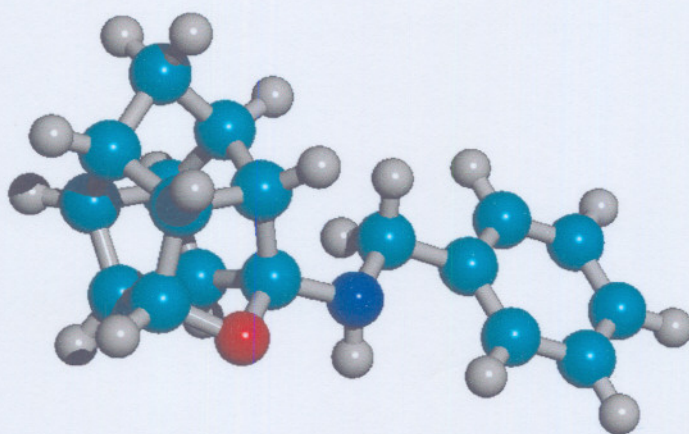


Biological Activity Profiles of Selected Polycyclic Cage Amines

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"Drug discovery is not a random event. The entire process can be planned and executed to optimize the chances for success."

- Franz Hefti (Koppal, T. 2003. Neuroscience research promises better drugs. Drug Discovery and development. 6, 30-34.)

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Abstract

Parkinson's disease (PD) is a severe and debilitating age-related neurodegenerative disease, with symptoms of resting tremor, akinesia and rigidity. The symptoms of PD are due to the selective loss of dopaminergic neurons in the substantia nigra. Three drug targets were identified for this study aimed at developing new drugs for the symptomatic treatment and/or prevention of PD. These included the dopamine transporter (DAT), monoamine oxidase (MAO) B enzyme and the *N*-methyl-D-aspartate (NMDA) receptor/ion channel complex. In addition, the parent compound, NGP1-01 (**1**), was evaluated in two *in vivo* models of neurodegenerative disease, which included the MPTP Parkinsonian mouse model (as a model for chronic neurodegenerative processes) and a stroke model - middle artery occlusion (MCAO) induced focal cerebral ischemia model - in mice (as a model for acute neurodegenerative processes).

Memantine is a polycyclic amine that is used clinically to treat PD and Alzheimer's disease. It is a low-affinity uncompetitive NMDA receptor antagonist and is well tolerated clinically. Antagonism of the NMDA receptor prevents Ca^{2+} overload in neuronal cells, thereby preventing neuronal cell death. The importance of Ca^{2+} in neuronal cell death led us to evaluate another polycyclic amine NGP1-01 (**1**) and its derivatives for possible neuroprotective activity. These compounds were previously characterized as L-type calcium channel antagonists.

NGP1-01 (**1**) and its derivatives were evaluated for their effects on [^3H]dopamine ([^3H]DA) release and uptake inhibition in murine striatal synaptosomes, as well as for inhibition of baboon liver monoamine oxidase (MAO) B. NGP1-01 (**1**) blocked the uptake of [^3H]DA with an IC_{50} of 57 μM , while another compound, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**9**), blocked uptake at an IC_{50} value

of 23 μM . These values were comparable to that of amantadine (IC_{50} ; 82 μM), another polycyclic amine that is currently used in parkinsonian therapy. MAO-B inhibition for this group was weak, with less than 50% inhibition at 300 μM for any of the compounds in the series.

NGP1-01 (1) and its derivatives were then further evaluated in a functional biochemical assay to assess NMDA antagonism. The IC_{50} values for the reference compounds MK-801 and memantine were 1.23 μM and 3.05 μM , respectively, for blocking NMDA-mediated $^{45}\text{Ca}^{2+}$ influx into whole brain murine synaptoneurosomes. NGP1-01 (1) proved to be the most potent experimental compound with an IC_{50} of 2.98 μM , while 8-amino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (8) had a similar IC_{50} of 4.06 μM . NGP1-01 (1) was further shown to be a noncompetitive NMDA antagonist.

Comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA) techniques were used to provide models to predict the PCP binding affinity of NGP1-01 and other polycyclic amines on the NMDA receptor. The resulted r^2 of the CoMFA and CoMSIA models were 0.976 and 0.883 respectively. Radioligand binding studies with [^3H]MK-801 or [^3H]TCP however, showed little or no displacement of the ligands by pentacycloundecylamines, indicating that they bind to a novel site on the NMDA receptor.

Since NGP1-01 (1) was shown to have NMDA receptor antagonist activity, it was evaluated in a battery of behavioural tests in the MPTP-mouse model using retired breeder C57BL/6 male mice, to determine whether this compound might alleviate behaviour deficits in this model. The behavioural tests included fore-paw stride length, pole test (bradykinesia), catalepsy, rearing and movement. Biochemical confirmation of MPTP-induced toxicity was done by measuring striatal DA levels using HPLC with electrochemical detection while the amount of DAT present was determined by Western Blot. NGP1-01 was found to attenuate bradykinesia and catalepsy to a small extent.

NGP1-01 (1) was also evaluated in the middle artery occlusion (MCAO) induced focal cerebral ischemia model in mice to evaluate the neuroprotective potential in

stroke. In this study, NGP1-01 (**1**), at a dose of 20 mg/kg ip injected 30 min before MCAO, reduced the edema volume by 87%, and the infarct volume by 58%, compared to control.

In conclusion, the pentacycloundecylamines represent a new class of compounds that might have utility in the prevention and/or treatment of neurodegenerative diseases through their interaction with a variety of drug targets involved in these diseases.

Opsomming

Parkinson se siekte (PD) is 'n ernstige en debilerende ouderdomsgekoppelde neurodegeneratiewe siekte, met simptome van rustende tremor, akinesia en rigiditeit. Die simptome is die gevolg van selektiewe verlies van dopaminergiese neurone in die substantia nigra. Drie geneesmiddeltekens is geïdentifiseer vir hierdie studie wat afgestem was op die ontwikkeling van nuwe geneesmiddels vir die simptomatiese behandeling en/of voorkoming van PD. Die tekens sluit in die dopamientransporter (DAT), die monoamienoksidase (MAO) B ensiem, en die NMDA- reseptor/ioon kanaalkompleks. Voorts is 'n bekende verbinding, NPG1-01 (1), ook geëvalueer in twee *in vivo* modelle van neurodegeneratiewe siektes, wat insluit the MPTP parkinsonisme muis model (verteenwoordigend van chroniese neurodegeneratiewe prosesse) en 'n beroerte model – die middel karotisarterie okklusie (MKAO) geïnduseerde fokale serebrale iskemie by die muis (verteenwoordigend van akute neurodegeneratiewe prosesse).

Memantien is 'n polisikliese amien wat klinies gebruik word om PD en Alzheimer se siekte te behandel. Dit is 'n lae-affiniteit nie-kompeterende NMDA-reseptor antagonist en word klinies goed verdra. Antagonisme van die NMDA-reseptor voorkom Ca^{2+} oorlading in neuronale selle en gevolglike neuronale seldood. Die belang van Ca^{2+} in neuronale seldood was die aanleiding vir die toets van NPG1-01 (1) en sy derivate as neurobeskermende middels. Hierdie verbinding is in vroeëre werk gekarakteriseer as L-tipe kalsiumkanaalantagoniste.

NGP1-01 (1) en sy derivate is geëvalueer vir hulle effekte op [^3H]dopamien ([^3H]DA) vrystelling en opname inhibering in muis striatale sinaptosome, asook vir inhibisie van bobbejaanlewer monoamienoksidase (MAO) B. NGP1-01 (1) het die opname van [^3H]DA geblokkeer met 'n IC_{50} van 57 μM , terwyl 'n ander verbinding, 8-fenieletiel-8,11-oksipentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undekaan (9), opname geblokkeer het met 'n IC_{50} waarde van 23 μM . Hierdie waardes was vergelykbaar met die van 'n ander polisikliese amien, amantadien (IC_{50} = 82 μM), wat tans gebruik word vir die behandeling van parkinsonisme. MAO-B inhibisie vir die groep verbindings was swak, met nie meer as 50% inhibisie teen 300 μM behaal vir enige van die verbindings in die reeks nie.

NGP1-01 (1) en sy derivate is verder geëvalueer in 'n funksionele biochemiese model om NMDA antagonisme te toets. Die IC_{50} -waardes vir die verwysingsverbinding MK-801 en memantien was 1.23 μM en 3.05 μM , onderskeidelik, vir blokkering van NMDA-gemedieerde $^{45}\text{Ca}^{2+}$ influks in heel-brein muis sinaptoneurosome. NGP1-01 (1) was die mees potente eksperimentele verbinding met 'n IC_{50} van 2.98 μM , terwyl 8-aminopentasiklo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undekaan (8) 'n vergelykbare IC_{50} van 4.06 μM het. Daar is verder gevind dat NGP1-01 (1) 'n nie-kompeterende NMDA antagonis is.

Vergelykende molekulêre veldanalise (CoMFA) en vergelykende molekulêre soortgelykheidsindeksanalise (CoMSA) tegnieke is gebruik om modelle te voorsien om die PCP bindingsaffiniteit van NGP1-01 en die ander polisikliese amiene op die NMDA-reseptor te voorspel. Die r^2 waarde van die CoMFA en CoMSA modelle was 0.976 en 0.883 respektiewelik. Radioligandbindingstudies met [^3H]MK-801 en [^3H]TCP het getoon dat daar min of geen verplasing deur die pentasikloundekielamiene plaasvind nie, wat mag dui daarop dat hierdie verbindings waarskynlik op 'n ander, unieke bindingsplek op die NMDA-reseptor bind.

Nadat aangetoon is dat NGP1-01 (1) NMDA-reseptorantagonisme toon, is die verbinding getoets in 'n reeks gedragstudies in die MPTP muismodel. Afgetrede C57BL/6 manlike teelmuis is gebruik om te bepaal of NGP1-01 dalk 'n verbetering in gedrags-inkorting in die model toon. Die gedragstoets het voorpoot-treelengte, paaltoets (bradikinesie), katalepsie, orentstaan en beweging ingesluit. Biochemiese bevestiging van MPTP-geïnduseerde toksiteit is gedoen deur die meting van striatale

DA-vlakke met HPLC en elektrochemiese deteksie. Die hoeveelheid DAT teenwoordig is bepaal deur van Western Blot-tegnieke gebruik te maak. NGP1-01 het bradikinesie en katalepsie in 'n geringe mate verminder.

NGP1-01 (1) is ook geëvalueer in die middel karotisarterie okklusie (MKAO) geïnduseerde fokale serebrale iskemie model in muis om die neurobeskermende potensiaal tydens beroerte te evalueer. In hierdie studie het NGP1-01 (1), nadat 'n dosis van 20 mg/kg ip ingespuut is 30 min. voor MKAO, die edeemvolume met 87%, en die infarkt volume met 58% verminder, vergeleke met die kontrole.

Ter samevatting bied die pentasiklo-undekielamiene 'n nuwe klas verbindings wat die potensiaal het om aangewend te word in die voorkoming en/of behandeling van neurodegeneratiewe siektes deur die interaksie van hierdie verbindings met 'n verskeidenheid van geneesmiddelteikens wat betrokke is in die ontstaan van hierdie siektetoestande.

Graphical Abstract

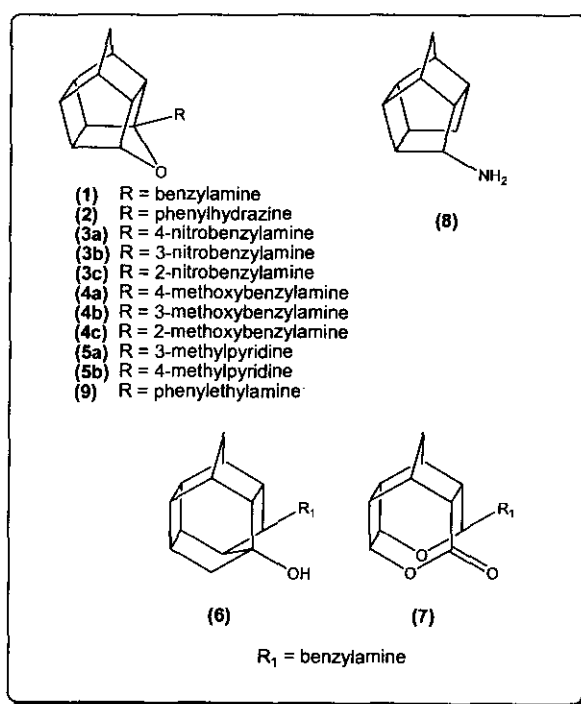


Figure 1. Polycyclic cage compounds used in this study.

Introduction

Chapter

1

1.1 Drug Discovery

The pharmaceutical industry is a hugely profitable global business with the total annual worldwide sales for all human therapeutic drugs in the region of \$350 billion (Flower, 2002). The discovery and development of any new drug is a long and expensive process with no more than 1% of entities in the discovery and development pipeline reaching the market place (Leach, 2001; Brenan & Morrison, 2003). Criteria for a new drug, are not only to produce the desired response, but also to be demonstrably better than existing therapies, with minimal side-effects. The two key steps in any drug discovery program is the identification of hit molecules ('hits') and lead series ('leads'). A hit is a molecule that has some reproducible activity in a biological assay, while a lead series comprises of a set of related molecules that usually share some common structural features, with discernable structure-activity relationships (SAR) (Leach, 2001). Lead optimization (synthetic modification of the lead series) may result in a drug candidate with the desired potency and selectivity, acceptable level of toxicity and the appropriate characteristics to enable it to reach its target *in vivo* (Leach, 2001; Flower, 2002).

Finding a novel lead can be a difficult process. In today's drug discovery process, serendipity has been largely replaced with an amalgamation of computational chemistry or molecular modelling, bioinformatics and cheminformatics, as well as technical processes such as combinatorial chemistry, high-throughput screening (HTS) and micro-arrays, to name but a few. Figure 1.1 gives a general flow of processes involved in the discovery of new drug entities. Since this process is one usually applied by big Pharma, the general procedure of target and drug discovery (on

a smaller scale) will be used in this study to determine the pharmacological profile of selected pentacycloundecylamines.

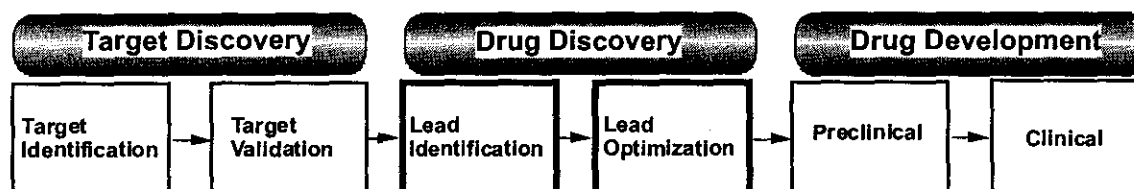


Figure 1.1. Current paradigm in drug discovery and development (Whittaker, 2003).

1.2 Rationale for study

Neurodegenerative diseases, such as Parkinson's (PD) and Alzheimer's diseases, are increasingly becoming a burden to society as the world's population is growing older. The need has therefore increased to find drugs to prevent these diseases from occurring or developing further, thereby increasing morbidity and mortality of patients. This has led pharmaceutical companies to focus on geriantopharmacology in drug discovery (Flower, 2002).

PD is the second most common neurodegenerative disease and is characterized by the progressive loss of dopaminergic neurons in the striatum. Currently, there is relatively good symptomatic therapy for PD, but no proven therapy that prevents cell death (neuroprotection), or restores sick neurons to a normal state (neurorescue) (Dawson & Dawson, 2002). The development of potential neuroprotective or neurorestorative therapies must be based on an understanding of the underlying molecular pathogenesis. Several drug targets have been identified that would act either as symptomatic and/or neuroprotective agents. These include the dopamine transporter (DAT), monoamine oxidase B (MAO-B), *N*-methyl-D-aspartate receptor (NMDAR), inhibitors of α -synuclein and inhibitors of neuronal nitric oxide synthase (nNOS), to name but a few.

Amantadine, an adamantane amine, is a polycyclic cage amine which is clinically used as adjunct treatment in PD in conjunction with L-Dopa (Deleu *et al.*, 2002). In addition to exhibiting symptomatic relief in the treatment of PD, amantadine and its dimethyl derivative memantine, have also been shown to have neuroprotective activity in animal models (Wenk *et al.*, 1995). These findings sparked interest in another series of polycyclic cage amines, the pentacycloundecylamines, and in particular NGP1-01 (8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]-undecane), a novel L-type calcium channel antagonist (Van der Schyf *et al.*, 1986).

In vivo neuroprotective activity has been shown for a small set of pentacycloundecylamines when studied in a pilot neuroprotection study, utilizing the well know MPTP parkinsonian mouse model (Geldenhuys *et al.*, 2003). This study was initiated due to the important role of calcium in neurodegeneration. In addition, there is a structural similarity that exists between the polycyclic cage amine derivatives of NGP1-01 (**1**) and the two adamantane amines (see Fig. 1.2). Therefore it would seem likely that the pentacycloundecylamines might be sharing similar symptomatic as well as neuroprotective profiles than the two adamantane amines.

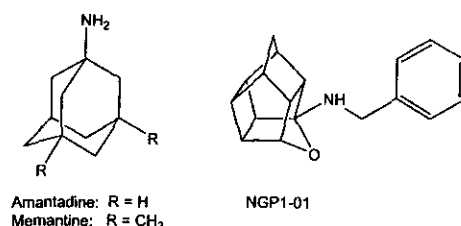


Figure 1.2. Polycyclic cage amines amantadine, memantine and NGP1-01 (**1**).

1.3 Aim of study

The aim of this study is to further elucidate the pharmacological profile of pentacycloundecylamines and to discover other possible candidates for the treatment or prevention of neurodegenerative diseases. Using NGP1-01 (**1**) (as the parent compound) and its derivatives, a battery of *in vitro* tests (i.e. biochemical assays) was performed as a primary screening process to identify "hit" compounds. The *in vitro* tests will focus on drug targets that may be implicated in the symptomatic and/or

neuroprotective treatment of PD (e.g. DAT, MAO-B and the NMDA receptor). These "hit" compounds will then become the lead compounds that will be tested further, *in vivo*. This approach is necessary, since *in vitro* activity does not always guarantee *in vivo* activity. Candidate compounds may also be potent *in vitro*, but their ADME/tox (adsorption, distribution, metabolism, excretion/toxicology) properties may be inadequate for *in vivo* activity. Principles of SAR analysis were used to determine likely "optimal compounds" for each drug target (Lutz & Kenakin, 1999). This process was supplemented by computer-assisted molecular modelling (*in silico*) to generate predictive models for future investigations.

Literature Review

Chapter

2

The purpose of this chapter is to give insight into the drug targets in Parkinson's disease (PD) as a typical neurodegenerative disease. An understanding of the molecular and biochemical pathogenesis of the disease helps to determine how the drug targets pertain to symptomatic treatment and/or prevention of the disease. Such an understanding of the pathogenesis is important in the development of potential drug candidates. The intent of this chapter is not to give a full review of all the available literature, but to provide a comprehensive, rather than an exhaustive overview of pertinent literature.

2.1 Neurodegeneration in PD

PD is a devastating neurological disease that affects at least four million people in the USA (Lotharius & Brundin, 2002). There is a preferential loss of dopaminergic neurons in the striatum and the nature of the pathologic process underlying clinical deterioration in PD remains elusive, although genetic mutations and environmental toxins have been implicated (Riederer *et al.*, 2001). PD is a terminal late-onset neurodegenerative disease that results in muscle rigidity, bradykinesia, resting tremor, and loss of postural reflex. Management of PD is classically divided into three categories: protective or preventive, symptomatic, and restorative or regenerative. The ultimate goal of developing effective protective or preventive therapy will probably depend on advances in our understanding of the etiology and pathogenesis of PD (Lozano *et al.*, 1998).

There are three main mechanisms of neuronal cell death. These may act separately or cooperatively to cause neurodegeneration (Araki *et al.*, 2000). This lethal triplet includes *metabolic compromise*, *oxidative stress* and *excitotoxicity* and combining these three events causes neuronal cell death that is both necrotic and apoptotic in nature. Necrosis is a form of cell death by "murder" that involves an injury from which the cell cannot recover and causes

it to lyse, while apoptosis is active programmed cell death whereby the cell activates a self-destruction mechanism which causes the cell to shrink and ultimately be phagocytosed.

Metabolic compromise may be caused – amongst other, less commonly encountered events – by stroke, asphyxiation, hypoglycemia and certain respiratory toxins. Neurotoxic toxins are usually mitochondrial poisons which include cyanide, carbon monoxide, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 3-nitropropionic acid (3-NP) to name but a few. These mitochondrial poisons result in loss of mitochondrial function and therefore depletion in cellular ATP. Due to the high concentration of mitochondria in the basal ganglia, there is a preponderance of neurodegeneration in these parts after toxic insult. Dysfunction of the mitochondria leads to loss of intracellular calcium buffering capacity and an increase in the production of damaging oxygen and nitrogen based free radicals, leading to oxidative stress and the activation of a host of calcium dependent proteases and lipases (Alexi *et al.*, 2000; Meldrum & Garthwaite, 1990).

Oxidative stress is caused by the actions of highly reactive free radicals or reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radical (OH) and the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$). The oxidizing actions of these reactive species destroy membrane lipids, proteins, and DNA. Excessive accumulation of these radicals in cells can be detrimental to cells and cause neurodegeneration. Production of free radicals can be enhanced by various substances including intracellular calcium, dopamine (DA), and nitric oxide synthase (NOS) (Alexi *et al.*, 2000; Mattson, 2003).

Excitotoxicity is a term coined to describe neuronal death due to the overstimulation of *N*-methyl-D-aspartate (NMDA) receptors, an event that causes an excessive influx of calcium into neuronal cells (Kemp & McKernan, 2002). Calcium overloading (Fig. 2.1) of neuronal cells can cause the activation of calcium-dependent signals to enzymes such as phospholipases and proteases, as well oxidative stress through ROS and RNS, leading to cell death (Mattson, 2003; Meldrum & Garthwaite, 1990; Alexi *et al.*, 2000). In addition, Ca^{2+} entering through voltage-dependent calcium channels (VDCC) are also implicated in calcium overload and mitochondrial disruption (Cano-Abad *et al.*, 2001; Mattson, 2003).

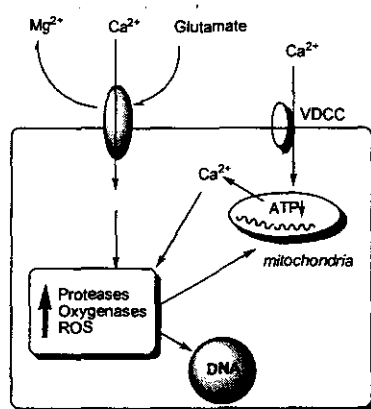


Figure 2.1. Opening of the ionotropic glutamate receptor, and the subsequent influx of Ca^{2+} , are the first steps in excitotoxicity. The increased Ca^{2+} levels can induce uptake of Ca^{2+} into mitochondria, which, if excessive, can induce the production of ROS and inhibit ATP production. In addition, increased Ca^{2+} levels activate proteases and induce oxidative stress, which leads to cellular death (Mattson, 2003). Ca^{2+} entering through voltage-dependent calcium channels (VDCC) is also implicated in calcium overload and mitochondrial disruption (Cano-Abad *et al.*, 2001).

2.2 Drug Targets in PD

2.2.1 Dopamine Transporter

The DA transporter (DAT; Fig 2.2) is a member of the family of Na^+, Cl^- -dependent transporters, mediating uptake of DA into dopaminergic neurons (Reith *et al.*, 1997). This transporter is situated in neuronal plasma membranes, localized on presynaptic sites, where they are crucial for the termination of DA transmission and maintenance of presynaptic DA storage (Torres *et al.*, 2003). Translocation of DA by the transporter is accompanied by co-transport of two Na^+ ions and one Cl^- ion. After release of DA inside the neuron, the transporter needs to be reoriented or 'returned' to the state with the substrate recognition site facing outward (Reith *et al.*, 1997). Abnormal DA signalling has been implicated in various diseases such as cocaine abuse, PD, and depression (Enyedy *et al.*, 2003).

