en afname van Parkinson symptomen, maar niet in verbetering van de hand-oog coördinatie. Er kan geconcludeerd worden dat beide stoffen

therapeutisch eigenschappen hebben en daarom een toevoeging kunnen zijn aan het huidige medicatiespectrum.

Neuroprotectieve effecten van modafinil en Δ⁹-THC

In de *hoofdstukken 6 en 7* staan het onderzoek naar de neuroprotectieve effecten van modafinil in het MPTP marmoset model voor de ziekte van Parkinson beschreven. Bij twee groepen marmosetten werden de Parkinson verschijnselen geïnduceerd met MPTP. Tegelijkertijd met de start van de ziekte inductie kreeg de ene groep een dagelijkse behandeling met modafinil gedurende 27 dagen. De andere groep kreeg in plaats van modafinil, vehicle toegediend. De ernst van de symptomen werd dagelijks gescoord en gedragstesten die de activiteit, hand-oog coördinatie, kleine snelle bewegingen, angst gerelateerde gedragingen en startle response meten werden een aantal maal gedurende de onderzoeksperiode toepast. Voor ziekte inductie en twee maal na ziekte inductie werden veranderingen in de hersenen gemeten met behulp van MRI scans. Aan het einde van het experiment werden de post-mortem hersenen gebruikt voor quantificatie van de overlevende neuronen door middel van immunohistochemie en analyse van de neurotransmitter niveaus in het striatum.

De toepassing van een dergelijk breed scala aan meetmethodes levert een veelheid aan informatie op binnen een dier. Metingen binnen een dier versterken ook de waarde van de uitkomst.

De modafinil behandeling resulteerde in een verhoogde overleving van de neuronen ten opzichte van de vehicle behandelde dieren. Het functioneren van de neuronen door middel van meting van de neurotransmitter niveaus toonden ook een betere neuronale functie van de modafinil behandelde groep. Deze resultaten zijn ook bevestigd met de MRI scans. Op gedragsniveau resulteerde de modafinil behandeling in verminderde aanwezigheid van de Parkinson symptomen.
Uit dit experiment kan geconcludeerd worden dat modafinil in staat is om de cellen in het MPTP model te beschermen en dus potentie heeft als neuroprotectieve therapie bij de ziekte van Parkinson.

Er is weinig bekend over het neuroprotectieve werkingsmechanisme van modafinil. Er wordt gesuggereerd dat modafinil direct ingrijpt op de celdood processen, maar modafinil kan ook indirect effect hebben door de genexpressie te beïnvloeden, welke effect heeft op de productie van eiwitten die een rol spelen in de celdood processen. In *hoofdstuk 8* is de mogelijkheid van neuroprotectie via indirecte effecten van modafinil onderzocht op twee manieren. De eerste methode is een onderzoek in het MPTP muis model waarbij 12 uur voor de inductie van de Parkinson verschijnselen modafinil is toegediend. Op dat tijdstip is modafinil uit het lichaam verdwenen en alleen secundaire effecten van modafinil zouden de schade door MPTP tegen kunnen gaan. De praktijk wees uit dat deze theorie niet klopte. De tweede methode is het meten van de effecten van modafinil op de genexpressie van humane astrocyten. Met behulp van microarrays zijn de mogelijke modafinil geïnduceerde veranderingen op cytokines en chemokines geïnventariseerd. De resultaten toonden dat modafinil geen duidelijke direct effecten heeft op de genexpressie. Uit deze twee experimenten kan geconcludeerd worden dat de neuroprotectieve effecten van

modafinil hoogstwaarschijnlijk niet via indirecte effecten wordt bewerkstelligd.

In *hoofdstuk 9* staat het onderzoek beschreven naar de neuroprotectieve werking van Δ⁹-THC in het marmoset MPTP model. De proefopzet was gelijk aan die van modafinil zoals is beschreven in hoofdstuk 6 en 7. De orale Δ⁹-THC (4 mg/kg) toediening was niet in staat de neuronen te beschermen tegen de schade door MPTP. Dit in tegenstelling tot onderzoek in een 6-OHDA rat model waarbij was aangetoond dat Δ⁹-THC dopaminerge neuronen in de substantia nigra kan beschermen tegen de neurotoxine 6-OHDA. Redenen voor het wegblijven van bescherming kunnen een combinatie zijn van een ernstiger schade model en een te lage dosering voor de anti-oxidatieve bescherming. Tevens kan het zijn dat dit onderzoek in een model dichterbij de mens, neuronale bescherming door Δ⁹-THC uitsluit.