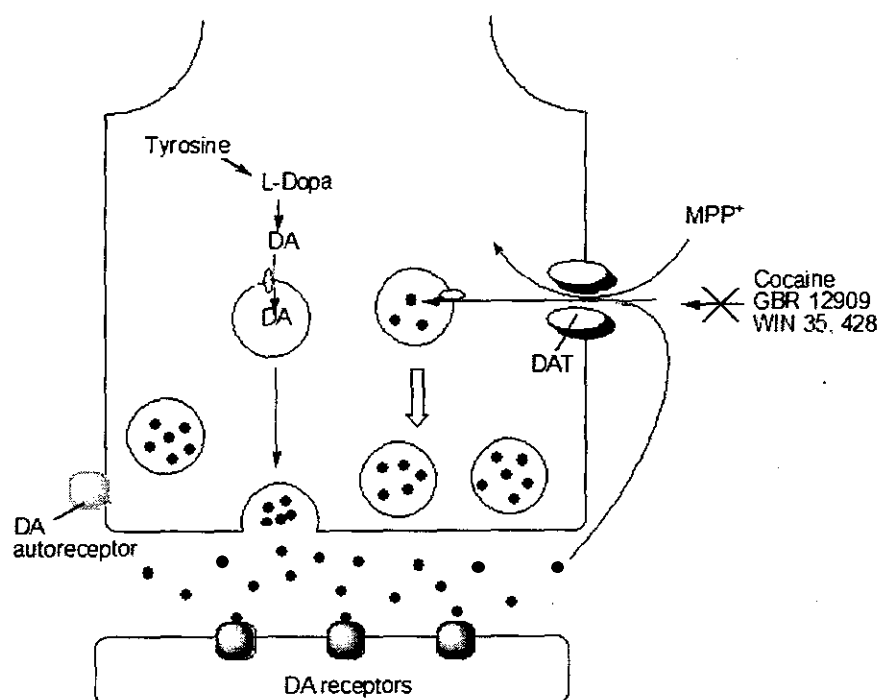


Figure 2.2. Schematic representation of the DA synaptic terminal (Torres *et al.*, 2003).

Although the DAT is essential for normal physiology of DA neurotransmission, it also renders the DA neuron susceptible to damage by toxins that can be transported by DAT. Because many potentially toxic substances resemble DA and are thus substrates for DAT, they can accumulate in the DA neurons (Miller *et al.*, 1999). Inside the neurons, these compounds can then react with vulnerable targets such as the mitochondrial function, for which MPTP (1-methyl-1,2,3,6-tetrahydropyridine) is a prime example (Fig. 2.3). MPTP's neurotoxic properties were discovered by its inadvertent administration by heroin addicts in the early 1980s (Langston *et al.*, 1983). Individuals exposed to the compound developed an acute form of parkinsonism with features of idiopathic PD, also including the specific death of DA-ergic neurons in the substantia nigra. MPTP is a highly lipophilic pro-toxin that penetrates the blood-brain barrier (BBB) and is converted in the brain to the toxin MPP⁺ by monoamine oxidase B (MAO-B) in glial cells (Speciale, 2002; Sedelis *et al.*, 2001). MPP⁺, on the other hand, does not cross the BBB due to its charge. MPP⁺ is then transported by DAT into the DA-ergic neurons, producing neurodegeneration in the DA-ergic nigrostriatal system, with all the symptoms of PD, including akinesia, rigidity, tremor and posture/gait abnormalities. The neuropathological features of the MPTP-parkinsonian mouse model are similar to idiopathic PD in that there is a dose-dependent decrease in striatal DA, a decrease

in striatal DA terminals, respiratory inhibition, and an increase of α -synuclein expression in surviving neurons (Przedborski & Vila, 2001; Speciale, 2002; Tillerson *et al.*, 2002). The full mechanism of toxicity of MPP⁺ is still being elucidated, but to date the neurotoxic effect is thought to be due to inhibition of mitochondrial complex I, producing oxidative stress and disturbing intracellular calcium homeostasis (Speciale, 2002; Sedelis *et al.*, 2001). The ensuing cell death can be either apoptotic or necrotic. In addition to exogenous toxins like MPTP, there are several putative endogenous toxins, such as DA itself and reactive metabolites of DA that can be transported by DAT. The metabolism of these compounds can lead to the production of free radicals that cause oxidative damage to the cell (Miller *et al.*, 1999).

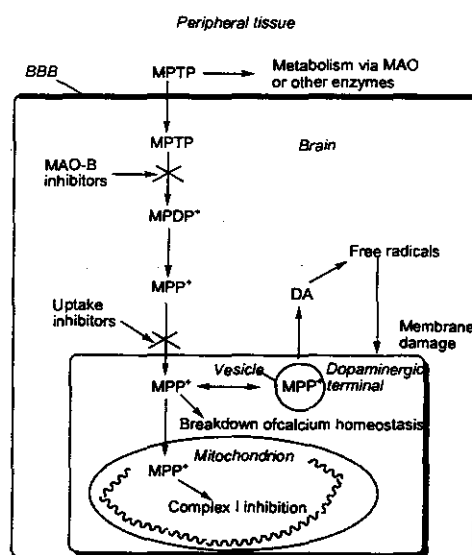


Figure 2.3. Schematic representation of the mechanisms of MPTP toxicity. Abbreviation not mentioned in the text: MPDP⁺ (1-methyl-4-phenyl-2,3-dihydropyridinium) (Sian *et al.*, 1999).

Substrates for the DAT gain access to the intracellular side of the plasma membrane by translocation whereas blockers are incapable of translocation. Classic uptake and binding experiments have generally indicated identical sites for the recognition of DA and uptake blockers such as cocaine, GBR 12909 and WIN 35,428 (Torres *et al.*, 2003). However, recent studies have suggested the involvement of distinct binding domains in addition to shared domains (Torres *et al.*, 2003).

Various pharmacophore models have been identified for DAT inhibitors, using classical structure-activity relationship (SAR) studies, combined with comparative molecular field analysis (CoMFA) (Carroll, 2002; Enyedy *et al.*, 2003). Figure 2.4 shows structural requirements for DAT inhibitors. It was found that substitution on the 3 β -phenyl ring greatly influences binding affinity and monoamine transporter selectivity, with 2 β -substitution required for the highest activity. Substitution at 2 β with various substituted esters (CO₂R), reverse esters (CH₂OCO₂R), amines (CO₂NR'R''), alcohols (CH₂OH), and several types of heterocyclic and aromatic rings all resulted in compounds with high affinity for DAT. Furthermore, nortropine analogs (N-methyl removed) showed a high affinity for DAT, but also showed a high affinity for the serotonin (5-HT) and noradrenaline transporters. The basic nitrogen in the tropane ring can also be substituted by an oxygen, which can function as a hydrogen bond acceptor, such compounds being equally effective for binding with DAT and inhibition of DA uptake. Therefore, the tertiary N (sp³) can also be replaced with a secondary N (sp²), which acts as a hydrogen bond acceptor.

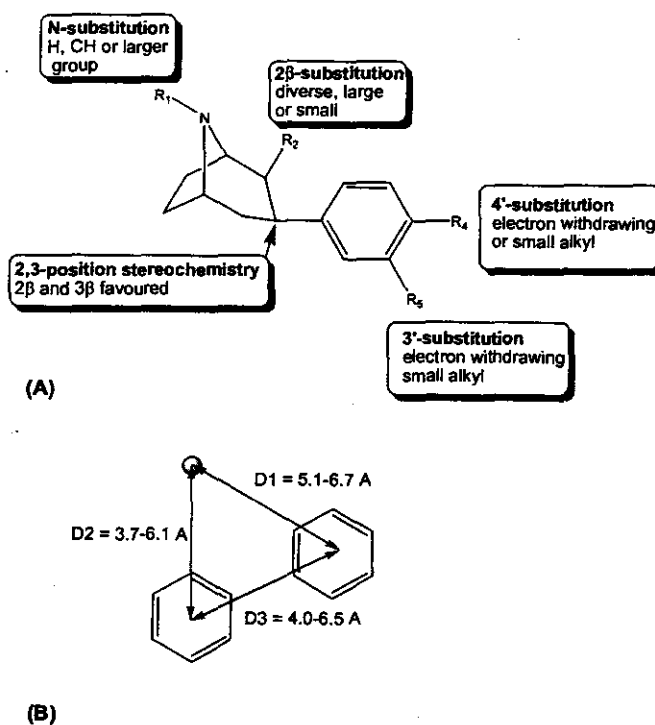
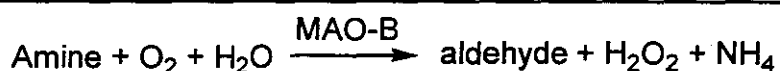


Figure 2.4. Pharmacophore model for (A) structural features that favour DAT binding (Carroll, 2003), and (B) distances (D), measured in Å, between important pharmacophore points (Enyedy *et al.*, 2003).

The characteristic PD symptoms described earlier are not observed until ~80% of striatal DA-ergic nerve terminals are lost (Tillerson *et al.*, 2002; Speciale, 2002). In parallel with the decrease in the number of DA nerve terminals in the striatum, an accompanying reduction in the density of the DA uptake sites is seen. This loss of DAT might be a passive compensatory mechanism that could counterbalance the loss of DA innervation in the early stages of PD when the disease remains undetected. The accompanying loss of DAT results in a reduction in the rate of DA re-uptake that, in turn, permits synaptic concentrations of DA to reach normal levels (Emilien *et al.*, 1999). This then illustrates the therapeutic intervention potential for using DA uptake blockers in PD therapy, with treatment resulting in a similar increase in synaptic DA concentrations to attain normal levels under such directed therapy.

2.2.2 MAO-B enzyme

Another drug target in PD, is the enzyme monoamine oxidase (MAO) B. MAO is a flavin-containing enzyme that catalyzes the oxidative deamination of a variety of amine-containing neurotransmitters such as DA, serotonin and norepinephrine, as well as other xenobiotic amines. The reactions yield aldehydes, H_2O_2 and ammonia (Salach & Weyler, 1987; Zhou & Panchuk-Voloshin, 1997).



This enzyme is located in the outer mitochondrial membrane of neuronal, glial, and other cells in the mammalian body. There are two distinct isoforms, designated MAO-A and MAO-B. Both these enzymes have been identified by their substrate specificity for selective inhibitors. MAO-A is inhibited by clorgyline and it acts preferentially on serotonin and norepinephrine. MAO-B is inhibited by *R*-deprenyl and it acts preferentially on 2-phenylethylamine and benzylamine.

Recently (in 2002), the crystal structure of the MAO-B enzyme was elucidated (Binda *et al.*, 2002; Binda *et al.*, 2003) and the crystal data deposited in the Protein Data Bank (<http://www.rcsb.org/pdb/>) code 1OJB. MAO-B binds to the membrane of the mitochondria through a C-terminal transmembrane helix and apolar loops located at various positions in the

amino acid sequence. Deprenyl, co-crystallized with MAO-B, was found to bind to the flavin atom N5. The active site consists of a 420 Å³-hydrophobic substrate cavity interconnected to an entrance cavity of 290 Å³. Therefore, a drug (substrate) needs to traverse through this entrance cavity before entering the substrate cavity. The recognition site for the substrate amino group is an aromatic cage formed by Tyr 398 and Tyr 435. The active site (cavity) is highly apolar, but hydrophilic areas exist near the flavin and this directs the amine moiety of the substrate for binding and catalysis. What came as a total surprise was that the enzyme exists with a two-domain architecture, therefore two binding cavities exist in one enzyme.

Deprenyl is used together with L-dopa, where it retards the breakdown of L-dopa and therefore prolongs the effect L-dopa in patients with PD (Aminoff, 1998). MAO-B is perhaps best known for its part in the bioactivation process associated with MPTP neurotoxicity (Fig. 2.3). It has played an important role in the development of animal models of PD to find new drug compounds for the treatment of this disease. In the early 1980s, a number of young adults were admitted to emergency rooms with symptoms of PD, which responded to treatments of L-dopa (Speciale, 2002; Langston *et al.*, 1983). As mentioned previously, this was due to the abuse of home-made heroin-related drugs, which contained MPTP. In addition, there is increasing evidence that oxidative stress through an MAO-B catalyzed mechanism may play a major role in the pathogenesis of PD, raising the possibility that MAO inhibition could be neuroprotective by inhibiting peroxide formation consequent to the oxidative metabolism of DA. With this in mind, deprenyl has been shown to be neuroprotective (Koller, 1997). Insights into the structure of the MAO-B enzyme should thus facilitate intelligent design of inhibitors with neuroprotective activity (Binda *et al.*, 2002).

MAO-B inhibitors therefore have a dual role to play in the pharmacological management of PD. First, to increase DA concentrations in the striatum (current strategy), and second, as possible neuroprotecting agents, aiming to protect surviving neurons and preserving their function (Foley *et al.*, 2000).

2.2.3 NMDA receptor

Glutamate is the main excitatory amino acid in the central nervous system. Glutamate receptors are divided into two main groups: ligand-gated ion channels and G-protein-coupled receptors. Glutamate-gated ion channels are further divided into three groups, based on their

selectivity for agonists. These include NMDA, kainate (KA) and AMPA [(*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid] receptors. Due to its particular involvement in excitotoxic neuronal degeneration, only the NMDA receptor will be discussed further. For an excellent review that includes detailed descriptions of the other two receptors, see Bräuner-Osborne *et al.* (2000). Functional antagonism of the NMDA receptor can be achieved at different recognition sites (Fig. 2.5). These include the primary transmitter site, the strychnine-insensitive glycine site (glycine), polyamine site, and the phencyclidine (PCP) site located inside the cation-transporting channel (Parsons *et al.*, 1999; Dingledine *et al.*, 1999; Bigge, 1993). NMDA receptors are heteroligomers formed by the co-assembly of the ubiquitous NR1 subunit and one or more of the NR2 subunits (NR2A-D) (Ferrer-Montiel *et al.*, 1998).

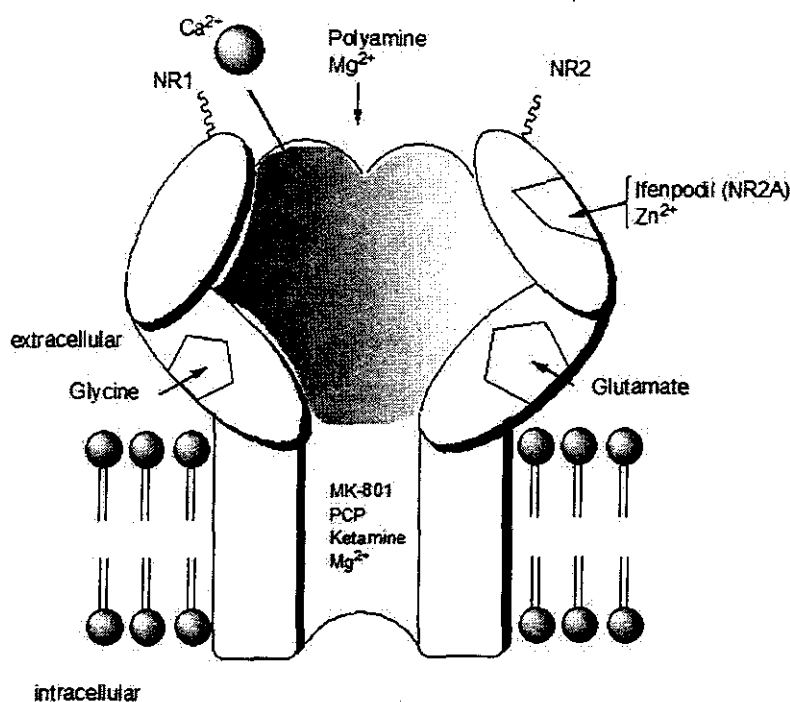


Figure 2.5. Schematic representation of the NMDA receptor/ion channel (Kemp & McKernan, 2002).

Activation of the NMDA receptor needs both binding of the agonist, glutamate, and of a co-agonist, glycine, thereby opening the ion-channel and allowing Ca^{2+} to enter the neuron. Mg^{2+} provides a voltage-dependent block of the ion-channel, which is removed by membrane depolarization. Under normal physiological conditions, activation of the NMDA receptor promotes signalling to the nucleus that culminates in CREB phosphorylation, multiple gene

activation and long term synaptic plasticity. Synaptic plasticity is the mechanism underlying learning and memory (Kemp & McKernan, 2002).

Under pathological conditions however, excessive and chronic stimulation of the NMDA receptor allows excess intracellular Ca^{2+} , causing neurons to die. This glutamate-dependent process is called excitotoxicity (Kemp & McKernan, 2002). This process therefore represents an attractive drug target. The entry of Ca^{2+} into neuronal cells under pathological conditions, may potentially be treated by compounds blocking/modulating the NMDA receptor. Neurodegenerative diseases, both acute (e.g. stroke, brain trauma) and chronic (Parkinson's, Alzheimer's diseases) are disorders where NMDA receptor antagonists would be beneficial. At first glance it might seem counter-intuitive to use such antagonists in a disease such as Alzheimer's, since the NMDA receptor is important for synaptic plasticity. Low affinity channel blocking (uncompetitive) NMDA receptor antagonists (e.g. memantine) however, have shown promise for clinical development due to their favourable tolerability (Rogawski, 2000; Palmer & Widzowski, 2000).

2.2.3.1 Memantine, a prototypical neuroprotective drug

Organic chemists have been interested in the chemistry of polycyclic cage compounds for the better part of this century (Marchand, 1995; Marchand *et al.*, 1989; Griffin *et al.*, 1989; Marchand, 1989). Interest in the pharmacology of polycyclic cage amines was stimulated by the finding that the adamantane amine, 1-amino-adamantane or amantadine (1), had antiviral activity against the influenza virus (Davies *et al.*, 1964). Polycyclic cage compounds such as adamantane are also known as “diamondoids”, and are considered to be the simplest unit of the cubic diamond lattice (Marchand, 2003). Amantadine hydrochloride was the first orally active antiviral drug to be used in the USA, and its initial use was restricted to prophylactic treatment for the prevention of respiratory illness due to susceptible influenza A2-Asian viruses. A congener of amantadine, rimantadine (3) [1-(1-aminoethyl)adamantane], also displays antiviral activity, and has been reported to have fewer side effects than amantadine (Tilley & Kramer, 1981).

The anti-parkinsonian activity of amantadine was found through the serendipitous observation of unexpected improvement of Parkinson's disease patients treated for influenza with amantadine (Schwab *et al.*, 1972). Amantadine's pharmacological mechanism as an

anti-parkinsonian agent has generally been attributed to its action on nigrostriatal DA-ergic neurons. Amantadine expresses its antiparkinsonian activity by increasing extracellular DA levels via DA re-uptake inhibition (Mizoguchi *et al.*, 1994) or DA release (Danysz *et al.*, 1997). Electrophysiological studies further indicated that amantadine acts via the PCP or MK-801 (dizocilpine) binding site located within the NMDA receptor/ion channel complex (Parsons *et al.*, 1999).

Interest in the pharmacology of polycyclic cage amines was further stimulated when the dimethyl derivative of amantadine, memantine, was later found to be a clinically well tolerated NMDA receptor antagonist (Danysz *et al.*, 1997; Parsons *et al.*, 1999; Palmer & Widzowski, 2000; Rogawski, 2000). Memantine is the 1-amino-3,5-dimethyladamantane derivative of amantadine and has also been referred to in older literature as D-145, DMA and dimethylamantadine (Tilley & Kramer, 1981). Memantine is currently under intense investigation and has been approved by the FDA as recently as October 17, 2003, for use in the USA in the treatment of moderate to severe Alzheimer's disease (Reisberg *et al.*, 2003; Jain, 2000; Vedantam, 2003). Memantine is a low-affinity uncompetitive NMDA antagonist. Such compounds show promise as neuroprotective drugs by preventing excessive influx of calcium into neuronal cells (Wenk *et al.*, 1995; Turski *et al.*, 1991; Zuddas *et al.*, 1992; Brouilliet & Beal, 1993). Potent uncompetitive NMDA antagonists such as the (non-cage) compounds MK-801 and PCP bind to the PCP binding site located within the ion-channel pore of the NMDA receptor (Dingledine *et al.*, 1999).

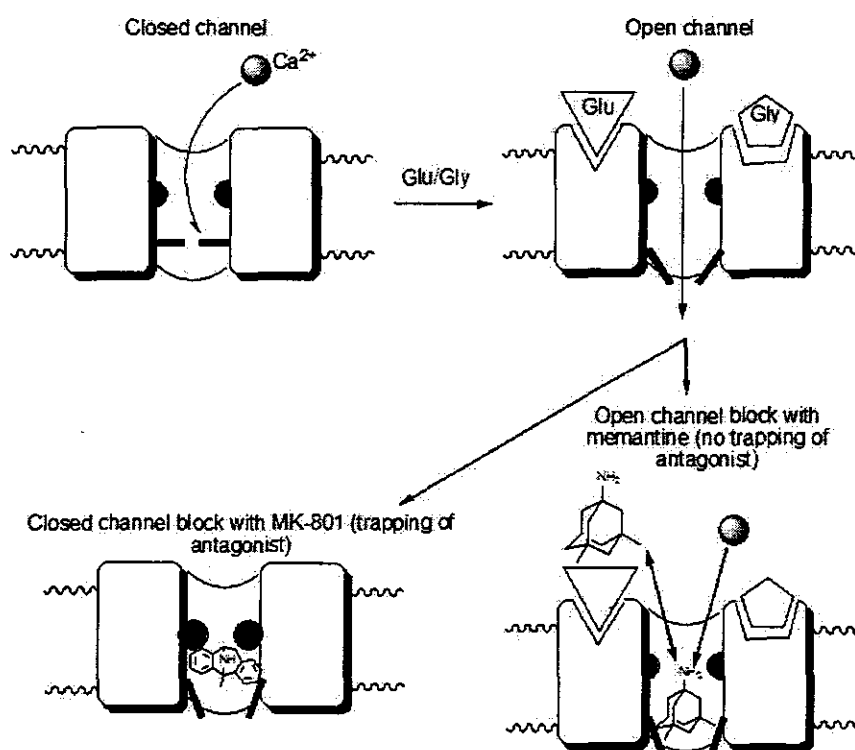


Figure 2.6. Concept of open- and closed-channel NMDA receptor block with memantine and MK-801 (Rogawski & Wenk, 2003). The processes involved are described in the text. The black filled dots and thick bars are cartoon tools to illustrate the concept of trapped block.

This antagonism is use-dependent in that the PCP site is only accessible when the ion channel pore is in an open or activated state (Fig. 2.6). The block is accelerated by increases in open channel probability. This phenomenon suggests that compounds having pronounced use-dependency will have greater affinity for brain regions where overstimulation occurs (Grauert *et al.*, 1997). Once bound, the blocker can be trapped by channel closure. Recovery from the trapped blocked state is generally slow. Block produced by MK-801 or PCP is difficult to reverse and MK-801 is long acting (Dingledine *et al.*, 1999). Unfortunately, use of these long duration antagonists is associated with adverse CNS side effects including hallucinations (Carter *et al.*, 1994), memory impairment (Tist, 2000), and neuronal vacuolization (Bigge, 1993). On the other hand, low-affinity use-dependent channel blockers (i.e. antagonists with a K_i above 200 nM) such as amantadine and memantine are generally not associated with the unacceptable side effects described above, are well tolerated (Parsons *et al.*, 1999; Kroemer *et al.*, 1998), and have shown promise for clinical development (Palmer & Widowski, 2000).

The favourable clinical toleration of memantine as opposed to MK-801 also lies in the fact that memantine exhibits rapid and strong voltage-dependent blocking kinetics (Parsons *et al.*, 1999). Partial trapping and release of memantine from NMDA channels favour the occurrence of strong block only during sustained receptor stimulation (such as may occur in excitatory neurotoxic events including ischemia and brain trauma) with more limited effects expected during normal synaptic transmission. There are clear differences in the pharmacology of amantadine and memantine in laboratory animals, probably due to the slight variance between the effects of each compound on various receptor systems. For example, amantadine, but not memantine binds to the σ_1 -site at therapeutically relevant concentrations (Danysz *et al.*, 1997). By taking the above into consideration, amantadine and memantine are thus ideal lead compounds for the development of other agents with favourable indices to treat NMDA-mediated neurodegenerative diseases.

Unfortunately, the three-dimensional structure of the NMDA receptor has not yet been determined experimentally. Therefore, indirect computer-assisted molecular modelling techniques are generally used to define a pharmacophore when designing uncompetitive drugs at the PCP binding site (Kroemer *et al.*, 1998; Elhallaoui *et al.*, 2002). The neuronal NMDA receptor has a pore dimension of 0.45 x 0.57 nm, with a pore area of 0.26 nm² (Dingledine *et al.*, 1999). A basic pharmacophore has been described where potent ligands generally contain an aromatic ring and a basic amine separated by areas of lipophilic bulk. Further studies have shown that aromatic rings are not strictly required. For example, hydrogenation of either of the aromatic rings of MK-801 was shown to render compounds that retained receptor affinity (Bigge, 1993). Another pharmacophore model recently constructed consists of the following geometrical properties (Fig. 2.7; Elhallaoui *et al.*, 2002):

- The gap between the nitrogen atom and the plane of the phenyl is 0.7 Å,
- The gap between the dummy atom (DA - representing the lone pair atoms on the nitrogen atom) and the plane of the phenyl ring is 3 Å,
- The distance between the phenyl centre and the nitrogen atom is 3.6 Å, and
- The dihedral angle DA-N-C(1)-C(2) is 45°.

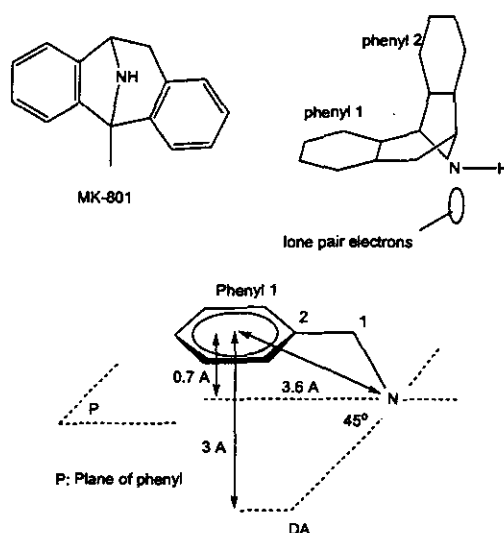


Figure 2.7. Geometric characteristics of a proposed pharmacophore model for the NMDA/PCP site, using MK-801 as example (Elhallaoui *et al.*, 2002). The dummy atom (DA) is used to evaluate the orientation of the lone pair atoms on the bridge nitrogen atom.

To give additional insight into the structural requirements for effective NMDA receptor antagonism, Kroemer *et al.* (1998) initiated a comparative molecular field analysis (CoMFA) study (Fig. 2.8). Firstly, a lipophilic region, corresponding to the area where the cyclohexyl moiety of PCP interacts with the receptor, was shown to exist. Compounds with only one bulky hydrophobic moiety such as adamantane amines appear to fit, but with a restriction on size. Adamantane amines are too bulky for this area and display reduced binding affinities and potentially higher K_{off} values. Secondly, there is another lipophilic area that appears to be important for high affinity binding and corresponds to the phenyl ring of PCP. Ideally, a compound should be able to interact with both these regions. The third requirement is the ability to form a hydrogen bond between the protonated amine in the ligand and a suitable residue in the NMDA ion channel.

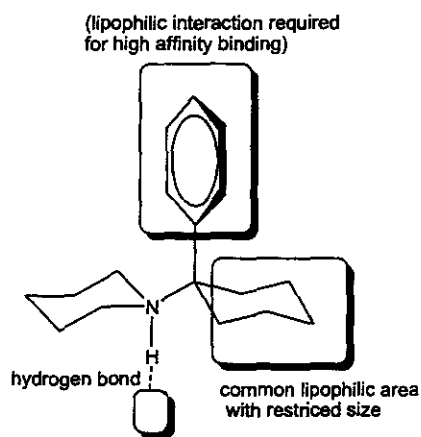


Figure 2.8. Main structural requirements for PCP binding site blockers (Kroemer *et al.*, 1998).

2.3 Pentacycloundecylamines

Pentacycloundecylamines form part of the polycyclic cage group of compounds (Marchand, 1995). There have been a variety of pharmacological activities reported for compounds of this type. These include antiviral (Oliver *et al.*, 1991a), antiparkinsonian (Oliver *et al.*, 1991a,b,c), antimicrobial (Liebenberg, 1989), calcium channel antagonism (Liebenberg *et al.*, 2000; Malan *et al.*, 2000; Malan *et al.*, 1997; Dockendorf, 1992; Van der Schyf *et al.*, 1986), sodium channel antagonism (Malan *et al.*, 2003), and sigma receptor antagonism (Liu *et al.*, 2001; Nguyen *et al.*, 1999, Kassiou *et al.*, 1996; Nguyen *et al.*, 1996).

The potential antiparkinsonian activity of the pentacycloundecylamines (Oliver *et al.*, 1991b) and D₃-trishomocubanes was demonstrated by Oliver *et al.* (1991b,c). Antagonism of reserpine induced catatonia (anticataleptic) and reduction of oxotremorine induced tremor and salivation were determined. Promising anticataleptic and mild to weak anticholinergic activities were observed, and compared favorably with that of amantadine. From these results the authors speculated that these compounds exhibited their antiparkinsonian activity due to an interaction with the DA-ergic system. Sigma receptor antagonism was reported for several pentacycloundecylamines (Kassiou *et al.*, 1996; Nguyen *et al.*, 1996 & 1999). Most sigma ligands also display significant cross reactivity at other binding sites such as DA, muscarinic and PCP receptors. The compounds studied (aza- and monoketo pentacycloundecylamines) by Kassiou *et al.* (1996) indicated high affinity and selectivity for sigma receptors with no cross activity for other binding sites. The calcium channel antagonism and sodium channel

antagonism described for compound **6** also indicate that these compounds might be potential neuroprotective agents (Malan *et al.*, 2000 & 2003).

2.3.1 Lead compound for this study: NGP1-01

Van der Schyf *et al.* (1986) first characterized NGP1-01 (8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane; **1**) as a novel calcium channel antagonist. Electrophysiological studies on NGP1-01 (**1**) and its derivatives showed L-type calcium channel antagonism for this class of compounds (Malan *et al.*, 1997). Although structurally unrelated to any of the known calcium channel antagonists, there are certain similarities with the dihydropyridine calcium channel antagonists. These similarities include a secondary N-atom, a phenyl ring and the geometric distance between these groups (Dockendorf, 1992).

Van der Schyf *et al.* (1986) postulated that the pentacycloundecane skeleton might only serve as a bulk contributor to the activity of NGP1-01 (**1**) and structure-activity relationships for NGP1-01 and its derivatives as a putative L-type calcium channel antagonists found that there was a domination of steric or geometric constraints rather than electronic considerations (Malan *et al.*, 2000). Aromatic substitution of NGP1-01 influenced activity significantly. *Ortho* and *meta* substitution led to more pronounced antagonism while increasing the cage size also led to better inhibition of the calcium current. From these studies, it appears that NGP1-01 (**1**) is the near optimal structure for calcium channel antagonism within the series studied.

2.3.2 Synthesis of NGP1-01 and its derivatives

The pentacycloundecylamines used in the current study are all derived from the well known Cookson's diketone (pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione; **15**), or so called 'bird-cage' compounds (Fig. 2.9) as the product of an intramolecular photocyclized Diels-Alder adduct between *p*-benzoquinone (**13**) and cyclopentadiene (**14**) (Cookson *et al.*, 1958).

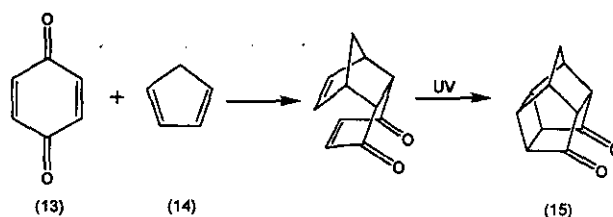


Figure 2.9. Synthesis of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (15) (Cookson *et al.*, 1958).

The structure of NGP1-01 (1) was at first erroneously assigned with an aza 8,11-bridgehead atom (Sasaki *et al.*, 1971; Sasaki *et al.*, 1974). Van der Schyf *et al.* (1989) and Marchand *et al.* (1989) correctly reassigned NGP1-01's structure indicating an oxa 8,11-bridge head atom by means of X-ray crystallography and NMR spectroscopy, respectively.

Synthesis of compounds 1-7 and 9 was conducted as previously described (Malan *et al.*, 2000; Geldenhuys *et al.*, 2003; Zah *et al.*, 2003) and is shown in Figure 2.10. Reductive amination of 15 followed by Dean-Stark dehydration and reduction with NaBH₄ furnished 1-5, and 9. Transformation of 15 by a boron trifluoride etherate reaction with ethyl diacetate, followed by subsequent decarboxylation, yielded 11, the starting ketone for 6. Similarly, the starting ketone (12) of 7 was prepared by Baeyer-Villiger oxidation of the diketone (15). Final compounds 6 and 7 were prepared by reductive amination. Compound 8 was prepared by lithium aluminum hydride reduction of the oxime, which was obtained by condensation of the mono ketone 13 with hydroxyl amine (Oliver *et al.*, 1991b). Compound 8 was purified as the HCl salt.

All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The ¹H and ¹³C spectra were recorded on a Varian VXR 300 spectrometer. Samples were dissolved in deuterated chloroform (CDCl₃) with tetramethylsilane (Me₄Si) as an internal standard. Infrared spectra were recorded as a neat film on KBr plates with a Shimadzu FT IR 4200 spectrophotometer. Mass spectra and HR-MS were recorded at 70 eV (EI) on a VG 7070E spectrometer. Elemental analyses were performed on a Perkin-Elmer model 240 instruments and data were all within acceptable limits ($\pm 0.04\%$).

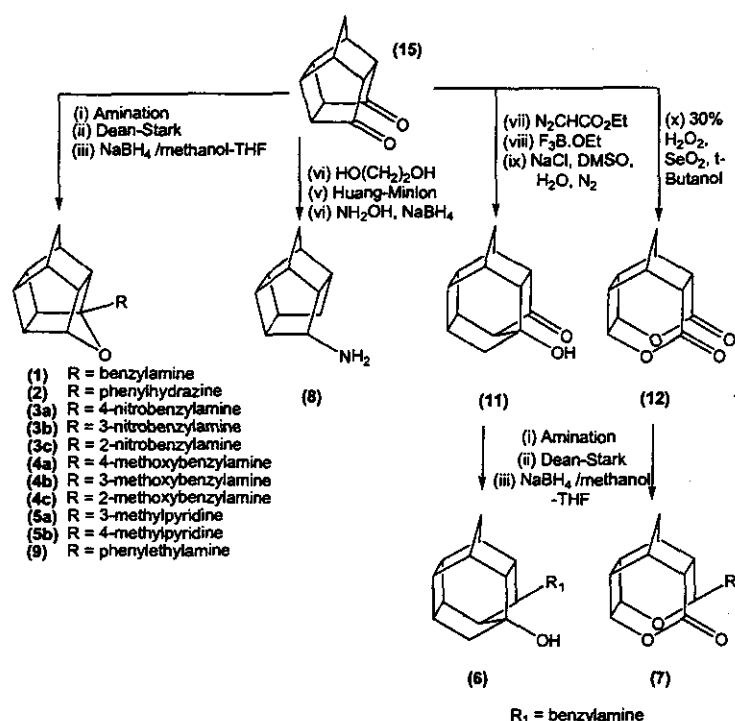


Figure 2.10. Synthesis of pentacycloundecylamine analogues (Malan *et al.*, 2000; Zah *et al.*, 2003; Geldenhuys *et al.*, 2003).

2.4 Conclusion

Three drug targets in PD have been identified in this chapter. These are targeted for the development of both symptomatic and/or neuroprotective therapy in our model of neurodegenerative disease and PD. The targets identified are DAT, MAO-B, and the NMDA receptor/ion channel complex. Inhibition of DAT would increase the concentration of DA in the synapse, thereby providing symptomatic relief in a state of decreased DA. In addition, because DAT is considered a gateway for xenobiotic toxins, inhibition of uptake through the DAT might prove to be neuroprotective. Inhibition of MAO-B results in both symptomatic relief, by increasing synaptic DA concentration, and may be neuroprotective through a mechanism that include inhibition of metabolic activation of neuronal toxins such as MPTP. Antagonism of the NMDA receptor inhibits the excessive influx of Ca^{2+} that causes neuronal cell death, therefore preventing excitotoxicity. Literature provides ample evidence for the potential benefits of polycyclic cage amines in the treatment of PD and other neurodegenerative diseases (especially low affinity use-dependent NMDA receptor antagonists show a lot of promise for clinical development). Two compounds used in the clinical practice, amantadine and memantine, especially stand out due to their favourable

tolerability. These facts have led us to evaluate NGP1-01 and its derivatives as possible therapeutic agents in the symptomatic treatment and/or prevention of neurodegenerative diseases. NGP1-01 (**1**) was chosen due to its calcium channel antagonism and the structural similarity (Fig. 2.11) that exists between this compound and the adamantane amines amantadine, and memantine.

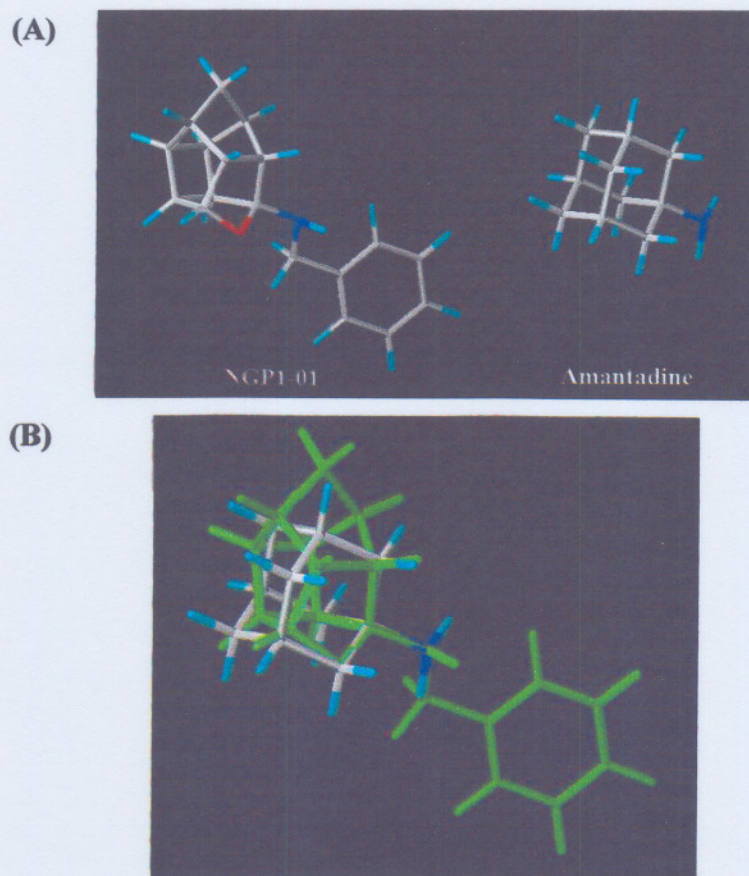


Figure 2.11. Structural similarity between minimum energy conformations (semi-empirical AM1 calculations through Hyperchem®) of NGP1-01 (**1**; green) and amantadine (atom colours). RMS fitting of **1** on amantadine was $< 0.1 \text{ \AA}$.

This current study aims to develop drugs with dual mechanism of action for the prevention and/or treatment of neurodegenerative diseases. Recently, there has been a change in attitude toward dual or multiple mechanism drugs. Earlier, such drugs were belittled as "dirty drugs", but it is better understood now that in certain cases a balanced activity of a singular drug at several targets might be better suited for effective therapy than high specificity (Kubinyi, 2003).

The Dopamine Transporter and MAO-B enzyme Studies

Chapter 3

3.1 Introduction

As described earlier, Parkinson's disease (PD) is a terminal, late-onset neurodegenerative disorder characterized by a progressive and relatively selective degeneration of DA-ergic neurons in the substantia nigra (Alexi *et al.*, 2000). To date, most therapies for PD have been symptomatic in nature, with no neuroprotective therapies currently approved for prevention of this chronic disease. Some therapeutic agents used in the early treatment of PD have, however, been shown to have neuroprotective activity in addition to their symptomatic effects, such as the DA agonist, pramipexole (Bennett & Piercey, 1999).

Recently, we reported an *in vivo* pilot evaluation of a small series of pentacycloundecylamines in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) parkinsonian mouse model and found that one of the compounds, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**9**), tested positively as a neuroprotective agent (Geldenhuis *et al.*, 2003). MPTP is a protoxin that requires activation by means of monoamine oxidase (MAO) B to form the neurotoxic pyridinium species, 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ is then transported into DA-ergic neurons via the DA transporter (DAT), where it inhibits mitochondrial complex I, or NADH-quinone oxidoreductase (Speciale, 2002). Neuronal energy deprivation in turn could alter the normal functioning of cell membranes and cause a partial depolarization leading to a release of the voltage-dependent Mg²⁺ block of the NMDA receptor ion channel. Removal of this Mg²⁺ block enables glutamate to excite the receptor persistently, therefore leading to excitotoxicity (Lange *et al.*, 1997).

On the basis of this information, we have chosen to evaluate a series of polycyclic cage amines for activity in the DA system as part of an ongoing investigation into the therapeutic potential of novel pentacycloundecylamines. Since the DAT and MAO-B are key elements in the DA depletion seen in the MPTP mouse model, we investigated the pharmacology of the compounds in this study for interaction with the DAT and MAO-B *in vitro*, aiming to select compounds for future *in vivo* studies. Moreover, because our future plans include analysis of effects on the NMDA receptor and MPTP/C57 mouse model, we included MK-801 as an established NMDA antagonist in the present study.

3.2 Materials and Methods

3.2.1 Materials

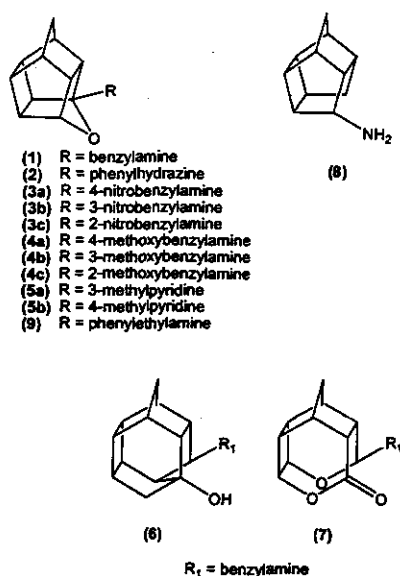


Figure 3.1. Pentacycloundecylamines used in this study.

Amantadine (hydrochloride salt) and memantine (hydrochloride salt) were purchased from Sigma Chemical Co. (St. Louis, MO). GBR-12909 (hydrochloride salt) and (+)MK-801 (maleate) were purchased from Research Biochemicals (Natick, MA, USA). [³H]DA (20.3 Ci/mmol) was purchased from New England Nuclear

(Wilmington, DE). (*R*)-Deprenyl and TMN were gifts from Dr. Neal Castagnoli Jr. at the Harvey W. Peters Centre, Department of Chemistry, Virginia Tech. Buffer constituents were obtained from other commercial sources.

3.2.2 Pharmacology

Evaluation of release and uptake inhibition of [³H]DA was carried out in murine striatal synaptosomes as described earlier (Wright *et al.*, 1998). Compounds were screened at 100 μ M, except for GBR-12909 ([³H]DA release, 10 μ M; inhibition of [³H]DA uptake, 2 μ M). Initial experiments identified compounds that blocked [³H]DA uptake without causing appreciable release ($\leq 40\%$ at 100 μ M), in order to ensure specificity. IC₅₀ values for inhibiting [³H]DA uptake were then determined for selected compounds. Inhibition of MAO-B was carried out in baboon liver MAO-B. The baboon liver contains MAO-B almost exclusively (Inoue *et al.*, 1999). All compounds were screened initially in this assay at 100 μ g/ml.

3.2.2.1 Animals

ICR male mice (20 – 26 g, 6 – 8 weeks old) were obtained from Harlan, Dublin, VA. All animal care and experimental protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee in accordance with guidelines established by the United States Public Health Service and Virginia Tech, USA.

3.2.2.2 Preparation of synaptosomes

Male ICR mice were sacrificed by cervical dislocation and the striata were rapidly removed. The striatal tissue was homogenized in 4 ml of sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4). The homogenate was then centrifuged for 10 min at 1000 $\times$ g. The supernatant was removed and centrifuged for 15 min at 10,000 $\times$ g. The resulting pellets were washed once and resuspended in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, CaCl₂·2H₂O, 10 mM sucrose and 50 mM Tris-HCl, pargyline 0.05 μ M, ascorbate 0.1 μ M, pH 7.4).

3.2.2.3 Dopamine release studies

After preparation, the final pellet of synaptosomes was resuspended in incubation buffer containing 100 nM [^3H]DA and incubated for 5 min at 37 °C, and then re-centrifuged at $10,000 \times g$ for 10 min. The supernatant was discarded and the pellets washed once with cold incubation buffer and resuspended. The synaptosomes were aliquoted into tubes, treated with drug, and incubated for 10 min at 37 °C. Test compounds (100 μM final concentration) were dissolved in DMSO, the final DMSO concentration in the incubations did not exceed 0.1%, with the controls receiving 0.1% DMSO alone. Synaptosomes then received 3 ml of wash buffer at 37 °C, filtered through 1 micron glass microfiber filters (Whatman GF/B) under vacuum and washed an additional 3 times with 37 °C buffer (125 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM sucrose and 50 mM Tris-HCl, pH 7.4), followed by liquid scintillation counting to measure the radioactivity of [^3H]DA within the synaptosomes. For each compound screened, fresh striatal tissue was used.

3.2.2.4 Dopamine uptake inhibition studies

Test compounds (screened at 100 μM final concentration, dissolved in DMSO as described above) were incubated with synaptosomes in a heat block for 10 min. at 37 °C. Following the incubation, the synaptosomes were incubated with [^3H]DA for 2 min. Uptake was terminated by the addition of ice cold wash buffer, followed by vacuum filtration through 1 micron glass microfiber filters (Whatman GF/B). These filters were washed 4 times with 3 ml ice cold wash buffer, air dried, followed by liquid scintillation counting to measure the radioactivity of [^3H]DA within the synaptosomes. For each compound screened for DA uptake inhibition and each dose-response replicate, fresh striatal tissue was used.