Algemene discussie

In *hoofdstuk 10*, de algemene discussie worden er verbanden gelegd tussen de bevindingen van modafinil en Δ⁹-THC in naïeve gezonde dieren en als anti-parkinson of neuroprotectief middel in dieren met Parkinson symptomen. Hieruit blijkt dat 100 mg/kg modafinil duidelijk effect heeft op de activiteit zowel bij slaap gedepriveerde dieren als bij MPTP behandelde dieren. De neuroprotectieve werking vindt plaats op cellulair niveau. In de discussie wordt uitgesloten dat modafinil ingrijpt op het metabolisme van MPTP in plaats van de veroorzaakte schade. De discussie gaat tevens in op de mogelijke neuroprotectieve werkingsmechanismen van modafinil. Dit zal hoogstwaarschijnlijk via directe effecten op de celdood processen gaan, zoals wordt ondersteund door de data uit hoofdstuk 8.

De gedragseffecten in marmosetten van Δ⁹-THC waren vergelijkbaar met de menselijke verschijnselen, maar op sommige punten afwijkend van de knaagdieren. Sommige effecten op knaagdieren zoals apathie waren ook waarneembaar bij de Δ⁹-THC behandelde marmosetten met Parkinson verschijnselen. Deze behandeling leidde ook tot symptoom verlagende effecten zoals waargenomen in de locomotor activiteit test en ook met de hand-oog coördinatie taak. Dit is een opvallend resultaat omdat er in de literatuur over knaagdieren juist positieve resultaten van cannabinoid antagonisten wordt verwacht. Deze waarneming bevestigt daarom de verschillen in het cannabinoid systeem tussen knaagdieren en niet-humane primaten en bevestigd tevens de potentie van Δ⁹-THC om Parkinson symptomen te onderdrukken. De potentiële neuroprotectieve werking van Δ⁹-THC is niet bevestigd in de marmoset MPTP model. Verder onderzoek moet de reden hierachter achterhalen. Het gebruik van Δ⁹-THC in de kliniek is niet geheel onomstreden met name door de bijwerkingen die de psychoactieve stof veroorzaakt en de discussie rondom mogelijke neurotoxische effecten.

In de discussie wordt ook ingegaan op de toepassing van neuroprotectieve medicatie bij patiënten met de ziekte van Parkinson. Ondanks de positieve vindingen in het experimentele onderzoek is er nog geen effectieve neuroprotectieve stof bij de mens gevonden. Oorzaken zijn gebrek aan goede klinische methoden om effectiviteit te bewijzen en de discrepantie tussen experimentele modellen en de ziekte in de mens.

Conclusie

Conclusie uit het onderzoek gepresenteerd in dit proefschrift is dat modafinil potentie heeft als neuroprotectieve en therapeutische stof bij de ziekte van Parkinson. Verder onderzoek is nodig naar achterliggende neuroprotectieve werkingsmechanisme. Dit kan een bijdrage leveren aan het onderzoek naar mechanismen om de celdoodprocessen te stoppen. Tevens heeft het geregistreerde geneesmiddel modafinil voldoende potentie om in een klinische studie met Parkinson patiënten te worden getest. Over Δ⁹-THC kan geconcludeerd worden dat het potentie heeft als therapeutische stof bij behandeling van de Parkinson symptomen. Het gebrek aan neuroprotectie door Δ⁹-THC in dit onderzoek toont dat verder onderzoek nodig is, vooral naar de mogelijke verschillen tussen de geteste Parkinson diermodellen.

Met de resultaten van modafinil en Δ⁹-THC gepresenteerd in dit proefschrift is er bijgedragen aan de ontwikkeling van behandelingen ingrijpend op de progressie van de ziekte van Parkinson, een ziekte die in loop van de tijd de kwaliteit van leven van zijn slachtoffers ernstig verstoord.

Author & co-authors

About the author

Sanneke van Vliet was born on May 15, 1979 in Pijnacker. In 1997 she received her VWO diploma at the Sint Stanislas college in Delft and proceeded to study Biology at the Leiden University. During her first research internship she participated in a project about decellularization of heart valves for longer survival after transplantation. The research was conducted at the department of Anatomy and Embryology of the LUMC in Leiden under supervision of dr. Marco de Ruiter. Her second research internship was about the effects of tryptophan depletion on the mood and cognition of depressive patients and patients in remission. The research was under supervision of Dr. Linda Booij and Prof. Dr. Willem van der Does of Clinical Psychology of the Leiden university. During that period she developed her interest in the neurosciences. In the summer of 2002, she graduated as medical biologist. In anticipation of the start of her PhD-research project at TNO, she taught biology and chemistry at a high school for children with minor speaking and hearing problems.

Her interest in the neurosciences came further to expression during her PhD research about neuroprotection in Parkinson's disease. The research was conducted at TNO Defence, Security and Safety and was supervised by Dr. Ingrid Philippens and promoter Prof. Dr. Berend Olivier of Utrecht University. The results of this research project are described in this thesis.