3.2.2.5 MAO-B Inhibition

Intact mitochondria from baboon liver served as the source for MAO-B (Ooms *et al.*, 2003). Mitochondrial fractions were prepared as described previously (Salach & Weyler, 1987). The mitochondrial homogenate was suspended in sodium phosphate buffer (100 mM, pH 7.4) containing 50% (w/v) glycerol. Protein was determined by

the method of Bradford (Bradford, 1976). The inhibition of MAO-B by the test compounds (100 µg/ml) was evaluated by incubating the substrate (1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP; 0.25 mM) with the mitochondrial homogenate (0.15 mg/ml). Test compounds were dissolved in DMSO and added to the buffered incubation mixture so that the final DMSO concentration did not exceed 4%. The final volume of the incubation mixtures was 500 µl (in sodium phosphate buffer, pH 7.4) and the samples were incubated at 37 °C for 15 min. The reaction was terminated by the addition of 20 µl perchloric acid (70% v/v), centrifuged (16,000 × g for 8 min) and the concentration of the enzyme-generated metabolite of MMTP determined spectrophotometrically at 420 nm in the supernatant fractions.

3.2.2.6 Statistical Analysis

DA uptake and release studies were analysed using Student's *t*-test (95% confidence level) in InStatTM (GraphPad Software, San Diego, CA), to find statistically significant effects. Concentration-response data were analysed by non-linear least squares fit to a four parameter logistic equation:

$$y = bottom + \frac{(Top - Bottom)}{(1 + 10^{\log EC_{50} - X})}$$

with resulting IC₅₀ values and goodness of fit statistics calculated using PrismTM (GraphPad Software, San Diego, CA).

3.2.3 Molecular modelling

The three most active compounds were selected for *in silico* evaluation of spatial arrangement and physicochemical properties. Molecular modelling to obtain a reasonable minimum energy conformation for each structure was performed by optimising the structures with the ChemplusTM extension of Hyperchem® modelling software (Release 4.5 for Windows 1994 Hypercube Inc. Ontario, Canada) using MM⁺ and AM1 with the Polak-Ribieré minimization procedure (Polak, 1971). The reasonably low energy conformer found for compound 3b and 4b were used to superimpose the structures. The amines of in all cases were unprotonated. The physicochemical properties of 9 were calculated to assist estimation of log BB

(Equation 3.1), and the predicted concentration of **9** in the central nervous system (CNS) following intraperitoneal treatment.

3.3 Results and Discussion

Table 3.1. Summary of effects of cage amines and related compounds on DA release (at 100 μM), inhibition of DA transport (at 100 μM , with selected IC_{50} values), and inhibition of monoamine oxidase B activity (at 300 μM).

Compound	DA Release, % Control $\pm\text{S.E.M. (n = 2)}$	DA Uptake, % Control $\pm\text{S.E.M. (n = 3)}$	IC_{50} , DA Uptake, $\mu\text{M}, \pm \text{S.E.M.}$	MAO-B Activity, % Control $\pm\text{S.E.M. (n = 2)}$
1	88.00 ± 23.00	$42.66 \pm 1.76^*$	57 ± 1.6	$71.77 \pm 0.002^*$
2	79.50 ± 16.50	54.66 ± 9.17	n. d.	$29.58 \pm 0.005^*$
3a	81.50 ± 0.50	86.00 ± 2.00	n. d.	$70.16 \pm 0.016^*$
3b	30.00 ± 12.00	25.66 ± 6.69	n. d.	$24.62 \pm 0.005^*$
3c	55.00 ± 6.00	41.00 ± 4.61	n. d.	$34.95 \pm 0.009^*$
4a	61.00 ± 1.00	43.00 ± 7.09	n. d.	$33.12 \pm 0.010^*$
4b	$22.00 \pm 2.00^*$	$7.00 \pm 1.73^*$	n. d.	$35.74 \pm 0.004^*$
4c	57.50 ± 3.50	$33.00 \pm 2.30^*$	n. d.	$77.97 \pm 0.021^*$
5a	71.50 ± 8.50	66.00 ± 1.00	n. d.	$83.67 \pm 0.010^*$
5b	62.50 ± 12.50	64.33 ± 5.84	n. d.	$88.82 \pm 0.003^*$
6	99.00 ± 4.00	$33.33 \pm 0.88^*$	55 ± 1.2	$88.33 \pm 0.011^*$
7	96.50 ± 6.50	$51.33 \pm 6.69^*$	199 ± 1.1	$97.64 \pm 0.016^*$
8	67.00 ± 14.00	$40.33 \pm 2.02^*$	n. d.	$91.73 \pm 0.023^*$
9	76.00 ± 3.00	$18.66 \pm 0.88^*$	23 ± 1.0	$56.09 \pm 0.013^*$
Amantadine	72.50 ± 3.50	$30.00 \pm 6.55^*$	82 ± 1.1	$86.09 \pm 0.005^*$
Memantine	74.50 ± 7.50	$49.66 \pm 7.33^*$	>100	$75.98 \pm 0.041^*$
MK-801	81.50 ± 5.50	$13.33 \pm 0.66^*$	18 ± 1.1	$92.64 \pm 0.017^*$
GBR 12909	69.57 ± 0.32^b	$6.00 \pm 2.51^{*c}$	0.007 ± 0.4^a	n.d.
Deprenyl	n.d.	n. d.	n. d.	$0.82 \pm 0.0002^*$
TMN	n.d.	n. d.	n. d.	$4.40 \pm 0.002^*$

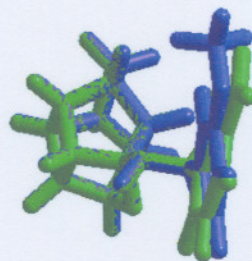
*Significantly different from control, $P < 0.05$; n.d. = not determined; ^areported IC_{50}

(Kolhatkar *et al.*, 2003); ^b10 μM ; ^c2 μM

Initial screening studies determined the compounds' ability to release DA, and compounds with significant activity were selected for further evaluation (Table 3.1). The compounds fell into three groups with respect to causing DA release; those that were able to elicit no more than 0 – 20 % release of label (1, 2, 3a, 6, and 7), those that elicited 20 – 40 % release of label (3c, 4a, 4c, 5a, 5b, 8, 9, memantine, and MK-801), and those that released >60% of label (3b and 4b). Because these studies served as a screen for activity, and to eliminate potentially toxic compounds, each assay was replicated only twice.

For [³H]DA release (Table 3.1) it was apparent that replacement of the benzylamine moiety of 1 by phenylhydrazine (2) did not alter the release of [³H]DA significantly, suggesting the importance of a linker atom spaced between the nitrogen and phenyl moieties in both these compounds. In addition, substitution on the aromatic ring seemed to influence the degree of [³H]DA release. Introduction of a nitro group (3a-c) on the phenyl ring of 1 resulted in varying [³H]DA retention. The *meta* nitro compound (3b) showed the least intracellular retention of [³H]DA while the *para* substituted nitro compound showed the highest intracellular retention of [³H]DA for these compounds. These data suggest that for nitro substitution the rank order of activity would be *meta* > *ortho* > *para*. For the methoxy compounds (4a-c), *meta* substitution also yielded the most active compound in the whole series, while *para* and *ortho* compounds elicited lower, but equipotent release of [³H]DA. For [³H]DA release it would seem that electron donating groups (e.g. methoxy) lead to slightly more active compounds, when compared to the electron withdrawing groups (e.g. nitro). However, this effect of electron donation seems to play only in a small (additive) role, with steric or conformational considerations more important, since it was not the *ortho* and *para* compounds (which would be expected to elicit a stronger electron effect, than either the *meta* positions on the phenyl ring), but the *meta* compounds in each series that showed the highest activity (i.e. caused more release), when comparing the nitro and methoxy compounds. Both the nitro and the methoxy groups occupy the same spatial volume as is clear when energy minimised structures 3b and 4b are superimposed. In addition, the oxygens from the methoxy and one of the nitro group, are oriented in the same direction away from the cage (Fig. 3.2).

(A)



(B)

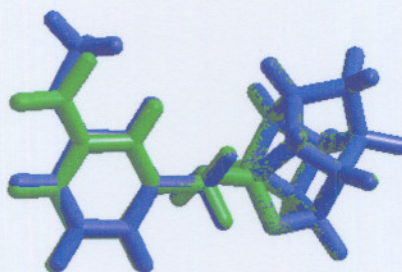


Figure 3.2. Superimposition of **3b** (green) and **4b** (violet) through the Chemplus™ extension of Hyperchem®, given in two views **A** and **B**.

The distance between these two oxygen atoms is only 1.77 Å, which suggests that these oxygens may be important for release of [³H]DA, and that the orientation of the methoxy oxygen of **4b** may be more preferable than that of either of the two oxygens of the nitro group of **3b**.

When a nitrogen is included in the aromatic ring of **1** to produce the methylpyridine compounds (**5a** and **5b**), retention of [³H]DA was only slightly less than that found with **1**. This finding emphasizes the importance of aromatic substitution and the spatial arrangement of the molecule. Enlargement of the polycyclic cage (**6** and **7**) resulted in decreased release of [³H]DA. In contrast, the primary amine **8**, afforded release of DA similar to that found with **1**. The three NMDA receptor antagonists exhibited equipotency in releasing [³H]DA, with amantadine slightly more active than memantine and MK-801. As was expected, the reference compound GBR-12909, a potent DAT inhibitor,²⁴ was not very active in this assay.

Structure-activity relationships similar to those for the release of [³H]DA were observed when the compounds were screened for inhibition of [³H]DA uptake (Tab.

3.1). Substitution of the aromatic ring of **1** resulted in a more pronounced influence on [³H]DA uptake inhibition than was seen for release of [³H]DA. Substitution of the aromatic ring with nitro groups (**3a-b**) resulted in the *meta* compound **3b** being the most active uptake inhibitor in this group. The inhibition of [³H]DA uptake by **3b** is three times greater than that of its *para* analog and one third more than that of the *ortho* compound **3c**. Substitution with methoxy groups on **1** resulted the most active inhibition of [³H]DA uptake to be found with the *meta* compound **4b**. The rank order of activity for both the nitro and methoxy compounds, was found to be *meta* > *ortho* > *para*. As was seen for [³H]DA release, **4b** was found to be the most active compound in this polycyclic amine series tested. The *meta* position seems to be more preferred for inhibition of [³H]DA uptake, than the *ortho* and *para* positions. This might suggest an optimal binding pocket where the substituent in the *meta* position fits into the DAT, compared to **1** with no substitution on the phenyl ring. The *para* position seems to be less favourable, especially with the nitro group. This might indicate a steric interaction that may keep the phenyl ring from forming a bond with the DAT. The methoxy group substitution on the phenyl ring also seems to be a more ideal group to increase inhibition of [³H]DA uptake, suggesting an additional bond formation with the DAT where the small electron donating effect of the methoxy group results in increasing hydrophobic/ π - π bonding stabilization of **3b**, compared to **4b**. For the inhibition of [³H]DA uptake, it would therefore seem that the same electronic effects are similarly important factors in activity, as was seen with [³H]DA release. Compounds which contained electron donating groups (e.g. methoxy) generally were more active, when compared with compounds containing electron withdrawing groups (e.g. nitro). However, as for DA release, this effect of electron donation seems to play only in a small (additive) role, with conformational considerations more important, since it was again not the *ortho* and *para* compounds but the *meta* compounds that showed the highest activity (i.e. caused more inhibition of uptake), when comparing the nitro and methoxy compounds. In fact, the *para* compounds actually exhibited the lowest uptake inhibition activity, supporting the theory of sterics overshadowing electronic effects.

Differences in inhibition of DA uptake were seen for the "enlarged cages" in that compound **6** was more active than **7** (Table 3.1). These differences could be attributed to several factors, however, including the presence of an hydroxyl group in

6 and the carbonyl group and ether ring linkages in 7, suggesting that different associations are relevant for the two compounds in their interactions with the DAT. The activity of the compounds fall in the same range as that of 1, with 6 slightly more active. Removal of the side chain and endocyclic ether of 1 to yield the primary amine (8) resulted in very little, if any, change in activity if compared to 1. Increasing the distance between the polycyclic cage and the aromatic ring seemed to increase activity for compound 9. The increased chain length instils more flexibility, which might improve fit at the site of interaction. Memantine exhibited less DA uptake inhibition than amantadine and 8. It was surprising that MK-801 exhibited such a potent inhibition of [³H]DA uptake. The reference compound GBR-12909, a potent DAT inhibitor (Torres *et al.*, 2003) was most active in this assay. Binding studies done by us using the cocaine analogue WIN 35,248 suggested that these compounds bind to a different site on the DAT than WIN 35,248 (data not shown).

From the initial experiments compounds could be identified that blocked DA uptake without causing appreciable release (<40% at 100 μ M) of the neurotransmitter. Selectivity for DA uptake inhibition is desirable since excessive release of DA is also thought to be neurotoxic (Kirby *et al.*, 2002). DA uptake inhibition arguably may also be beneficial to patients in the early stages of PD, because decreasing DA uptake might not only result in increased synaptic and decreased intracellular DA concentrations, but also may help slow the progression of the disease. Moreover, in cases where endogenous or exogenous toxins use the DAT as a gateway (e.g., MPP⁺), uptake inhibitors would be ideal neuroprotective compounds.

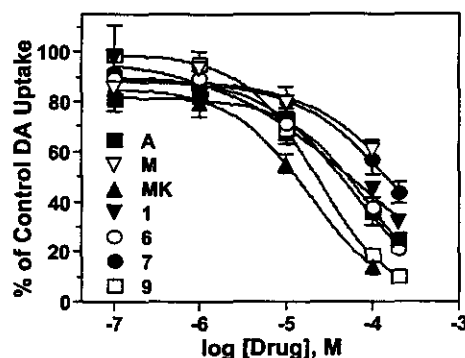


Figure 3.3. Dose-response relationships for the inhibition of [^{3} H]DA transport by selected compounds. Each data point represents the mean ($\pm$ S.E.M.). Lack of error bars indicates that the S.E.M. resides within the size of the symbol. Abbreviations are: Amantadine (A), Memantine (M) and MK-801 (MK).

To this end, we undertook further dose-response studies (Fig. 3.3) with compounds **1**, **6**, **7**, **9**, as well as the reference compounds amantadine, memantine and MK-801. Compounds **3b** and **4b** were excluded, because although potent in inhibition uptake assays, they are also potent releasers of DA. We thus focused on compounds that would be neuroprotective against endogenous and exogenous toxins, by selectively inhibiting DAT. The IC_{50} values ($\pm$ SEM) of compounds **1**, **6**, and **9** were similar in magnitude to those measured for the reference compounds amantadine and MK-801 (Table 3.1). A higher IC_{50} value was observed for **7** and memantine seemed to have a similar activity profile ($IC_{50} > 100 \mu M$, Fig. 3.3). The shallower dose-response slopes of **1**, **6**, **7**, and memantine (Fig. 3.3) suggest that these compounds might be therapeutically more acceptable (Meltzer *et al.*, 2000), as a steeper dose-response curve such as those found with **9** and MK-801 (Fig. 3.3) indicate a narrow index of therapeutic activity that might be compromised *in vivo* by individual differences in pharmacokinetics. It is therefore likely that compounds such as **1**, **6**, and **7** may be more viable for future *in vivo* studies. The slight difference in IC_{50} values between MK-801 and **9** may be explained by the steeper slope of the dose-response curve of MK-801 (Fig. 3.3).

The dose-response curves generated for **1**, **6**, **7**, amantadine, and memantine (Fig. 3.3) show incomplete blockade of [^{3} H]DA uptake ($\leq 80\%$). Possible explanations for this may be low drug penetration to the DAT, affinity differences, or enantiomeric

preference for DAT binding with these compounds. In addition, multiple ligand acceptor sites have been reported to exist on the DAT (Meltzer *et al.*, 2000), and these may account for the differences in blocking activity. In contrast, **9** and MK-801 exhibited complete inhibition of [³H]DA uptake.

The activity of **9** as an uptake inhibitor of DA in these experiments might offer an explanation for its neuroprotective activity as seen in the MPTP parkinsonian mouse model (Geldendhuys *et al.*, 2003). The degeneration of DA-ergic neurons by MPTP is initiated by accumulation, via the DAT, of the MAO-B generated pyridinium metabolite of MPTP, MPP⁺. Blocking the DAT might underlie this neuroprotection in striatal neurons by preventing the action of agents or toxins similar to MPTP/MPP⁺ that require access to DA-ergic cells through the DAT (Kirby *et al.*, 2002; Koller, 1997). Based on this hypothesis, compounds **1** and **6** would additionally be ideal candidate compounds for screening in the MPTP parkinsonian mouse model.

Monoamine oxidases (MAOs) are thought to be involved in the etiology of PD (Volz, & Gleiter, 1998). For example, MPTP is converted by MAO-B to MPP⁺, the active neurotoxin. Inhibition of this enzyme prevents the neurotoxicity of MPTP and increases the amount of free DA that is still present in the synaptic cleft (Fukuda, 2001). We therefore screened the pentacycloundecylamines, the three NMDA receptor antagonists amantadine, memantine, and MK-801, and the two MAO-B inhibitors deprenyl (a mechanism-based irreversible inhibitor; Heikkila *et al.*, 1984) and 2,3,6-trimethyl-1,4-naphthoquinone (TMN, a reversible inhibitor; Castagnoli *et al.*, 2001) in a robust MAO-B inhibition assay (Table 3.1). Although some of the compounds elicited marginal inhibition, none of the pentacycloundecylamines that were found to be selective DAT inhibitors, inhibited MAO-B in any significant way (inhibition > 50%) at therapeutically relevant concentrations. As expected, the known MAO-B inhibitors deprenyl and TMN resulted in complete (99%+) inhibition of the enzyme.

During the drug discovery process it is important to consider the pharmacokinetic properties of the drugs evaluated (Lin & Lu, 1997). Due to the central nervous system location of action of the drugs screened in this study, it is important to mention

that the pentacycloundecylamines are capable of crossing the blood-brain barrier. Permeation into the brain was observed in a study done by Zah *et al.* (2003). These researchers developed a physiochemical prediction model from *in vivo* studies, to predict blood-brain distribution of pentacycloundecylamines.

$$\text{Log BB} = 4.56796 - 0.52226(\log P_{\text{oct}}) + 0.0052(\text{Energy}) + 0.05559(\text{SV}) - 0.33873(\text{MR}) \quad (\text{Eq. 3.1})$$

Where $\log \text{BB} = \log (\text{Concentration}_{\text{brain}}/\text{Concentration}_{\text{serum}})$; Energy = minimum energy conformation; SV = solvent accessible molecular volume; and MR = molar refractivity.

Using this predictive equation (Eq. 3.1) of Zah *et al.*, and assuming therapeutic serum levels (Danysz *et al.*, 1997) are similar to that of amantadine (9 μM), a predicted CNS concentration of 17 μM is calculated for **9**, which is higher than the therapeutic concentration range for amantadine (4 – 9.5 μM). This predicted concentration (17 μM) is near the IC_{50} value (23 μM) for DAT inhibition by compound **9**, and suggests that compound **9** is a good lead for further drug development as potential neuroprotective therapy in PD.

3.4 Conclusions

In this chapter, we described *in vitro* biological studies (DA release, interaction with the DAT and the enzyme MAO-B) of a group of polycyclic cage amines, in order to identify promising compounds for future neuroprotection studies. The cage compounds proved to be effective inhibitors of DA uptake, with IC_{50} values comparable to that of amantadine. The most active compound (**9**) had an IC_{50} value of 23 μM . In structure-activity studies, we investigated both aromatic substitution as well as increased size of the cage moiety. Most of the polycyclic cage amines showed greater activity for blocking DA uptake than for causing release of DA. For cage compounds with aromatic substituents, little or no selectivity between uptake inhibition and release of DA was observed. Increasing the cage moiety size enhanced selectivity for DA uptake inhibition. The distance between the aromatic moiety and the cage appears to play a vital role since **9** (phenylethylamine, the compound with the largest distance), was also the most active DAT inhibitor in this series. Structure-

activity relationships for this group of compounds therefore appear to be influenced predominantly by geometric and steric considerations, rather than by electronic characteristics. Results from the *in vitro* assays suggest that inhibition of the DAT might underlie the neuroprotective effect of **9** in the MPTP parkinsonian mouse model seen in earlier work (Geldenhuys *et al.*, 2003). Inhibition of MAO-B activity by these compounds occurred in the high (several hundred) micromolar range and likely would not contribute to any protection against MPTP. A primary goal of future PD therapy is the development of neuroprotective strategies that will retard or prevent neuronal death (Koller, 1997). The spectrum of activity observed within these pentacycloundecylamines makes them candidates for future exploration as possible lead compounds for symptomatic and neuroprotective therapies against PD.

3.5 Acknowledgements

The help of Dr. T. Murugesan of the Dept. of Chemistry, Virginia Tech, Blacksburg VA, USA with the MAO-B assay is hereby gratefully acknowledged. The other experiments were performed in the laboratory of Dr. J. R. Bloomquist, Neurotoxicology Laboratory, Dept. of Entomology, Virginia Tech, Blacksburg, VA, USA.

The NMDA receptor

Chapter

4

4.1 Introduction

The NMDA receptor has been suggested as a drug target through its involvement in neurodegenerative disorders such as Parkinson's and Alzheimer's disease (Kemp & McKernan, 2002). Overstimulation of the NMDA receptor by an excess of the endogenous neurotransmitter, glutamate, during pathological conditions, leads to an excess influx of calcium into neuronal cells resulting in cell death. Excitotoxicity is the term used to describe this glutamate-dependent mechanism of neuronal cell death. In addition to excitotoxicity, calcium entry through L-type calcium channels may cause both calcium overload and mitochondrial disruption that will lead to the release of mediators responsible for the activation of the apoptotic cascade and cell death (Cano-Abdad *et al.*, 2001).

Uncompetitive NMDA antagonists show promise as neuroprotective agents by preventing excessive influx of calcium into neuronal cells (Wenk *et al.*, 1995; Brouillet & Beal, 1993; Turski *et al.*, 1991; Zuddas *et al.*, 1992). Uncompetitive NMDA antagonists such as the potent MK-801 and PCP bind to the PCP binding site located within the ion-channel pore of the NMDA receptor (Dingledine *et al.*, 1999). This antagonism is use-dependent in that the PCP site is only accessible when the ion channel pore is in an open, or activated, state. The block is accelerated by increases in open channel probability. This phenomenon means that compounds having pronounced use-dependency will have greater affinity for the brain region where overstimulation occurs (Grauert *et al.*, 1997). Once bound, the blocker can be trapped by channel closure. Recovery from the trapped blocked state is generally slow (Dingledine *et al.*, 1999). Block produced by MK-801 or PCP is difficult to reverse and MK-801 exhibits a very long duration of action (Carter, 1994). Unfortunately,

use of these long duration antagonists are associated with adverse CNS side effects including hallucinations (Trist, 2000), memory impairment (Bigge, 1993), and neuronal vacuolization (Grauert *et al.*, 1997). Low-affinity use-dependent channel blockers (i.e. antagonists with a K_i above 200 nM) however, are usually not associated with the unacceptable side effects and are well tolerated (Parsons *et al.*, 1999; Kroemer *et al.*, 1998).

The adamantane amine, memantine, is a low-affinity, clinically well-tolerated uncompetitive NMDA antagonist (Parsons *et al.*, 1999). The reason for the better toleration of memantine as opposed to MK-801 is that memantine exhibits rapid and strong voltage-dependent blocking kinetics. Partial trapping and release of memantine from NMDA channels favour the occurrence of strong block only during sustained receptor stimulation, which may occur during brain trauma, with more limited effects expected on normal synaptic transmission (Dingledine *et al.*, 1999). Memantine has been used clinically to treat Parkinson's and Alzheimer's disease (Parsons *et al.*, 1999) and is therefore an ideal lead compound in the development of agents with a favourable therapeutic index to treat NMDA-mediated cell death diseases.

4.2 Materials and Methods

4.2.1 Materials

Amantadine and memantine were purchased as the hydrochloride salts from Sigma Chemical Co. (St. Louis, MO). (+)MK-801 (maleate) was purchased from Research Biochemicals (Natick, MA, USA). $^{45}\text{CaCl}_2$ (12 mCi/ml) was purchased from ICN Biochemicals CA, (USA). $[^3\text{H}]$ MK-801 (17.1 Ci/mmol) and $[^3\text{H}]$ TCP (45.2 Ci/mmol) were purchased from Perkin-Elmer (Boston, MA, USA). Buffer constituents were obtained from other commercial sources. The pentacycloundecylamines used in the current study (Fig. 4.1) were prepared as reported previously (Malan *et al.*, 1996; Malan *et al.*, 2000) with the exception of 8 and 9, which were prepared according to Oliver *et al.* (1991; for compound 8), Geldenhuys *et al.* (2003) and Zah *et al.* (2003) for compound 9, respectively.

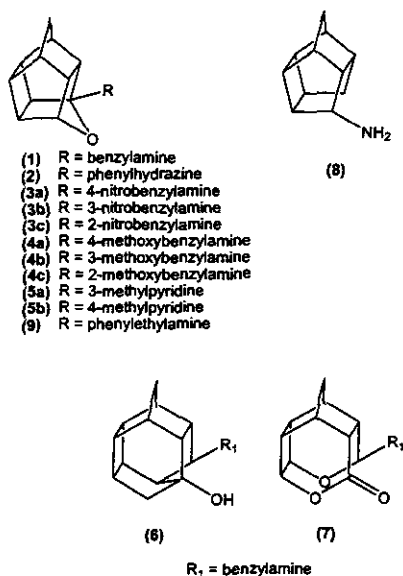


Figure 4.1. Pentacycloundecylamines used in this study.

4.2.2 Calcium Flux Assay

Synaptoneurosomes were prepared as described previously (Bloomquist & Soderlund, 1985) with minor modifications. Whole brains of male ICR mice (Harlan) were dissected out on ice and coarsely minced with scissors. The tissue was homogenized in magnesium-free incubation buffer by hand using a Dounce homogenizer. The incubation buffer contained 118 mM NaCl, 4.7 mM KCl, 0.1 mM CaCl₂, 20 mM HEPES and 30.9 mM glucose. The tissue was then centrifuged for 15 min at $928 \times g$ and 0 °C. After centrifugation, the pellet was resuspended in fresh incubation buffer by hand using a Pasteur pipette and then centrifuged again as described above. We found that two centrifugation steps were absolutely required for achieving consistent levels of background $^{45}\text{Ca}^{2+}$ uptake, as well as a reproducible estimation of antagonist activity (Fig. 4.2).

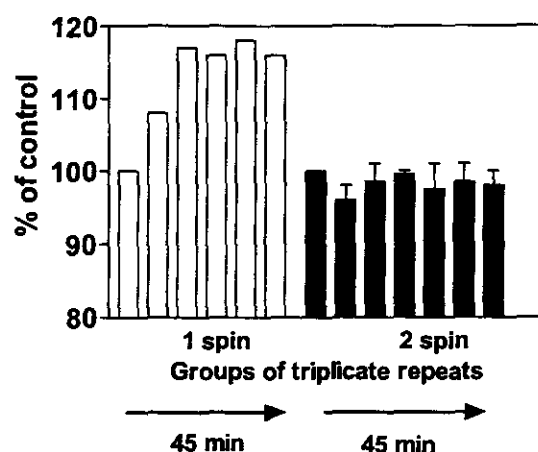


Figure 4.2. Difference in $^{45}\text{Ca}^{2+}$ uptake into murine synaptoneurosomes using one or two centrifugation steps. There was a time-dependent increase in $^{45}\text{Ca}^{2+}$ uptake when the tissue was centrifuged only once. After a second centrifugation step was used, consistent levels of $^{45}\text{Ca}^{2+}$ uptake were seen. The total time for the experiment, from test tube one to twenty-one, was 45 min. In the figure, the white bars are representative of a typical experiment.

The final pellets were resuspended in a volume of incubation buffer adequate for experimental usage. The tissue containing synaptoneurosomes was then exposed to drug dissolved in DMSO. The pentacycloundecylamines have been previously reported as L-type calcium channel blockers (Van der Schyf *et al.*, 1986; Malan *et al.*, 1996; Malan *et al.*, 2000), so we added 10 μM of the dihydropyridine L-type calcium channel blocker, nitrendipine, to all the incubations as a precaution. Control incubates received DMSO in addition to the nitrendipine. All drug treatments were pre-incubated for 10 min at room temperature. Stock solutions of NMDA and Gly were prepared in incubation buffer. Treated synaptoneurosomes were then exposed to incubation buffer containing $^{45}\text{CaCl}_2$ (3 μM final concentration) and NMDA/Gly for 5 sec. The final concentrations of NMDA and Gly were 100 μM each for the screening assays, and for the NMDA dose-response curves the concentration NMDA varied, while Gly was kept constant at 100 μM . Flux was terminated by the addition of 4 ml ice cold wash buffer. The wash buffer contained 118 mM choline chloride, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.18 mM MgSO_4 , 20 mM HEPES and 27 mM glucose, pH 7.4. The incubate was poured onto a Whatman GF/C glass fiber filter under vacuum and then washed twice with 4 ml of wash buffer. The filters were then air dried,

scintillation cocktail added, and the amount of trapped $^{45}\text{Ca}^{2+}$ was measured by liquid scintillation spectrometry. Treatments were repeated two to four times on different tissue preparations with three determinations in each replicate.

4.2.3 Molecular Modelling

Molecular modelling of minimum energy conformations and volume for **9** was obtained by optimizing them with the ChemplusTM extension of Hyperchem[®] modelling software (Release 4.5 for Windows, 1994, Hypercube Inc. Ontario, Canada) using MM⁺ and AM1 with the Polak-Ribieré minimization procedure (Polak, 1971). For the MOLCAD surface area analysis, the structures of compounds **1** and **6** used in the study were built using SYBYL 6.9 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA) molecular modelling software on a silicon graphics Octane computer. Both the molecules were energy minimized using the standard Tripos molecular mechanics force field and Gasteiger-Huckel charges were applied, with a 0.005 kcal/mol energy gradient convergence criterion.

4.2.4 Data Analysis

Calcium flux and radioligand binding data were analysed with InStat 2.0 and plotted with Prism 2.0 software (both from GraphPad Software, San Diego CA, USA). Concentration-response data were analyzed by non-linear least squares curve fitting, with IC₅₀ values for blockers or EC₅₀ values for NMDA/Gly calculated using PrismTM. The bottom value for the antagonism dose-response curves was held constant at 100% of control (Tab. 4.1). Student t-tests were performed on mean responses of test compound vs. controls in the screening studies and on curve fit parameters in the dose-response studies. A value of $p < 0.05$ was considered statistically significant.

4.3 Results

Effects of the pentacycloundecylamines on background (no agonists) or NMDA/Gly-mediated ^{45}Ca influx into synaptoneurosomes are shown in Fig. 4.3. There was significant inhibition of NMDA-mediated ^{45}Ca influx by compounds **6** and **8**, with

complete inhibition ($\leq 100\%$ of control) by compounds **1**, **3b**, **3c**, **4b**, **9** and the reference compounds memantine and MK-801. Compounds **2**, **3a**, **4a**, **4c**, **5a**, **5b**, **7**, and the reference compound amantadine blocked NMDA-mediated ^{45}Ca influx into synaptoneurosomes only weakly. Because the pentacycloundecylamines are reported to be L-type calcium channel blockers (Van der Schyf *et al.*, 1986; Malan *et al.*, 1996; Malan *et al.*, 2000), we evaluated the effect of the pentacycloundecylamines on the background ^{45}Ca influx as well by screening the compounds in the absence of NMDA/Gly. All the pentacycloundecylamines showed only modest inhibition of background calcium influx, with only MK-801 having a statistically significant antagonist effect (Fig. 4.3).

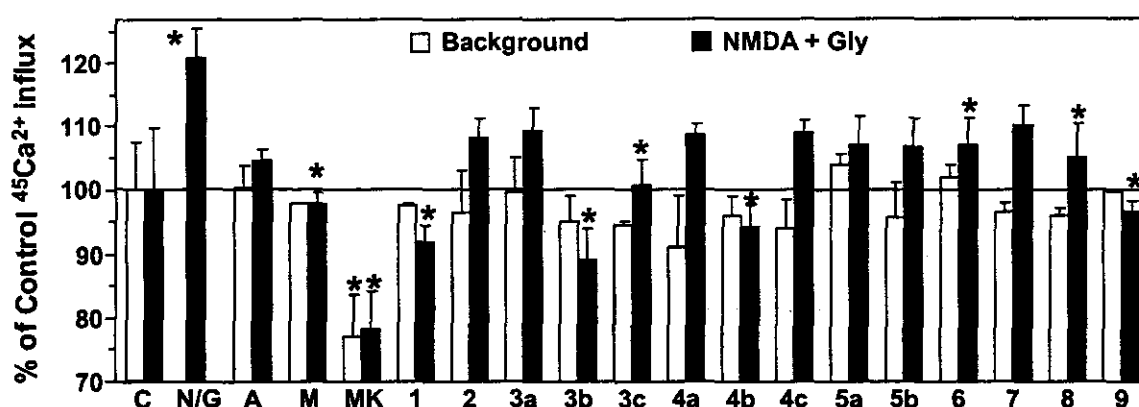


Figure 4.3. Screening of pentacycloundecylamines (100 μM) for antagonism of background or NMDA + Gly-mediated ^{45}Ca influx into synaptoneurosomes. Each bar represents mean percentage of control values $\pm$ S.E.M. Abbreviations are: Control (C) of both the group untreated with NMDA/Gly and the group treated with NMDA/Gly, NMDA/Gly (N/G), amantadine (A), memantine (M) and MK-801 (MK). Statistical analysis was performed on raw data, with asterisks signifying significant inhibitory effect ($p < 0.05$) when compared to NMDA + Gly (if black bar is labelled) or vehicle-treated background (if white bar is labelled).

Because of the unique effect of MK-801 on background uptake Ca^{2+} influx, we characterized its potency in dose-response curves in the presence and absence of agonists (Fig. 4.4). MK-801 potently antagonized the NMDA-mediated ^{45}Ca influx, with the effect of NMDA completely blocked at about 5 μM . In contrast, antagonism of background flux in the absence of NMDA had a threshold near 10 μM , and

therefore was of lower potency than the effect on NMDA/Gly. Inhibition was maximal at about 80% of control in both cases, although solubility limits prevented us from testing higher concentrations. These results suggest that the high potency effect was on NMDA/Gly mediated Ca^{2+} uptake, with a low potency action on another site or process requiring higher concentrations of MK-801.

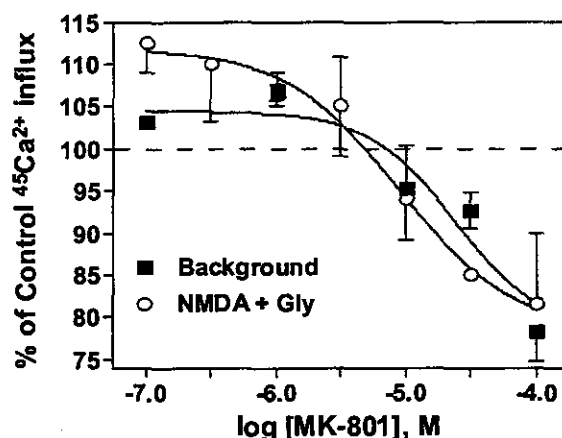


Figure 4.4. Dose-response curve of MK-801 in the absence (Background) and presence of agonists (100 μM). Results shown are the means (% of control) $\pm$ S.E.M. of 3 determinations, performed in triplicate. Dashed line is 100% of uptake referenced to untreated flux levels.

Dose-response curves were performed on selected compounds to determine IC_{50} values, Hill slopes, and goodness of fit or r^2 values (Table 4.1). All fittings of dose-response relationships to a sigmoid curve were good, with r^2 values of no less than 0.94 and typically near 0.99. MK-801 was the most potent compound, having an IC_{50} value near 1 μM in this assay. The other compounds fell into three potency ranges. Memantine and compounds **1** and **8** had a 3-fold lower potency. The other compounds were less active (IC_{50} s of 20 – 40 μM), with the following rank order of potency: **4b** = **9** > **3b** = **6** = **3c**. Hill slope values showed a trend related to inhibitory potency (Table 4.1). The Hill slopes for compounds with high affinity as NMDA antagonists (MK-801, memantine, and **1**) were close to unity, with lower potency compounds having higher Hill slope values.

Table 4.1. IC₅₀ values for selected compounds calculated from dose-response studies.

Compound	IC ₅₀ ± S.E.M. (μM)	Hill Slope	r ²
1	2.98 ± 1.0	-0.89	0.99
3b	32.78 ± 1.2	-4.62	0.99
3c	37.62 ± 1.0	-3.05	0.99
4b	20.65 ± 1.1	-1.97	0.99
6	36.22 ± 1.2	-1.21	0.99
8	4.06 ± 1.1	-0.40	0.94
9	23.5 ± 1.5	-1.74	0.97
MK-801	1.23 ± 1.4	-1.09	0.99
Memantine	3.05 ± 1.7	-1.03	0.99

Paired agonist dose-response curves for NMDA/Gly in the absence and presence of compound **1** are shown in Fig. 4.5. NMDA significantly ($p < 0.05$) increased the amount of ⁴⁵Ca taken up by the synaptoneurosomes. The threshold for NMDA-stimulated influx into synaptoneurosomes was approximately 10 μM and a maximal effect was observed at 1 mM. Higher concentrations of NMDA resulted in decreased uptake of ⁴⁵Ca (data not shown). Compound **1** was selected as representative of this group, as well as being one of the most potent polycyclic cage amines, to explore the mechanism of antagonism. The maximal effect of NMDA-mediated ⁴⁵Ca influx was depressed in the presence of **1** (2 μM) from 113.4% to 108.9% of control. The NMDA-curve shifted slightly to the right in the presence of **1**, although there was no statistical significance between the EC₅₀ of the NMDA dose-response curve in the presence (68 μM) and absence (49 μM) of **1**.

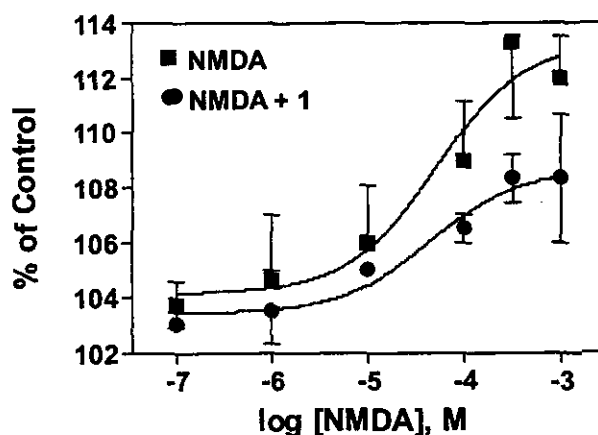


Figure 4.5. Concentration-response curve of NMDA in the absence and presence of 1 (2 μ M). Gly was kept constant at 100 μ M. Symbols are mean % of control $\pm$ S.E.M. of three determinations, performed three times. Where not visible, the error bars are contained within the size of the symbol.

4.4 Discussion

The $^{45}\text{Ca}^{2+}$ flux assay used in this study provided a convenient method for measuring effects of antagonists on NMDA receptor function with apparently little contribution from L-type calcium channels. $^{45}\text{Ca}^{2+}$ uptake stimulated by NMDA/Gly was sensitive to block by the classical antagonist MK-801 and had an agonist potency ($\text{EC}_{50} = 49 \mu\text{M}$) similar to that reported for mouse hippocampal microsacs (30 μM ; Daniell, 1991). When the experiments were done in the presence of 10 μM nitrendipine, only MK-801 showed a statistically significant inhibition of background $^{45}\text{Ca}^{2+}$ uptake, suggesting that this action did not contribute to the observed effects of the pentacycloundecylamines. Upon completion of these studies, we reinvestigated the literature and found several papers claiming a specific inhibitory effect of nitrendipine on NMDA receptors in cerebellar granule cells (Skeen *et al.* 1993), as well as inhibition by nitrendipine of MK-801 binding (Filloux *et al.*, 1994), an action not shared by other L-type channel blockers such as verapamil. Accordingly, we ran additional $^{45}\text{Ca}^{2+}$ uptake studies in the absence and presence of 10 μM nitrendipine or verapamil. We observed little or no effect of either compound on NMDA/Gly-stimulated $^{45}\text{Ca}^{2+}$ uptake compared to agonists alone (Fig. 4.6). We therefore conclude that the reported effects of nitrendipine on NMDA receptors had little impact on the results of the present study.

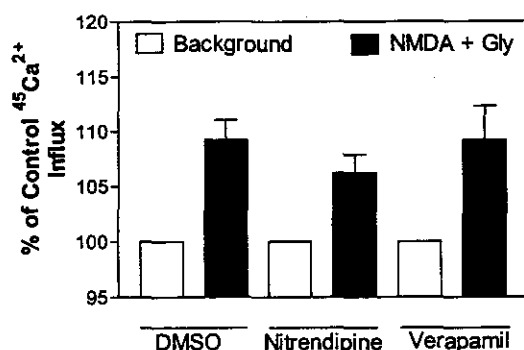


Figure 4.6. Effects of two calcium channel antagonists on NMDA + Gly (100 μM) stimulated $^{45}\text{Ca}^{2+}$ influx into murine whole brain synaptoneurosomes. Each bar represents the mean percentage of the control influx $\pm$ S.E.M. Vehicle control is with DMSO. Error bars not visible denote that S.E.M. resides within the printed bar line.

Dose-response studies done with MK-801 on background or NMDA/Gly-stimulated $^{45}\text{Ca}^{2+}$ uptake resulted in inhibition well below 100% of control at high concentrations (Fig. 4.3; Fig. 4.4). Thus, high concentrations of MK-801 are either blocking another route of calcium entry or stimulating efflux via an action at a different site. Among the established antagonists tested, MK-801 was by far the most potent antagonist of NMDA/Gly-specific $^{45}\text{Ca}^{2+}$ uptake, followed by memantine, and then amantadine, a rank order of potency that agrees with previous studies (Danysz *et al.*, 1997). The potency of MK-801 in the present study (IC_{50} ca. 1 μM) is greater than that observed by Daniell (1991), who reported that 100 μM MK-801 was required to block 50% of $^{45}\text{Ca}^{2+}$ uptake stimulated by NMDA/Gly in mouse hippocampal microsacs. We note that Daniell (1991) used only a single centrifugation step for harvesting synaptoneurosomes, whereas we found that two were required for obtaining consistent levels of background $^{45}\text{Ca}^{2+}$ uptake, as well as reliable estimation of antagonist activity (Fig. 4.2). This difference in technique may account for the differences in observed potency of MK-801 between these two studies.

Screening studies on the pentacycloundecylamines indicated they were effective antagonists of NMDA-mediated $^{45}\text{Ca}^{2+}$ influx into synaptoneurosomes. Structure-activity relationships for the pentacycloundecylamines appear to be determined primarily by geometric factors, with a small influence of electronic effects (Fig. 4.3; Table 4.1). The polycyclic cage amine seems to be the most important

pharmacophoric element required by the pentacycloundecylamines to interact with the NMDA receptor. The increased potency of **1** compared to **8** (Table 4.1), suggests that the phenyl ring in **1** adds to the interaction and therefore increases NMDA antagonism for pentacycloundecylamines. This was, however, not the case for the methylpyridine compounds, **5a-b**. The oxygen bridge atom, linking C-8 and C-11, does not seem to be important for an interaction with the NMDA receptor/ion channel complex, when comparing the activity of **8** to **1**. The length of the linker atoms between the amino and phenyl rings does play a role, since there was an 8-fold decrease in potency (Table 4.1) with the increase from a methyl (**1**) to ethyl (**9**).

Lower potencies were observed for compounds with substitutions on the phenyl ring (Table 4.1). These moieties might be too bulky for the “phenyl pocket” with steric hindrance causing reduced affinity and therefore reduced NMDA antagonism. However, the steric interaction seems to favour the *meta* position because the *para* substituted compounds were observed to have the lowest activity in the series of compounds with phenyl ring substituents (Fig. 4.3). Electronic effects impact on these phenyl substituents as well, since a lower IC₅₀ value (Table 4.1) was observed for compounds containing the electron donating methoxy moiety as opposed to compounds containing groups with electron withdrawing effects such as those with the nitro moiety. The donation of electrons makes the phenyl ring more lipophilic and would increase affinity for a π - π or hydrophobic interaction.

Compounds **6** and **7** were synthesized to investigate the effect of increased cage size, and both were of reduced effectiveness compared to **1** and **8**. Research done by Sobolevsky *et al.* (1999) indicated that the affinity of adamantane amines for the NMDA channel diminished with an increase in molecular size and was raised with an increase in hydrophobicity. The 12-fold higher experimental IC₅₀ value that was obtained for compound **6** (containing a larger cage moiety) than that obtained for **1** (smaller cage moiety), would thus be consistent with this, although the orientation of the phenyl side chain could also be an issue in this case. Fig. 4.7 shows the root mean square fitting of **1** and **6**, comparing the orientation of the side chains in these two compounds. For compound **6** the side chain is bent closer to the cage than for **1**. This might be due to intramolecular hydrogen bonding, making the atoms involved less able to form interactions with the NMDA ion channel.

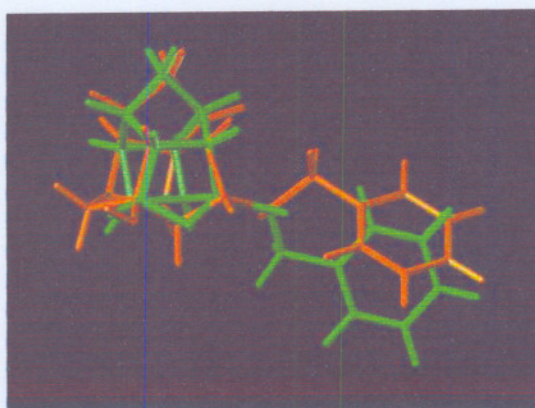


Figure 4.7. Overlay of **6** (orange) on **1** (green), indicating the energy-minimized positions of the phenyl side chains relative to the cage.

Log P calculations predict a lower Log P for **6** (1.78) than for **1** (2.57). It is expected that the larger cage would increase the hydrophobicity, but the free hydroxyl group in **6** apparently compensates for such increase in cage size. From the MOLCAD surface area maps of **1** and **6** (Fig. 4.8), there seem to be additional hydrogen bonding areas for **6**, which would account for this compound's lower calculated Log P, and thus lower activity on the NMDA channel compared to **1**. This result is similar to the finding of Sobolevsky *et al.* (1999), with diminished affinity for the NMDA channel associated with a decrease in hydrophobicity. This decrease in hydrophobicity, in conjunction with the increased molecular size, could explain the lower potency of **6** on the NMDA channel.

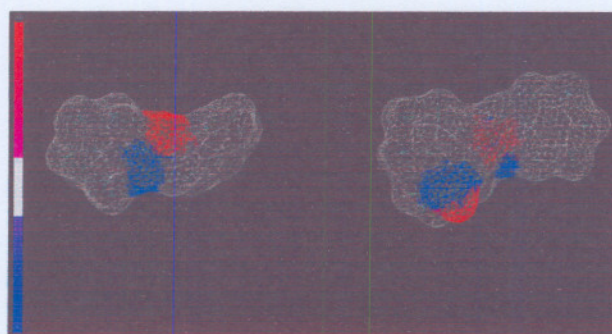


Figure 4.8. MOLCAD surface maps of **1** (left) and **6** (right) indicating hydrogen-bonding regions. Colour code indicates blue as high capacity for H-acceptor areas and red high capacity for H-donating areas.