Over de auteur

Sanneke van Vliet is geboren op 15 mei 1979 in Pijnacker. In 1997 ontving ze haar VWO diploma op het Sint Stanislas college in Delft en ging vervolgens Biologie studeren aan de Universiteit Leiden. Gedurende haar eerste onderzoeksstage heeft ze deelgenomen aan een project over het celvrij maken van hartkleppen ter bevordering van overleving na transplantatie. Het onderzoek is uitgevoerd bij de afdeling Anatomie en Embryologie van het LUMC onder begeleiding van Dr. Marco de Ruiter. Haar tweede onderzoeksstage ging over de effecten van tryptofaan depletie op stemming en cognitie van patiënten met een depressie of in remissie. Het onderzoek was onder begeleiding van Dr. Linda Booij en Prof. Dr. Willem van der Does van klinische psychologie, Universiteit Leiden. Gedurende deze periode ontwikkelde ze haar interesse in de neurowetenschappen. In de zomer van 2002 studeerde ze af als medisch bioloog. In afwachting van de start van haar aio-onderzoek op TNO heeft ze biologie- en scheikundelessen gegeven op een middelbare school voor kinderen met lichte spraak- en gehoorsproblemen.

Haar interesse in de neurowetenschappen kwam verder tot uiting gedurende haar aio-onderzoek naar neuroprotectie bij de ziekte van Parkinson. Het onderzoek is uitgevoerd bij TNO Defensie en Veiligheid onder begeleiding van Dr. Ingrid Philippens en promotor Prof. Dr. Berend Olivier van de Universiteit Utrecht. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

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Van Vliet SA, Vanwersch RA, Jongsma MJ, Olivier B, Philippens IH (2006)
Neuroprotective effects of modafinil in a marmoset Parkinson model: Behavioral and neurochemical aspects. Behavioral pharmacology 17(5-6):453-62.

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Van Vliet SA, Blezer EL, Jongsma MJ, Vanwersch RA, Philippens IH (2007)
Neuroprotective effects of modafinil in a non-human primate Parkinson model.
Poster at the Joint Annual Meeting ISMRM-ESMRMB, Berlin, Germany (presenter EB)

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Anti-Parkinson effects of modafinil and Δ9-THC in the marmoset MPTP model.
Poster at the Annual meeting of the society for Neuroscience, Atlanta, GA, USA

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Co-authors and their affiliations *(in order of appearance)*

Drs. Sanneke A.M. van Vliet
Department of Diagnosis and Therapy, TNO Defence, Security and Safety, Rijswijk
Department psychopharmacology, Faculty of Science, Utrecht University
Utrecht Institute of Pharmaceutical Sciences, Utrecht University
Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht

Ing. Marjan J. Jongsma
Department of Diagnosis and Therapy, TNO Defence, Security and Safety, Rijswijk

Raymond A. P. Vanwersch
Department of Diagnosis and Therapy, TNO Defence, Security and Safety, Rijswijk

Prof. Dr. Berend Olivier
Department psychopharmacology, Faculty of Science, Utrecht University
Utrecht Institute of Pharmaceutical Sciences, Utrecht University
Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht
Department of Psychiatry, Yale University School of Medicine, New Haven, USA
PsychoGenics inc., Tarrytown, NY, USA

Dr. Ingrid H.C.H.M. Philippens
Department of Diagnosis and Therapy, TNO Defence, Security and Safety, Rijswijk

Dr. Jan van der Gugten
Department psychopharmacology, Faculty of Science, Utrecht University
Utrecht Institute of Pharmaceutical Sciences, Utrecht University
Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht

Dr. Erwin L.A. Blezer
Image Sciences Institute, University Medical Center Utrecht

Dr. J.M. (Hans) van Noort
Carla Persoon-Deen
Department of Biosciences, TNO Quality of life, Leiden

Dankwoord

Berend, bedankt dat je promotor wilde zijn op een voor jou niet zo'n gangbaar onderwerp. Ik wil je bedanken voor je opbouwende kritiek, prettige gesprekken en snelle reactie op de manuscripten.

Ingrid, ik wil jou bedanken voor de tijd en energie die je hebt gestopt in het opleiden van je eerste aio. De afgelopen vier jaar waren zeer afwisselend en leerzaam, niet in de laatste plaats door jouw creativiteit, inventiviteit en doorzettingsvermogen.

Marjan, muchos gracias voor de helpende handen, het luisterend oor en de vrolijke gezelligheid die je om je heen creëert. Veel succes met je nieuwe leven onder de Spaanse zon!

Raymond, zonder de gouden combinatie van je inventiviteit en pragmatiek zouden de gedragstesten niet bestaan hebben en bij tegenslag zo weer aan de praat waren. Jouw kijk op het leven en de bijpassende humor waren amusant.