Compound 1 reduced the maximal uptake of $^{45}\text{Ca}^{2+}$ stimulated by NMDA/Gly with little effect on potency (Fig. 4.5). This finding suggests noncompetitive antagonism as the mechanism of action of the pentacycloundecylamines. Previous studies of memantine and amantadine defined their mechanism of action as open channel block of the NMDA receptor (Dingledine *et al.*, 1999), but electrophysiological studies will be needed to confirm a similar mode of action for the pentacycloundecylamines. Hill slopes for compounds with high affinity as NMDA antagonists (MK-801, memantine, and 1) were near unity, suggesting that one molecule of the antagonist is necessary to block the NMDA-mediated influx of $^{45}\text{Ca}^{2+}$ through one NMDA channel. Compounds with higher Hill slopes suggest a positive cooperativity for these compounds (Gibb, 1996).

4.5 Conclusion

The presented pentacycloundecylamines represent a novel group of NMDA receptor antagonists. The non-competitive nature of the NMDA receptor antagonism, in addition to their L-type calcium channel blocking effects, suggests that these compounds might be useful as neuroprotective agents for the treatment of neurodegenerative disorders of the CNS. Further study is however needed to elucidate the exact mechanism of NMDA receptor complex block by the pentacycloundecylamines tested in this study, as well as the site they occupy on the NMDA receptor/ion channel complex.

4.6 Acknowledgments

The experiments were performed in the laboratory of Dr. J. R. Bloomquist, Neurotoxicology Laboratory, Dept. of Entomology, Virginia Tech, Blacksburg, VA, USA.

NMDA receptor binding – predictive modelling and radioligand binding studies

Chapter

5

5.1 Introduction

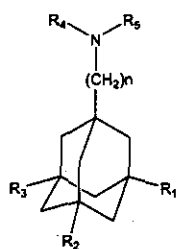
In the previous chapter it was described that NGP1-01 and its derivatives were found to antagonize NMDA-mediated $^{45}\text{Ca}^{2+}$ influx into murine whole brain synaptoneurosome. NGP1-01 (**1**) was found to be a noncompetitive antagonist, with an IC_{50} value of 2.9 μM , comparing favourably to that found for the reference compound memantine ($\text{IC}_{50} = 3.05 \mu\text{M}$).

One potential site for drug action is the phencyclidine (PCP) binding site located within the ion channel associated with the NMDA receptor. Uncompetitive NMDA antagonists such as memantine, MK-801, PCP, and ketamine are known to bind to this site (Grauert *et al.*, 1997; Kroemer *et al.*, 1998) and are suggested to be the most promising drugs to prevent events that most likely lead to excitotoxicity (Mattson, 2003).

Correlating the physicochemical properties of compounds with their biological activities is believed to provide a powerful tool in designing new ligands, therefore minimizing the number of compounds necessary to synthesize and screen pharmacologically (Cha *et al.*, 2003). In the absence of a crystal structure of the binding site for a drug, comparative molecular field analysis (CoMFA) is traditionally used to predict binding affinity of drugs to a binding site. CoMFA is one of the well known 3-D quantitative structure activity relationship (QSAR) techniques which have been used regularly to produce three dimensional models based on the assumption that each compound acts via a common binding site. In addition, comparative

molecular similarity index analysis (CoMSIA) further describes the hydrophobic and hydrophilic interactions of functional groups. We used these techniques because the crystal structure of the NMDA receptor remains to be determined (Kroemer *et al.*, 1998). To predict the binding affinity of the polycyclic cage amines used in this study, we utilized structurally similar adamantane amines (Table 5.1) to build the CoMFA contour maps. This CoMFA model can then be used to predict the binding affinity of novel compounds for the PCP binding site of the NMDA receptor, with the CoMSIA model giving further insight as to where functional groups for future compounds should be placed.

Table 5.1. Structures of the CoMFA and CoMSIA training set (Kroemer *et al.*, 1998).



Compound	R ₁	R ₂	R ₃	R ₄	R ₅	n
MRZ 2/16	H	H	H	H	H	1
MRZ 2/23	H	H	H	H	H	3
MRZ 2/24	H	H	H	H	H	4
MRZ 2/138	Me	Me	H	H	H	2
MRZ 2/150	Et	Et	H	H	H	0
MRZ 2/169	Me	Me	H	Me	H	0
MRZ 2/170	Me	Me	H	Me	Me	0
MRZ 2/171	Me	Me	H	Et	H	0
MRZ 2/174	H	H	H	H	H	2
MRZ 2/175	Et	H	H	H	H	0
MRZ 2/177	i-Pr	H	H	H	H	0
MRZ 2/180	C ₆ H ₅	H	H	H	H	0
MRZ 2/184	C ₆ H ₅	Et	H	H	H	0
MRZ 2/187	H	H	H	Me	Me	0
MRZ 2/188	H	H	H	Me	H	0
memantine	Me	Me	H	H	H	0
amantadine	H	H	H	H	H	0

5.2 Materials and Methods

5.2.1 Structure building

The structures used in the study were built using SYBYL 6.9 (Tripos, St Louis, MO) molecular modelling software on a silicon graphics Octane computer. All the molecules were energy minimized for the CoMFA and CoMSIA analysis using the standard Tripos molecular mechanics force field. Gasteiger-Huckel charges were applied, with a 0.005 kcal/mol energy gradient convergence criterion.

5.2.2 Molecular alignment

All of the adamantane amines (Table 5.1) were entered into a database and then aligned using the ALIGN DATABASE function in SYBYL. The polycyclic cage of amantadine was used as the reference template.

5.2.3 CoMFA and CoMSIA Studies

The CoMFA analysis was carried out using the standard Sybyl/QSAR routine. The results of this analysis are presented in Figure 5.1-5.4 and in Tables 5.2 and 5.3. The CoMFA grid spacing was 2.0 Å in the X, Y and Z directions, and the grid region was automatically generated by the CoMFA routine to encompass all molecules with an extension of 4.0 Å in each direction. A sp^3 carbon for sterics and a charge of +1.0 for electrostatics were used as probes to generate the interaction energies at each lattice point, and the default energy cut-off was 30 kcal/mol. Partial least squares (PLS) regression analysis was performed from the resulting field. Cross-validated and non-cross validated PLS analysis were performed.

5.2.4 Radioligand Binding Assay

Procedures similar to those in published studies were used to measure [^3H]MK-801 (Filloux *et al.*, 1994) and [^3H]TCP (Vignon *et al.*, 1983) binding. Murine synaptoneurosomes were prepared as described for the $^{45}Ca^{2+}$ functional assay (Chapter 4). The reaction mixture consisted of 380 μ l of membrane preparation

containing, typically, 0.38 mg protein and [^3H]-MK-801 (5 nM) or [^3H]-TCP (5 nM) as radioligand. Unlabeled MK-801 and other test compounds were applied at 100 μM final concentration and were dissolved in DMSO. The reaction was initiated by the addition of 20 μl incubation buffer containing NMDA (100 μM) and Gly (100 μM) to the membrane preparation. Incubation of the mixture was for 1 h at 25 $^{\circ}\text{C}$. The reaction was terminated by the addition of ice cold wash buffer and filtration on Whatman GF/B filters. Filters were pretreated with polyethyleneimine (0.05%) (Vignon *et al.*, 1983) for [^3H]-TCP binding. Following air drying, the filters were placed into scintillation liquid and radioactivity retained on the filters was determined by liquid scintillation counting.

5.3 Results

The optimized CoMFA of the 17 adamantane amines gave a good non-cross validation with the optimum number of components of 6 and $r^2 = 0.976$, (Fig. 5.2; Table 5.2). The relative steric and electrostatic contributions were 0.467 and 0.533 respectively, indicating almost equal importance of both for designing future compounds. These relative contributions were represented as a 3D coefficient map (Fig. 5.3) with the favoured 80% steric (in green) and electrostatic (in blue) effects and the 20% disfavoured steric (in yellow) and electrostatic (in red) effects. The green coloured part of the map indicates that a sterically bulky group at that position could result in enhancement of the PCP binding affinity. The blue colour indicates a region where more electronegative groups could result in better binding affinity. Using this model, we used the PREDICT PROPERTY in the QSAR module and predicted the binding affinity of the polycyclic cage compounds to be in the low micromolar range. The model predicted (Table 5.3) the binding affinity to be in the same low μM range as was found experimentally for experimental $^{45}\text{Ca}^{2+}$ flux assay data (Chapter 4).

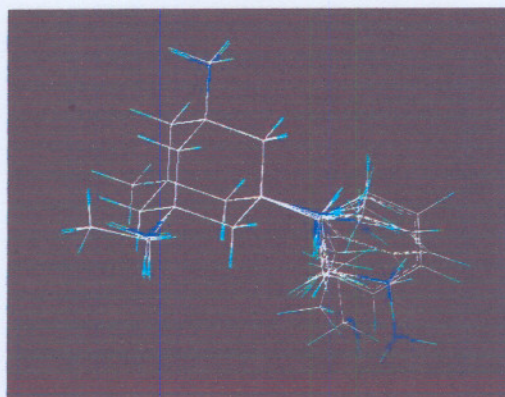


Figure 5.1. Superposition of 17 adamantane amines for CoMFA studies.

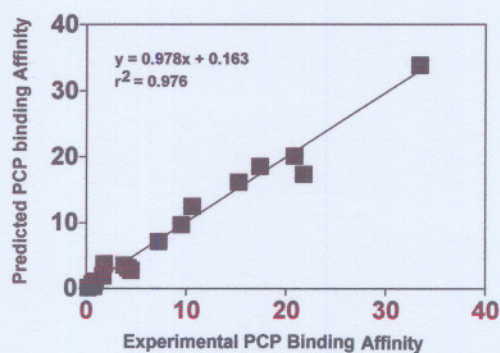


Figure 5.2. Experimental PCP binding affinities versus predicted affinity for the CoMFA model of the training set (adamantane amines).

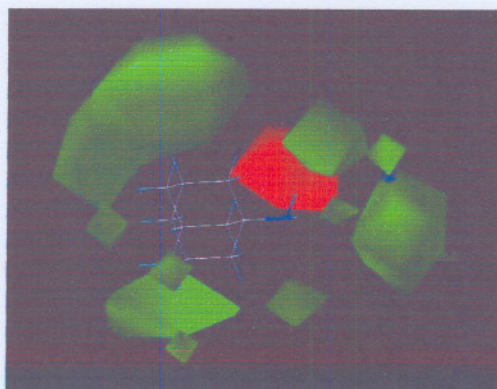


Figure 5.3. CoMFA contour map of the training set. Sterically favoured/disfavoured areas are shown in green/yellow colour. Electrostatic favoured/disfavoured areas are shown blue/red colour. Amantadine is shown docked.

Table 5.2. Experimental and predicted binding affinity (μM) for the PCP binding site.

Compound	Experimental K_i (μM) ^a	Predicted K_i
MRZ 2/16	33.3	34.0
MRZ 2/23	9.50	9.82
MRZ 2/24	17.4	18.5
MRZ 2/138	0.79	1.2
MRZ 2/150	0.19	0.5
MRZ 2/169	1.61	2.0
MRZ 2/170	4.44	2.7
MRZ 2/171	1.72	3.7
MRZ 2/174	3.80	3.6
MRZ 2/175	0.60	0.2
MRZ 2/177	7.21	7.1
MRZ 2/180	15.2	16.1
MRZ 2/184	4.08	3.1
MRZ 2/187	20.8	20.1
MRZ 2/188	21.7	17.4
memantine	0.54	0.6
amantadine	10.5	12.6

^a as reported previously in postmortem human brain tissue
(Kroemer *et al.*, 1998)

Table 5.3. Predicted PCP binding affinities (μM) of selected polycyclic cage amines compared to experimental $^{45}\text{Ca}^{2+}$ flux assay data (Chapter 4).

Compound	Predicted PCP binding affinity (Ki)
1	4.5
3b	15.7
4b	-1.8
6	17.8
8	21.7
9	21.2

To estimate the hydrophobic contributions of the adamantane amines at the PCP binding site, hydrophobic similarity index fields were constructed by use of the standard CoMSIA option in Sybyl (Fig. 5.4). This was done to give additional insight

regarding functional group placement. The CoMSIA model gave an r^2 of 0.884 with 6 components. QSAR analysis done previously on the PCP binding site with adamantane amines, suggested that compounds fit into a common lipophilic area and that compounds with a bulky hydrophobic moiety appear to fit better into this area. The relative contributions were represented as a 3D map with hydrophobic favoured areas (in yellow) indicating where hydrophobic substitutions are preferable to increase potency and disfavoured areas (in white) indicating where hydrophilic substituents would be beneficial to binding affinity.

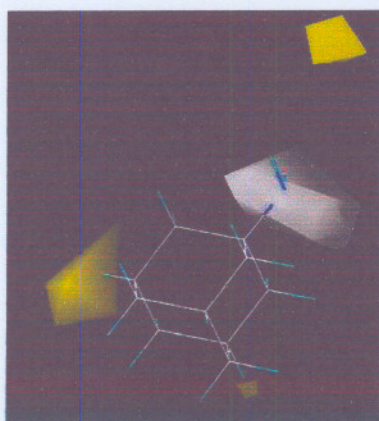


Figure 5.4. CoMSIA contour map with yellow contours where hydrophobic substituents are favoured and white contours where hydrophilic substituents are favoured. Amantadine is shown docked.

Figure 5.5 shows the results of binding studies done with [^3H]MK-801 and [^3H]TCP. The reference compounds memantine and MK-801, displaced 90% and 73%, respectively, of [^3H]MK-801 binding. On the other hand, the pentacycloundecylamines (**1**, **8** and **9**) at 100 μM did not displace bound [^3H]MK-801 significantly. Memantine and MK-801 displaced [^3H]TCP binding markedly (67% and 65%, respectively). Compound **1** and **9** displaced [^3H]TCP weakly (20% and 44%, respectively), but were significantly different from the control and each other, while **8** caused no displacement of [^3H]TCP. In addition, no displacement by **1** and **8** were found for [^3H]glutamate binding to synaptoneurosomes (data not shown).

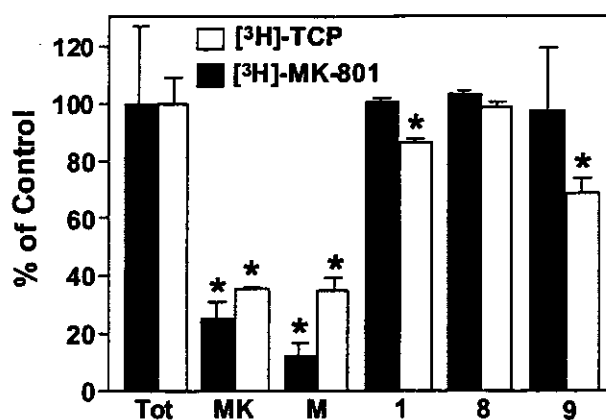


Figure 5.5. Competition binding of compounds 1, 8 and 9 with [³H]MK-801 and [³H]TCP. Values are expressed as % of control $\pm$ S.E.M. of 2-5 separate experiments. Abbreviations are: Total binding (Tot); or binding in the presence of cold MK-801 (MK), memantine (M), and compounds 1, 8 and 9, all at 100 μ M. Statistical significance compared to the total binding in a t-test, * $P < 0.05$.

5.4 Discussion

A 3D-QSAR analysis was performed on a set of adamantane amines for PCP binding affinity in the NMDA receptor, using previously published PCP binding data. This was done in part to support future synthesis and pharmacological evaluation of the parent compound, NGP1-01 (1) as well as other polycyclic cage amines as possible NMDA receptor antagonists. A satisfactory CoMFA model (Fig. 5.3) was obtained with a non-cross validated r^2 value of 0.976 and a CoMSIA model (Fig. 5.4) with $r^2 = 0.884$. The model predicted the binding affinity of the tested compounds to be in the low μ M range (Tab. 5.3).

Binding studies with [³H]-MK-801 and [³H]-TCP were used in an attempt to identify the binding site of the pentacycloundecylamines experimentally (Fig. 5.5). Compounds 1, 8 and 9 were chosen for these assays, due to their potent antagonism of NMDA stimulated ⁴⁵Ca²⁺ influx into murine synaptoneurosomes (Chapter 4). Since memantine and the pentacycloundecylamines used in this study share a similar polycyclic cage amine template, we speculated that these compounds would interact with the PCP binding site within the ion channel of the NMDA receptor. In addition, compound 1 exhibited noncompetitive antagonism in the functional ⁴⁵Ca²⁺ flux assay, which further suggests a mode of action (uncompetitive antagonism) similar to MK-

801, PCP, and memantine (Dingledine *et al.*, 1999). The binding studies with [^{3} H]MK801 and [^{3} H]TCP indicates that these compounds do not bind to the MK-801 binding site and only weakly to the TCP binding site at 100 μ M. This is an exciting finding, since it appears that the pentacycloundecylamines interact with a different, as yet undiscovered binding site in the NMDA receptor/ion channel complex.

One possible explanation for the functional antagonism of the cage compounds (except for **8**), is that the phenyl ring undergoes a π - π aromatic interaction with (an) aromatic amino acid(s) located at the entrance of the NMDA receptor channel (Fig. 5.6). This anchors the molecule in such a way that the cage can “dangle” into the channel lumen at a depth allowed by the “spacer” between the nitrogen and the phenyl ring. The significantly greater activity of **9** for displacing [^{3} H]TCP binding would arise from its longer ethyl linkage, compared to the methyl spacer in **1** and **8**. The blocking action of compound **8**, without any amino-substitution, may be different from the compounds with a phenyl side chain, and its mechanism requires further investigation.

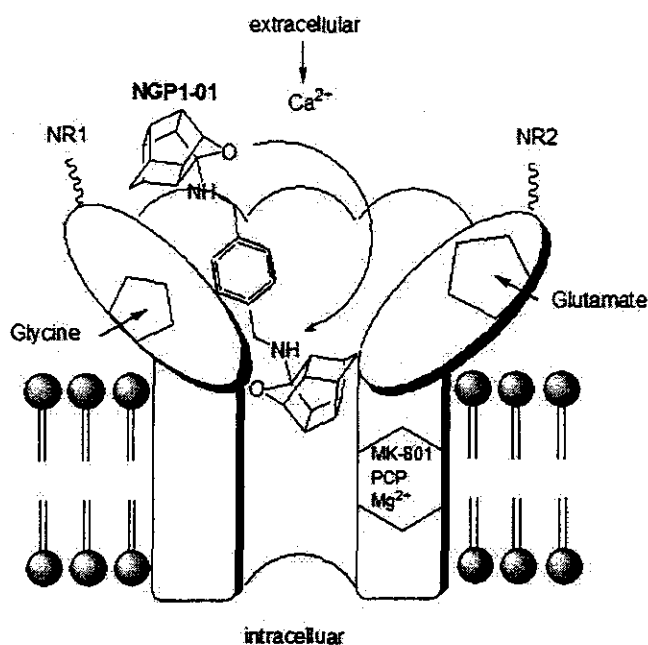


Figure 5.6. Schematic representation of possible mechanism of NMDA antagonism by NGP1-01 (**1**).

The binding results in this study are similar to that found for a set of azapentacycloundecylamines studied by Kassiou *et al.* (1996). They investigated sigma opiate receptor and PCP binding for a set of pentacycloundecylamines based on amantadine's interaction with the sigma binding site (Kornhuber *et al.*, 1993). These compounds included aza-bridgehead compounds, in contrast to the oxa-bridgehead compounds used in the present study. These authors found high selectivity for sigma binding sites, with no cross-reactivity with the PCP site ($K_i > 10 \mu\text{M}$).

Although binding data suggests little interaction with the PCP binding site, the pentacycloundecylamines studied here were functional antagonists of the NMDA receptor, highlighting the importance of functional assay screens.

5.5 Conclusion

We developed a 3D-QSAR model to predict the binding affinity for the cage compounds used in this study. The experimental data indicated that these compounds do not bind to the PCP binding site and therefore the model could not be validated in this paradigm. However, it does appear that these compounds bind at a novel binding site within the NMDA channel pore. Because compound 9 showed slightly more binding than 1, by displacing [^3H]TCP, future studies should focus on evaluating compounds with an increased linker chain between the cage and the phenyl moiety to probe this putative new binding site. The mechanism of antagonism by these compounds thus requires further investigation.

5.6 Acknowledgements

The authors gratefully acknowledge the use of the Sybyl 6.9 (Tripos) program of Dr. F. Etzkorn at the Department of Chemistry, Virginia Tech, Blacksburg USA. The remainder of the experiments were performed in the laboratory of Dr. J. R. Bloomquist, Neurotoxicology Laboratory, Dept. of Entomology, Virginia Tech, Blacksburg, VA, USA.

Effect of NGP1-01 on restoring behaviour in the MPTP mouse model

Chapter

6

6.1 Introduction

Parkinson's disease (PD) is a debilitating age-related progressive neurodegenerative disease, which produces symptoms of resting tremor, akinesia and rigidity (Przedborski & Vila, 2001). The symptoms of PD are due to the selective loss of dopaminergic (DA-ergic) neurons in the substantia nigra (Marino *et al.*, 2003; Speciale, 2002; Castagnoli *et al.*, 2001).

Evaluation of the functional circuitry of the basal ganglia, show the neurotransmitters involved in PD (Fig. 6.1). During PD, there is a degeneration of the pars compacta of the substantia nigra, which results in an overactive indirect pathway and increased glutamatergic activity by the subthalamic nucleus. By decreasing the glutamatergic activity i.e. by the use of glutamatergic antagonists, at the substantia nigra (pars reticulata), balance may be restored between the direct and indirect pathways (Marino *et al.*, 2003). Previous studies support the use of *N*-methyl-D-aspartate (NMDA) receptor antagonists (such as amantadine and memantine) alone (Lange *et al.*, 1997; Marino *et al.*, 2003), or as adjunct therapy together with L-dopa in PD (Lange *et al.*, 1997; Archer *et al.*, 2002).

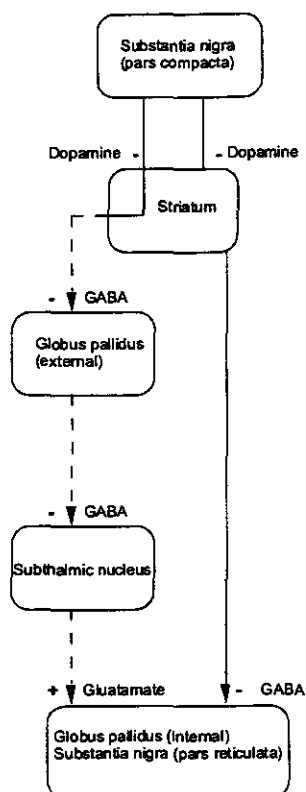


Figure 6.1. A simplified schematic representation of basal ganglia circuitry (Aminoff, 1998). The direct pathway is shown with a line, and indirect pathway with a dashed line. Symbols indicate stimulation (+) and inhibition (-).

Earlier in this study NGP1-01 (1) was found to exhibit noncompetitive NMDA antagonism ($IC_{50} = 2.9 \mu M$) against NMDA-mediated $^{45}Ca^{2+}$ influx into murine whole brain synaptoneurosome, *in vitro* (Chapter 4). In addition, NGP1-01 (1) and its derivatives have been evaluated previously for neuroprotective activity in the MPTP parkinsonian mouse model (Geldenhuys *et al.*, 2003). Mice treated with NGP1-01 (1), together with MPTP, showed neuroprotection reflected by increased levels of striatal dopamine (DA) compared with mice treated with MPTP alone, although this increase was found not to be statistically significant. Further investigation found that NGP1-01 (1) inhibits DA uptake with an IC_{50} value of $57 \mu M$ in murine synaptosomes (Chapter 3).

With the discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can be used to induce a parkinsonian syndrome in laboratory animals, MPTP animal models of PD became available to evaluate new treatments for PD (Marino *et al.*, 2003;

Castagnoli *et al.*, 2001; Speciale, 2002; Sedelis *et al.*, 2001). The neuropathological features of the MPTP-parkinsonian mouse model are similar to idiopathic PD in that there is a dose-dependent decrease in striatal DA, a decrease in striatal DA terminals, respiratory inhibition, and an increase of α -synuclein expression in surviving neurons (Przedborski & Vila, 2001; Speciale, 2002; Tillerson *et al.*, 2002). Although it is generally difficult to correlate DA depletion and behaviour in mice, Sedelis *et al.* (2001) and Tillerson *et al.* (2002), described a method to determine persistent and significant MPTP-induced behavioral deficits in retired breeder C57BL/6 mice. These researchers found that the measurement of the front paw stride length in mice demonstrated a dose-dependent deficit following MPTP injections, with significant improvement after treatment with L-Dopa.

Taking the above into account, we proceeded, in a pilot study, to evaluate the effect of NGP1-01 (1) in restoring normal behaviour in the MPTP-parkinsonian mouse model, possibly by ameliorating the glutamatergic overdrive in the indirect pathway of the basal ganglia circuitry.

6.2 Materials and Methods

6.2.1 Animals

Retired breeder (ca. 18 months old) C57BL/6 male mice (Harlan) were used for these experiments. Animals were housed in separate cages and given access ad libitum to standard animal chow and water. All experiments were performed during the animals' dark cycle.

6.2.2 Biochemical assessment of MPTP toxicity

6.2.2.1 HPLC Analysis

The HPLC method of Jussofie *et al.* (1993) was used to determine DA and its metabolite (DOPAC and HVA) levels in the striata of the animals. The right striatum of each mouse was taken for HPLC analysis. After dissection, the striatum was weighed, and homogenized in the mobile phase. The mobile phase consisted of 0.1 M

sodium acetate, 25 mM citric acid, 134 μ M ethylenediaminetetraacetic acid (EDTA), 6% (v/v) methanol and 230 μ M octanesulfonic acid. When this mobile phase was used during the homogenization process, L-isoprenaline (1 μ M) was added as internal standard. Homogenates were centrifuged for 15 min at $10\,000 \times g$. The supernatant was then removed and stored at $-70\text{ }^{\circ}\text{C}$ until the day of analysis. A Macherey-Nagel ODS Nucleosill 100 250 x 4 mm column was used with electrochemical detection at 0.35 V.

6.2.2.2 Western Blot (WB) Analysis

Striatal synaptosomes from treated C57BL/6 mice were prepared as follows. Mice were sacrificed by cervical dislocation and the striata were rapidly removed, with the right side used for DA analysis, and the left side used for Western blotting. The striatal tissue was homogenized in 4 ml of sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4). The homogenate was then centrifuged for 10 min at $1000 \times g$. The supernatant was removed and centrifuged for 15 min at $10,000 \times g$. The final membrane pellets were resuspended in KRH buffer (NaCl: 118 mM, KCl: 4.8 mM, CaCl_2 : 2.5 mM, MgSO_4 : 1.2 mM, HEPES: 20 mM; pH: 7.5), stored at $-70\text{ }^{\circ}\text{C}$, and thawed at the time of assay. The thawed tissue was mixed with 25% of sample buffer (Tris: 50 mM, SDS: 2%, DTT: 100mM, bromophenol blue: 0.001%; pH: 6.8) and heated at $80\text{ }^{\circ}\text{C}$ for 5 minutes. 10% SDS gel was used for the analysis of the DA transporter (DAT) and tyrosine hydroxylase (TH) analysis. A sample of 10 μ g and 5 μ g protein was loaded for DAT and TH, respectively. SDS gel was run at 150 V for 1 hour. The resulting protein was transferred to a nitrocellulose membrane, and the complete transfer was verified by Ponceau S staining. The membrane was incubated in non-fat milk (5%) at room temperature for 1 hour, followed by incubation with either rat monoclonal anti-DAT or mouse monoclonal anti-TH at $4\text{ }^{\circ}\text{C}$. After overnight incubation, the membrane was washed with TBST buffer (Tris: 20 mM, NaCl: 137 mM; pH: 7.6), followed by a 1 hr incubation with peroxidase-linked secondary antibody (Sigma Chemical Co.) at room temperature. After washing three times with TBST buffer for 5 min each time, the membrane was developed using the ECL Amersham Chemiluminescence kit and exposed to ECL hyperfilm for an

appropriate time. After washing with water and air drying, the protein content on the film was quantitated using a Kodak EDAS 290 system.

6.2.3 Forepaw Stride Length

The method for measuring the forepaw stride length during walking (Fig. 6.2) was adapted from Tillerson *et al.* (2002). In short, the front paws of the mice were dipped in blue ink and the animal allowed to walk through a PVC tube, 10 cm in diameter and 40 cm long, lined with standard photocopier paper. The PVC tube was elevated at a 5° angle. The mice were immediately returned to their cages upon completion of the task. Stride lengths of mice were determined by measuring the distance between each step on the same side of the body, measuring from the middle toe of the first step to the heel of the second step. The mean of three such step measurements was calculated for each mouse, and five mice comprised a treatment group.

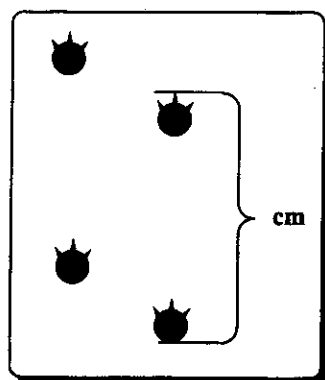


Figure 6.2. Cartoon of the forepaw stride length in C57BL/6 mice measured in this study.

6.2.4 Catalepsy

Mice were tested for catalepsy according to the method of Miller *et al.* (1991). The front paws of a mouse were placed on a 2.5 cm high block and the time it took for the mouse to move both front legs off the block was recorded.

6.2.5 Bradykinesia

The pole test adapted from Ogawa *et al.* (1987) was used to determine the degree of bradykinesia. Mice were placed on the top of a pole (1 cm in diameter and 40 cm in height). The time it took for the mouse to climb down to the base of the pole was recorded, and if a mouse fell at any point, it was recorded as a fall. The same test was repeated three times with each mouse. Mice were tested with the pole test pre-NGP1-01 (1) injection (14 days after MPTP injection), 30 min later, after NGP1-01 (1) injection and again 12 hours after NGP1-01 (1) injection. Mice were not tested before MPTP injections, because we have found a learning curve for the mice in accomplishing the task.

6.2.6 Movement and rearing

The mice were placed in an open-field apparatus for two minutes, where the floor was demarcated into a 3×2 matrix. Each time the mouse entered a square (documented when the two front paws crossed into a new block) was counted as one movement. Rearing was counted each time the mouse stood on its hind paws and not grooming (whereas grooming was defined when the mouse was rubbing its front paws over its snout).

6.2.7 Experimental Design

A schematic representation of the experimental design is given in Figure 6.3. The animals were divided randomly into three groups of 5 mice each. On day 1, mice in the control (C) and NGP1-01-only (N) groups were treated with saline (100 μ l ip) injection, while the MPTP (M) group was injected (ip) with MPTP (1 $\times$ 30 mg/kg in 100 μ l sterile saline) (Sigma). On day 14 post-injection of MPTP, control mice received triethylene glycol dimethyl ether (MTG) (10 ml ip), the NGP1-01 group as well as the MPTP group received 1 $\times$ 20 mg/kg NGP1-01 (10 μ l ip) dissolved in MTG. Behavioural tests were performed on the mice on day 1, before receiving any treatments and again on day 14, before receiving any treatments, 30 min after MTG vehicle and NGP1-01 injection, and again 12 hours after the NGP1-01 or vehicle injection. Mice were sacrificed on day 15 after MPTP injection for biochemical

assessment of the MPTP effect on DA and the dopamine transporter (DAT). The two striata were excised, with the right side used for DA analysis, and the left side used for DAT Western blotting. The mice treated with MPTP and then NGP1-01 served as their own control, since the NGP1-01 would either improve behaviour or not.

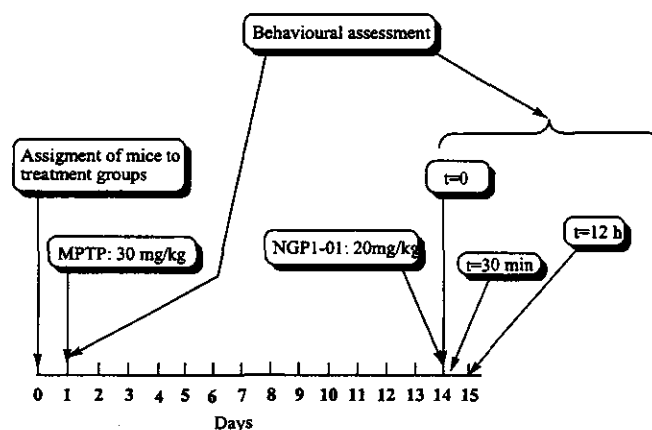


Figure 6.3. Experimental design timeline for the behavioural study of the MPTP mice. See text for further explanation.

6.3 Results

6.3.1 Biochemical assessment of MPTP toxicity

To confirm/verify whether the MPTP treatment reduced striatal DA levels, HPLC analysis (Table 6.1) was used to determine the striatal DA levels in the mice. In addition, WB (Fig. 6.4) of DAT was also done to confirm that the DA-ergic neurons in the striatum were reduced due to the MPTP treatment. MPTP caused a significant ($P < 0.05$) decrease in striatal DA levels of about 70% in mice treated with MPTP, as compared to mice treated with vehicle alone. This decrease was also seen with the metabolites of DA indicating that there was significant loss of DA-ergic neurons two weeks after MPTP injection.

Table 6.1. HPLC with electrochemical detection of DA and its metabolites in the three groups of animals studied. Mean (pmol/mg wet tissue) $\pm$ standard deviation (S.D.) of DA and its metabolites from one striatum. Statistical significance ($P < 0.05$) indicated with asterisk (*), compared to control. $n = 5$ mice per group, with one striatum per mouse.

	DA	DOPAC	HVA
Control (Vehicle)	95.65 $\pm$ 7.52	4.79 $\pm$ 0.93	7.45 $\pm$ 0.956
NGP	96.26 $\pm$ 14.27	5.06 $\pm$ 1.09	4.68 $\pm$ 1.23*
MPTP	27.87 $\pm$ 13.09 *	1.07 $\pm$ 1.07*	2.58 $\pm$ 1.19*

The WB analysis indicated a decrease in the amount of DAT expressed following MPTP toxicity and death of DA neurons. MPTP treatment (30 mg/kg) resulted in significantly decreased ($P < 0.05$) DAT expression compared to control mice. Mice treated with NGP1-01 alone on day 14 (20 mg/kg), showed a slight increase in DAT expression ($P < 0.05$).

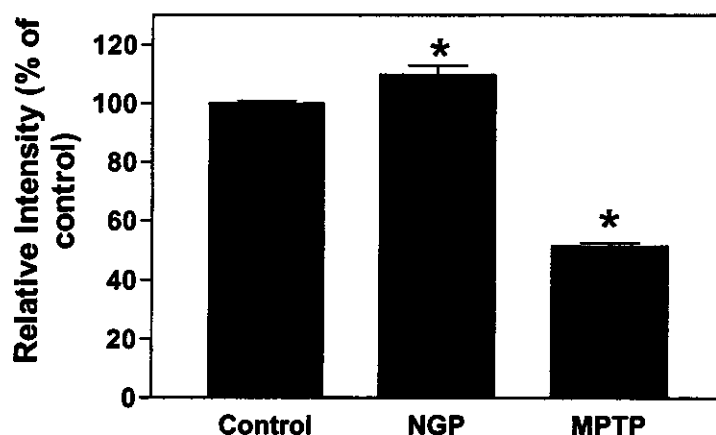


Figure 6.4. Western Blot data of DAT expression represented as relative intensity (% of control). Abbreviations are: mice receiving only NGP1-01 (NGP) on day 14, mice treated with MPTP (MPTP) for 14 days. $n = 5$, with one striatum per mouse. Statistical significance ($P < 0.05$) indicated with asterisk (*), compared to vehicle control.

6.3.2 Forepaw stride length

The results of the forepaw stride length measurements are shown in Fig. 6.5. No statistically significant differences were seen in the decrease of the fore paw stride length on day 14 in the mice treated with MPTP as compared to the vehicle control mice. The forepaw stride length was decreased in MPTP-treated mice 30 min after NGP1-01 injection. After 12 hours, the forepaw stride length of mice treated with MPTP and NGP1-01, returned to that of the vehicle control. A similar pattern of the stride lengths was seen in the animals treated with vehicle on day 1 and NGP1-01 on day 14, but not for the vehicle only treated group.

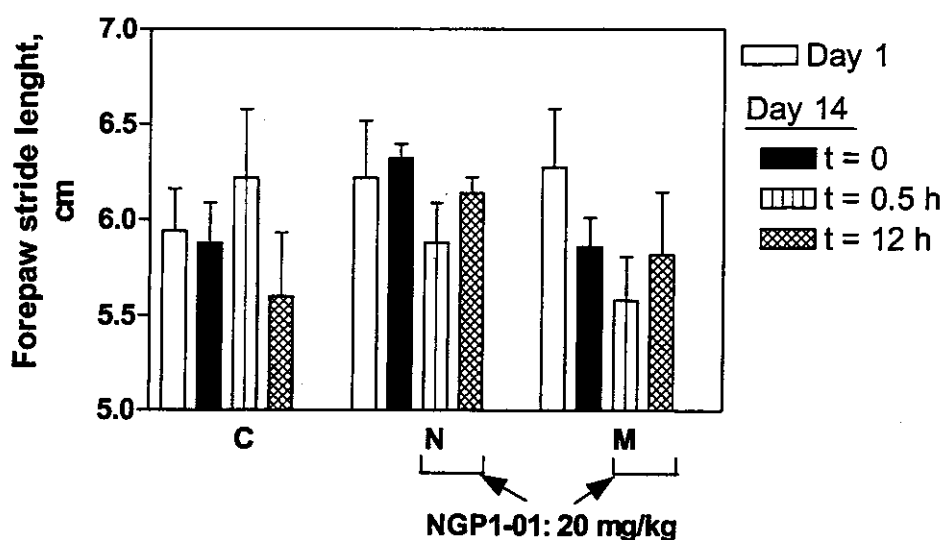


Figure 6.5. Forepaw stride lengths of C57BL/6 mice. Mice were observed on day 14 ($t = 0$) after either vehicle (C and N) and MPTP (M) were injected (day 1) and then injected with either drug (NGP1-01 in the N and M groups) or vehicle (C). The mice were then re-evaluated 30 min ($t = 30$) and 12 hours ($t = 12$ h) later. Abbreviations are: vehicle control (C), NGP1-01 (N) and M (MPTP).

6.3.3 Catalepsy

The three groups were evaluated on day 14, after vehicle or MPTP was injected, and the results are shown in Fig. 6.6. In the catalepsy experiment, no change was seen in the time it took for mice to take their front paws off the block in the MPTP treated animals. A small but not statistically significant decrease was seen in the time for

mice to take their paws off the block 30 min after NGP1-01 injection in the MPTP group, but a significant increase in the catalepsy time was seen in the MPTP group 12 hours after NGP1-01 injection ($P < 0.05$), compared to the MPTP group before NGP1-01 injection. In the NGP1-01-only group, there was also a small increase 30 min after NGP1-01 injection, which remained increased 12 hours after NGP1-01 injection.

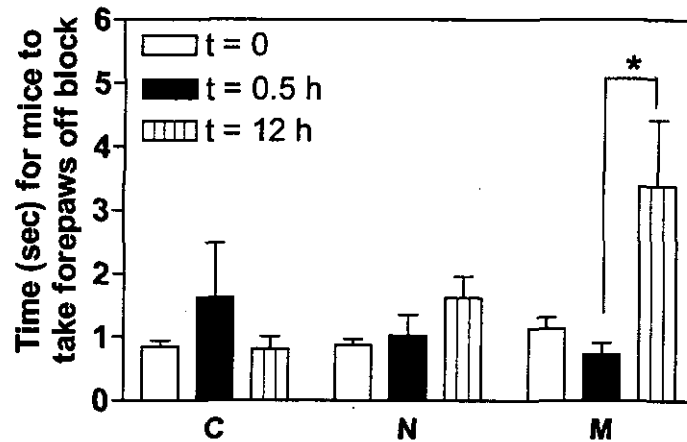


Figure 6.6. Test for catalepsy in C57BL/6 mice by timing mice taking their forepaws of a block. Mice were observed on day 14 ($t = 0$) after either vehicle (C and N) and MPTP (M) were injected (day 1) and then injected with either drug or vehicle. The mice were then re-evaluated 30 min ($t = 30$) and 12 hours ($t = 12$ h) later. Statistical significance ($P < 0.05$) indicated with *, compared to MPTP mice evaluated 30 min after NGP1-01 injection. Abbreviations are: vehicle control (C), NGP1-01 (N) and M (MPTP).

6.3.4 Bradykinesia

Results of the pole test (Fig. 6.7) are shown as a percentage % ratio of falls/successful descents down the pole, in the mice when observed on day 14. Mice treated with MPTP showed an increased incidence of falling while climbing down the pole ($P < 0.05$) when compared to the untreated (vehicle) mice. Mice treated with on day 1 with vehicle and then on day 14 with NGP1-01 tended to fall less 30 min after being treated with NGP1-01, as well as 12 hours later. In mice treated with MPTP on day 1 and then with NGP1-01 on day 14, the incidence of falling decreased 30 min after

NGP1-01 injection ($P < 0.05$), in this group, as compared to before NGP1-01 injection was injected. This improvement remained 12 hours after NGP1-01 injection.

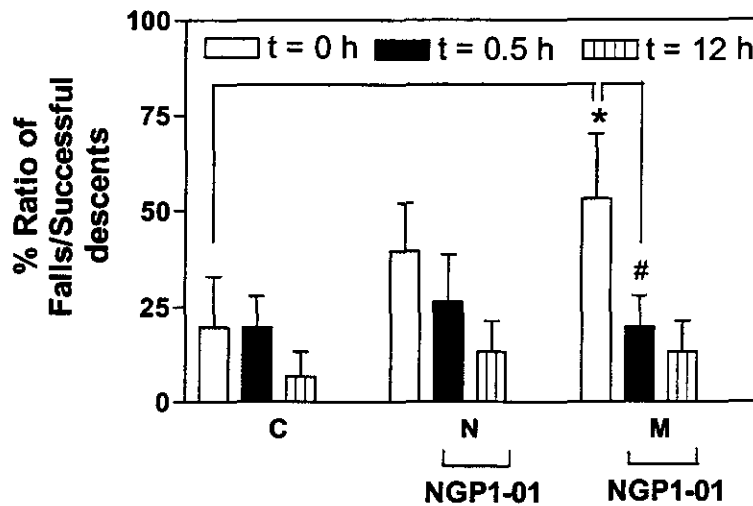


Figure 6.7. Results of pole test (bradykinesia test) in C57BL6 mice shown as a percentage % ratio of falls/successful descents down the pole. Mice were observed on day 14 ($t = 0$) after either vehicle (C and N) and MPTP (M) and then injected with either drug (NGP1-01 in the N and M groups) or vehicle (C). The mice were then re-evaluated 30 min ($t = 30$) and 12 hours ($t = 12$ h) later. Statistical significance ($P < 0.05$) indicated with *, compared to control, and # 30 min after NGP1-01 treatment, compared to MPTP treated mice. Abbreviations are: vehicle control (C), NGP1-01 (N) and M (MPTP).

6.3.5 Movement and Rearing

The results of the rearing and movement observations are given in Fig. 6.8. On day 1 the mice in all three treatment groups showed the same behaviour. Fourteen days later, a decrease in the both movement and rearing was seen in the vehicle treated mice ($P < 0.05$). The same decrease in behaviour was seen in the group treated with vehicle on day 1 and NGP1-01 on day 14 (Fig. 6.8 B), as for the group treated with MPTP on day 1 and then with NGP1-01 on day 14 (Fig. 6.8 C). Generally, the mice in the vehicle treated group showed less rearing and movement on day 14, than the other two groups. Mice treated with MPTP on day 1 and then with NGP1-01 on day 14, showed similar rearing as the vehicle group 30 min after the NGP1-01 treatment.

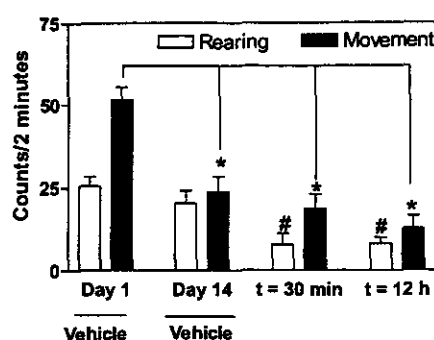
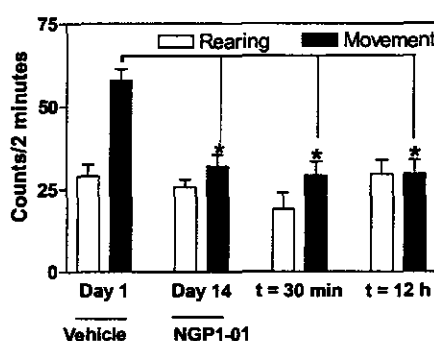
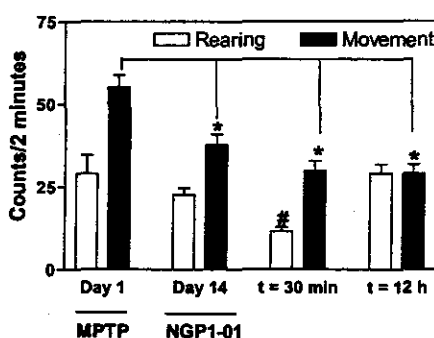
(A) Vehicle control**(B) NGP1-01 only****(C) NGP1-01
treatment of MPTP
mice**

Figure 6.8. Results of the rearing and movement observations in the C57BL/6 mice. Three groups were observed: (A) mice treated with vehicle alone on day 1 and 14; (B) mice treated with vehicle on day 1 and NGP1-01 (20 mg/kg) on day 14; (C) mice treated with MPTP (30 mg/kg) on day 1 and with NGP1-01 (20 mg/kg) on day 14. Mice were observed on day 1 (first treatment) and again on day 14 (second treatment), then 30 min after the second treatment ($t = 30 \text{ min}$) and again 12 hours later after the second treatment ($t = 12 \text{ h}$). *Statistical significance $P < 0.05$ compared to day 1 movement; # $P < 0.05$ compared to day 1 rearing.

6.4 Discussion

Glutamate seems to play an important role in PD, both in the pathophysiology and neurodegeneration (e.g. by means of excitotoxicity) of the disease (Marino *et al.*, 2003; Lange *et al.*, 1997). The resulting loss of DA-ergic tone in the direct pathway of the basal ganglia circuitry is accompanied by excessive basal ganglia efflux. Overstimulation in the indirect pathway results in higher inhibition of the thalamus (Lange *et al.*, 1997). In PD, the tonic inhibition of the basal ganglia output structures would therefore be exacerbated, which may account for the slowness of movement associated with PD.

Antagonists of NMDA receptors have shown promise in PD therapy in both preclinical and clinical studies (Marino *et al.*, 2003). The restorative effects of NMDA antagonists also seem to be important in ameliorating the “wearing-off” effect of L-Dopa treatment (Archer *et al.*, 2002; Jansen *et al.*, 2003). Combination therapy of L-Dopa with NMDA receptor antagonists would therefore allow for a reduction of the L-Dopa dose and therefore postpone side effects associated with long term L-Dopa treatments (Lange *et al.*, 1997). Amantadine is an open-channel NMDA receptor antagonist (similar to its dimethyl derivative memantine), which is used as adjunct therapy in PD to treat dyskinesia induced by L-Dopa and might also be beneficial against “wearing-off” fluctuations (Jansen *et al.*, 2003; Archer *et al.*, 2002).