Nelleke, fijn dat ik mijn werk kon delen met een opvolger. Geniet van het onderzoek doen en andere rariteiten die het aio-zijn met zich meebrengen. Zet vooral ook je socio-activiteiten door!

Gitte, ik wil je bedanken voor het napluizen van het manuscript en de andere paranimfklusjes in plaats van de dagelijkse trein sudoku. Gedeelde smart is halve smart en na regen komt zonneschijn om de cliché's de ruimte te geven!

Roland, je hebt je rol als de nieuwe Marjan goed op je genomen. Alleen hondenbrokken zijn niet echt mijn favo snoepje!

Martin, Bianca en Ingeborg, ik wil jullie bedanken voor de goede verzorging van de dieren.

Daan en Ruud, bedankt voor het kritisch doornemen van mijn manuscripten. De frisse blik uit een ander veld zorgden voor motivatie en verbetering.

Jan, je zei bij de sollicitatie dat ik niet alleen kennis van de wetenschap zou opdoen, maar ook vooral over mezelf. Beide zijn (gelukkig) nog niet afgerond! Bedankt voor deze motiverende woorden.

Nelleke, Dorien, Marloes, Jeroen en Margo. Het is al een aardig rijtje TNO-aio's aan het worden. De aio-meetings van het afgelopen jaar waren lekker en leerzaam. Fijn dat we lief en leed konden delen en veel succes met jullie onderzoek en promotie.

Overige TNO-collega's Desiree, Jitse, Floris, Helma, Herma vd W, Herman, Linda, Marcel A., Marcel vd S., Marijke, Martine, Netty, Roos M., Roos W., Ton, Wendy, Willem, mensen aan de diverse lunchtafels, collega's van Sander, ondersteunende diensten: bedankt voor de fijne tijd bij TNO!

Erwin, bedankt voor je hulp, ook bij de verwerking van de data. Herinneringen die bij me op komen zijn de lange dagen in de scannerruimte, nee geen metaal!, de elegante groene pakken en de opluchting als het experiment goed afgerond was. Voor het laatste wil ik ook de staf van het GDL bedanken voor hun professionele aanwezigheid bij het scannen van de dieren.

Kees van der Wulp, ik wil jou bedanken voor de plezierige samenwerking in de ontwikkeling van het de MRI-analyse software.

De Leidse TNO-collega's, Hans, Sipke en Carla, onze samenwerking binnen het Tender project was prettig en het heeft uiteindelijk geresulteerd in een gezamenlijk hoofdstuk. Bep, bedankt voor de tips bij het coupes snijden en Erik, bedankt voor de vele AAS gecoate glaasjes en je overige histologische hulp.

Beste mensen van Psychopharmacology, ik ben altijd met veel plezier langsgekomen om te werken of naar de lunchmeetings te gaan, maar ook vanwege de levendige discussies tijdens de pauzes. Ik heb me er altijd welkom gevoeld.

Jan van der G. en Wim bedankt voor de kritische blik en de HPLC analyse.

Monika, bedankt voor de hulp bij het opzetten van de tyrosine hydroxylase kleuring.

Jan V, bedankt dat ik van je neuroanatomische kennis gebruikt mocht maken en voor de goede gesprekken.

Ronald en Lucianne, bedankt voor de interesse in mijn werk en de bijbehorende kritsche vragen. Ronald, nog bedankt voor de aanzet tot het muizenexperiment.

Marga, bedankt voor de afhandeling van mijn UU-papierwinkel.

Utrechtse aio's en ex-aio's: Trynke (logeren in een chique hotel en de lekkere Neuroscience-cocktails), Meg vd B (bedankt voor de spoedcursus muizenbrein), Filip, Remco, Johnny ('How are you?'), Meg B (thanks for the 'gezellige' tea conversations and the tasty 'I-cannot remember-the-official-name' cannelloni), Marieke (succes met de laatste loodjes!) en Liesbeth, bedankt voor de gezelligheid bij cursussen en congressen.

Er is een groepje mensen die me langer dan de vier aio-jaren met raad en daad bijstaan. Bedanken voor specifieke dingen is daarom lastig en het is meer een gevoel ('ik kan het niet uitleggen') van verbondenheid die me steunen en motiveren. Daarom kort, maar daarom niet minder gemeend:

Pap en mam, bedankt voor de interesse, steun en de gegeven keuzevrijheid.

Geertjan en Jostein, jullie allerliefste zusje zegt dank voor de lol en de kritische noot. GJ bedankt voor het paranimf zijn.

Lieve Sander, bedankt dat je er voor me bent. Ik ben blij dat we al lange tijd samen zijn en dat het nog lang mag duren! (tidadididodidadidido!)

Sanneke