Biochemical analyses were performed to confirm the neurotoxic effects of MPTP in these studies (Fig. 6.4 and Tabel 6.1). Earlier studies of the neuroprotective effect of NGP1-01 in the MPTP mouse model (Geldenhuys *et al.*, 2003), showed very little protective effect. As expected, MPTP depleted striatal DA levels by about 70% in the C57BL/6 mice. A depletion of this magnitude is important, since it is commonly assumed that a loss of > 80 % is necessary to observe symptoms across species (Sedelis *et al.*, 2001). In addition, the Western Blot data (Fig. 6.4) indicated that there was a decrease in the amount of DAT expressed, that corresponded to a decrease in DA synaptic terminals and DA-ergic cells death following MPTP treatment. The mice treated with NGP1-01 alone, showed a small but significant increase in the amount of DAT expressed. NGP1-01 was shown to block DA uptake in murine

synaptosomes (Chapter 3). Although this might have contributed to the upregulation, the time frame of 12 h makes it unlikely that NGP1-01 led to an increase in DAT expression in mice treated with it.

Tillerson *et al.* (2002) reported an easy and useful method to detect behavioural impairments in the MPTP mouse model, as these are associated with loss of motor control in PD. The forepaw stride length test (Fig. 6.5) used by these authors correlated significantly with biochemical deficits in C57BL/6 mice. In our study, the stride length measured in mice treated with MPTP decreased after two weeks, when compared to vehicle control mice, although this decrease was not statistically significant. Injection with NGP1-01 further decreased the stride length in mice treated with NGP1-01 alone, and in MPTP mice, measured 30 min after NGP1-01 injection. There was a return to pre NGP1-01 status 12 hours later. NGP1-01 therefore did not seem to ameliorate the effect of MPTP on stride length, but in fact seemed to decrease the stride length. This could indicate that either NGP1-01 might have been injected in a too high dose, thereby decreasing the indirect pathway so much that a balance was not reached in the basal ganglia circuitry, or that NGP1-01 had no effect on this model in any way (which might be considered if it shares the same mechanism of action as memantine, since memantine produces little or no anti-Parkinsonian effects (Jansen *et al.*, 2003; Archer *et al.*, 2002)).

The catalepsy test (Fig. 6.6) is classified as a "simple" neurological test (Sedalis *et al.*, 2001). There was an initial decrease in catalepsy measured 30 min after NGP1-01 injection in the MPTP group, which increased 12 hours later to more than twice the values measured immediately before NGP1-01 injection. This suggests that NGP1-01 ameliorated the cataleptic effect of MPTP only to a small extent, although not in a statistically significant manner, but this observation is probably not therapeutically meaningful; since no change was seen in the time it took for the mice to take their paws off the block in the MPTP mice when compared to the vehicle treated mice. The substantial increase in catalepsy measured 12 hours after NGP1-01 injection in the MPTP group is likely not due to NPG1-01 per se, since at that time very little if any drug is still present in the animal. Although there has not been any formal metabolism studies done on NGP1-01, one study estimated an optimal brain

concentration of NGP1-01 after 1 hour of ip administration, with a decrease in concentration as time increases (Zah *et al.*, 2003).

The pole test (Fig. 6.7) was originally introduced as a test for bradykinesia (Sedelis *et al.* 2001). We adapted this test and counted the fall times of mice after being placed on the pole. MPTP treated mice showed a significant increased incidence of falling off the pole while descending, when compared with the control group ($P < 0.05$). These MPTP treated mice then received NGP1-01 on day 14 showed a decreased tendency to fall while descending ($P < 0.05$), 30 min after the NGP1-01 treatment. The decrease in falling tendency in MPTP treated mice after NGP1-01 treatment, approached control group values measured at the same time. It has to be kept in mind that a step wise decrease in the % ratio of falls/successful descents down the pole could be seen in all three groups. This suggests that there was a small learning curve exhibited by the mice. It seems that the difference in the group of mice treated with MPTP and then NGP1-01 was big enough to suggest that a small but significant beneficial effect on bradykinesia was seen with the treatment of NGP1-01 to the animals.

Rearing and movement (Fig. 6.8) were also investigated to measure any alterations in behavior. There was a significant decrease ($P < 0.05$) in rearing and movement in all the groups of mice 2 weeks after MPTP treatment, with the decrease in rearing being much more pronounced than was observed for movement. The mice probably became used to the environment and this could account for the decrease in exploratory behaviour (i.e. rearing and movement). Rearing was reduced in both the NGP1-01-only group and the MPTP/NGP1-01 group 30 min after NGP1-01 injection, but there was no similar decrease found for movement. The group treated with MPTP on day 1 and then again with NGP1-01 on day 14, showed similar rearing behaviour as the vehicle group. Rearing returned to the same levels as at the beginning of the experiment (before MPTP injection) 12 hours after NGP1-01 injection, while movement remained constant to before NGP1-01 injection, with only a small decrease in the MPTP group as compared to pre NGP1-01 injection. NGP1-01 thus did not seem to influence movement in any manner, but rearing behaviour was reduced to a small extent, similar to that of the vehicle group at the same time point. The same

reason that was given for the forepaw stride length decrease with NGP1-01 treatment probably holds true for this experiment as well.

6.5 Conclusion

NGP1-01 (1) was found to be a putative NMDA receptor antagonist, and we proceeded to investigate this polycyclic cage amine in the MPTP mouse model to determine whether it might alleviate behavioural deficits found in the MPTP mouse model of PD. Weak anticataleptic and antibradykinetic effects were seen for NGP1-01 (1) suggesting that NGP1-01 (1) may have potential as a possible antiparkinsonian agent. These results also suggest that it might be useful as an adjunct therapy together with L-Dopa, as seen in the forepaw stride length and open field test data. These results appear similar to those seen with amantadine (Archer *et al.*, 2002; Jansen *et al.*, 2003), another drug used as adjunct therapy together with L-Dopa. Further studies, especially studies using additional dosing regimens, are needed to determine the effect of NGP1-01 (1) in monotherapy, as well as assess its value when used as adjunct therapy together with L-Dopa.

6.6 Acknowledgements

The help of Jinghong Kou at Neurotoxicology Lab., Dept. of Entomology, Virginia Tech, Blacksburg, VA, USA for doing the Western Blot Analysis is gratefully acknowledged. All of the experiments were performed in the laboratory of Dr. J. R. Bloomquist, Neurotoxicology Laboratory, Dept. of Entomology, Virginia Tech, Blacksburg, VA, USA.

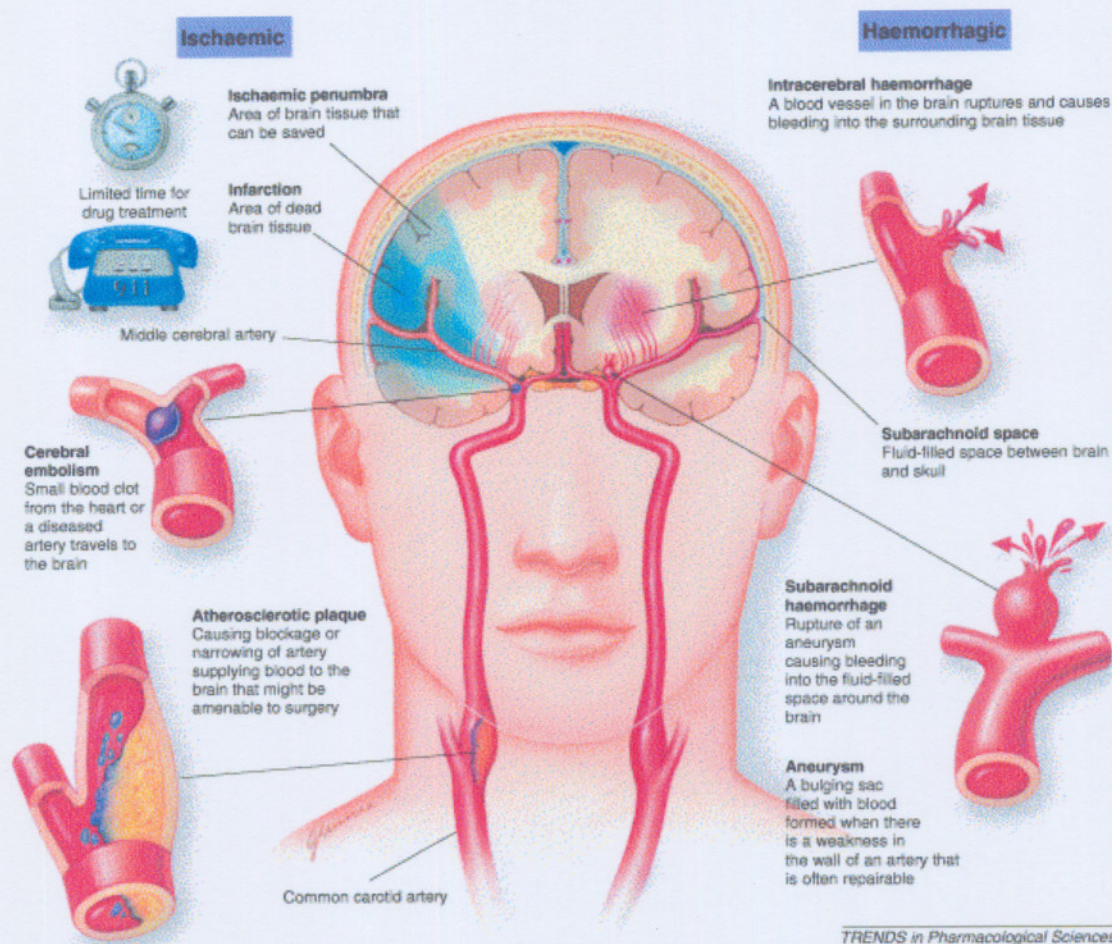
**Effect of NGP1-01 in the middle
cerebral artery occlusion (MCAO)
murine stroke model**

Chapter

7

7.1 Introduction

Stroke is the third leading cause of death and disability in major industrialized countries, with an incidence of ~350 per 100,000 persons of a population aged 45-89 (Green *et al.*, 2003; Ovbiagele *et al.*, 2003; Suzuki *et al.*, 2003). Approximately 20% of stroke patients do not survive the first month and >30% who are alive 6 months later will be dependent on others (Green *et al.*, 2003). Substantial efforts have been made in the past 20 years to understand the biochemical mechanisms involved in ischemia-induced cerebral damage. Most strokes are ischemic (~85%) and the majority of ischemic strokes result from an occlusion of a major cerebral artery by a thrombus or an embolism, which leads to loss of blood flow in a specific region. The remaining strokes are hemorrhagic, where a blood vessel bursts either in the brain or on its surface (Fig. 7.1; Green *et al.*, 2003).



TRENDS in Pharmacological Sciences

Figure 7.1. The major types of stroke (Green *et al.*, 2003. Animal models of stroke: do they have value for discovering neuroprotective agents? *Trends Pharmacol. Sci.* 24(8), 402-408).

Currently, only intravenous recombinant tissue plasminogen activator is approved by the US Food and Drug administration for therapy of acute ischemic stroke (Ovbiagle *et al.*, 2003; Green *et al.*, 2003). This approach tries to establish reperfusion of the compromised region by the dissolution of the clot using trombolytic drugs (Green *et al.*, 2003). Another approach is to develop neuroprotective agents. These compounds include therapies that block the cellular, biochemical and metabolic elaboration of injury that leads to neuronal cell death in patients with acute ischemic stroke (Fig. 7.2; Ovbiagle *et al.*, 2003). The basis for the use of neuroprotective drugs is that the core area of damage (infarction) is assumed not salvageable, but that the area that surrounds the core (the penumbra), despite being compromised with low blood flow, can be saved by either reflow or by a neuroprotectant. Evidence suggests that if the

penumbral region is left untreated, it too will become part of the core region (Green *et al.*, 2003). Saving the penumbral region will therefore likely attenuate many of the clinical problems associated with stroke, such as motor disability.

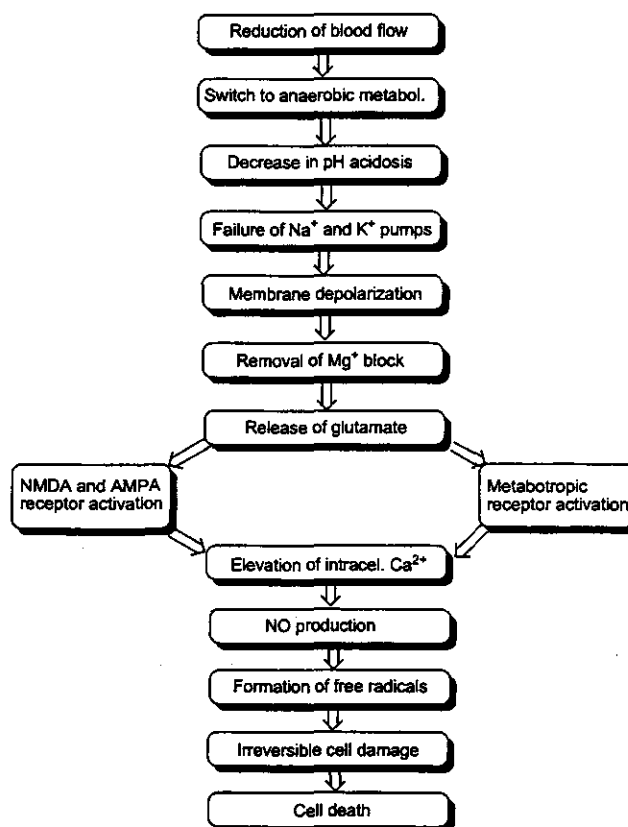


Figure 7.2. Ischemic cascade in patients with acute ischemic stroke (Ovbiagele *et al.*, 2003). Abbreviations are: metabolism (metabol.) and intracellular (intracel.). Each step along the path represents a possible target for therapeutic intervention.

Several studies have shown that compounds acting at excitatory amino acid receptors (including NMDA receptors), as well as L-type Ca²⁺ antagonists display neuroprotective effects in animal models of ischemia (Gerriets *et al.*, 2003; Le & Lipton, 2001; Palmer, 2001; Görügülü *et al.*, 2000; O’Niell *et al.*, 1997).

Based on these mechanisms, the present study was undertaken to evaluate the neuroprotective potential of NGP1-01 (1) in the middle cerebral artery occlusion (MCAO) induced focal cerebral ischemia model in mice. NGP1-01 has been shown

to exhibit both NMDA receptor antagonism (Chapter 4), as well as L-type calcium channel antagonism (Van der Schyf *et al.*, 1986).

7.2 Materials and Methods

7.2.1 Animals and Treatment

Female CD-1 mice were obtained from Harlan (USA) weighing approximately 30 g each. Female mice were chosen because their weight changed less with time than that of male mice. We observed a correlation between the length of suture used to induce MCAO and the weight of mice, and therefore aimed to only enroll mice in our studies with identical weights, thereby limiting interanimal variability in our experiments. Mice were divided into two groups ($n = 6$ in each group): vehicle control (sham) receiving 10 μ l DMSO and the remainder NGP1-01 (1) dissolved in DMSO and injected (10 μ l ip) at a dose of 20 mg/kg, 30 min before MCAO.

7.2.2 Middle cerebral artery occlusion induced focal cerebral ischemia

Cerebral ischemia was produced by middle cerebral artery occlusion (MCAO) in anesthetized female mice, weighing 30 g each, according to previously reported methods (Thiyagarajan *et al.*, 2004). Induction of anesthesia was in a plexiglass box with 4% isoflurane, and, during surgery a face mask administering 1% isoflurane was used, via a Surgivet vaporizer. The surgery was done under optical guidance with a Zeiss OPMI pico *i* surgical microscope on a S100 suspension system. The microscope is equipped with a 3CCD-digital camera (Toshiba) connected to a high definition LCD TV via a Pinnacle movie box. A midline incision on the ventral side of the neck allowed access to the left common carotid artery. The occipital, superior thyroid and pterygopalatine arteries were then cauterized and cut. A suture was placed around the external carotid artery above its bifurcation with the internal carotid artery. Five mm above the bifurcation the artery was cauterized to leave a small stump. An incision was then made in this stump and a 6-0 Nylon thread (20 mm long with pointed tip) was inserted. The stump was rotated 180° in the caudal direction to bring the external and internal carotid arteries in alignment. The nylon thread was

then carefully advanced in the cranial direction for about 10 mm, while changes in the cerebral blood flow were monitored using a laser Doppler Perfusion Monitor (Moor Instruments). In our experience, a reduction of > 90% is indicative of successful MCAO. The nylon thread was left for permanent occlusion (24 h) and the skin closed up. The mice were sacrificed 24 hours after MCAO.

7.2.3 Histological staining

Analysis of the stroke size was done by using the mitochondrial stain triphenyl tetrazolium chloride (TTC; Sigma) and representative slices are shown in Figure 7.3. The brain was cut coronally at 1 mm intervals using a tissue chopper (McIlwain) and the slices were soaked in a 2% TTC (0.1 M phosphate-buffered saline pH 7.4) solution for 10 min at 37 °C in a water bath. The stained slices were then fixed in a 10% formalin solution and refrigerated. Images of these slices were acquired by use of a digital camera and the images analyzed using Adobe Photoshop 6 and ImageJ software (ImageJ 1.30, running under Microsoft Windows 2000, downloadable from <http://rsb.info.nih.gov/ij/download.html>), according to a protocol adapted from Rorden & Brett (2000). The unstained area (stroke) was measured and calculated as a percentage of the total slice area. Thus, measurements were made on each slice to calculate the size of the lesion and correct for the effects of brain swelling (edema) where: a = area of the infarct (mm^2), b = area of the infarcted (ipsilateral) hemisphere (mm^2), c = area of the non-infarcted (contralateral) hemisphere (mm^2), d = brain swelling (mm^2) = $(b - c)$ and t = slice thickness. The volume (V) of the lesion (mm^3), corrected for swelling, can then be derived from the equation $V = \Sigma(a.t) - \Sigma(d.t)$. In summary, the edema volume was calculated as a ratio of ipsilateral hemisphere area/contralateral hemisphere area (Fig. 7.4), and the stroke (infarction) volume as a ratio of infarct area/total brain slice area (Fig. 7.5).

7.2.4 Statistical analysis

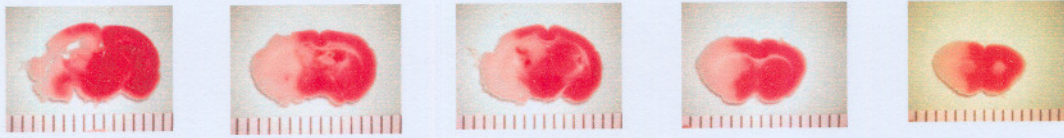
Statistical analysis was done using Prism 2.0 software (GraphPad Software, San Diego CA, USA). Student t-tests were performed on mean areas of treated brain

slices vs. vehicle controls. A value of $p < 0.05$ was considered statistically significant.

7.3 Results

Figure 7.3 shows representative brain slices of the vehicle control group as well as the NGP1-01 treated group. The TTC stains living mitochondria red, leaving the dead tissue (dead mitochondria = stroke area) as white areas in the slices. Visual inspection shows that there is a decrease in the infarct (stroke) volume in the slices treated with NGP1-01 (20 mg/kg). The reduction was most visible in the cortical area, with the internal core still affected by ischemia.

Vehicle control



Treatment with 20 mg/kg NGP1-01

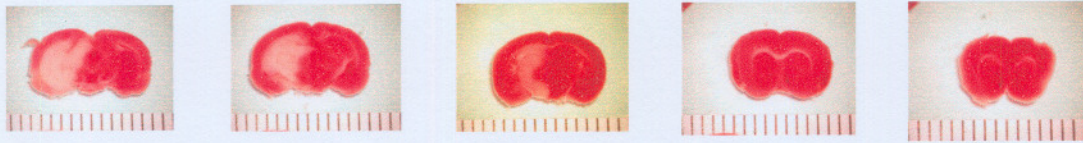


Figure 7.3. Representative murine brain slices (1 mm thick) 24 h after MCAO of vehicle control and treated (20 mg/kg NGP1-01) animals.

Brain slices treated with NGP1-01 (20 mg/kg) showed a decrease in edema volume, from 1.198 ± 0.027 in vehicle control to 1.043 ± 0.014 in NGP1-01 treated animals, a 13% reduction, which was statistically significant ($p < 0.001$), compared to vehicle treated controls.

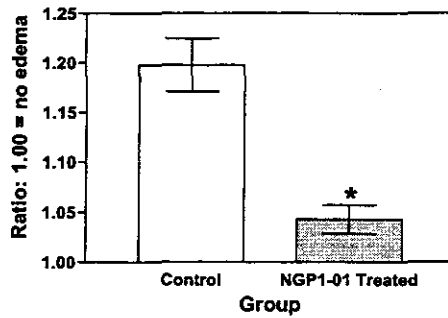


Figure 7.4. Edema volume after 24 h of MCAO in vehicle control and NGP1-01 treated (20 mg/kg) calculated as a ratio of ipsilateral hemisphere area/contralateral hemisphere area. Results are mean $\pm$ S.E.M. $n = 6$. Statistical significance * $P < 0.001$, compared to vehicle control.

Stroke (infarct) volume was calculated as a ratio of infarct area/total brain slice area. The infarct volume (Fig. 7.5) measured in the murine brain slices treated with NGP1-01 showed a decrease in volume from 0.290 ± 0.015 (vehicle control) to 0.17050 ± 0.017 in NGP1-01 treated animals, a 42% reduction, which was statistically significant ($p < 0.05$), compared to vehicle control.

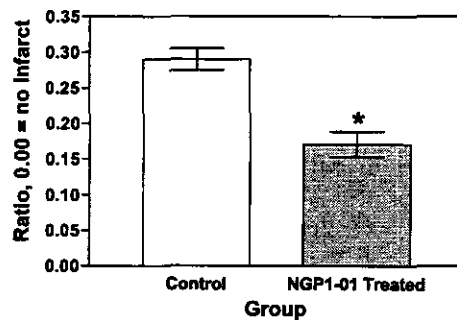


Figure 7.5. Stroke (infarct) volume after 24 h of MCAO in vehicle (sham) and NGP1-01 treated (20 mg/kg) calculated as a ratio of infarct area/total brain slice area. Results are mean $\pm$ S.E.M. $n = 6$. Statistical significance * $P < 0.05$, compared to vehicle control (sham).

7.4 Discussion

In the present study we showed that NGP1-01, at a dose of 20 mg/kg ip, reduced the edema volume by 13% and the stroke (infarct) volume by 42% in the MCAO murine model of ischemic stroke. During ischemia, glutamate is released in large amounts,

leading to activation of the NMDA receptor as well as other glutamatergic receptors, resulting in an increased influx of Ca^{2+} into neurons. Ca^{2+} also enters neurons through voltage-dependent Ca^{2+} channels, which respond to cellular depolarization. The net effect is an accumulation of Ca^{2+} inside neuronal cells, essentially 'overloading' them, which leads to activation of proteases, nucleases, phospholipases, nitric oxide synthase and other degradative enzymes that lead to free radical formation and cell death (Ovbiagele *et al.*, 2003; O'Neill *et al.*, 1997). Previous *in vitro* studies showed that NGP1-01 blocks both NMDA receptors and L-type calcium channels (Chapter 4; Van der Schyf *et al.*, 1986). This led us to evaluate the efficacy of NGP1-01 as a neuroprotective agent in the MCAO induced focal cerebral ischemia model of stroke in mice.

NGP1-01 produced a 13% decrease in edema volume and a 42% reduction in the infarct (stroke) area when administered at a dose of 20 mg/kg ip, 30 min before MCAO was induced. These results are in agreement with other studies that have shown NMDA receptor antagonists and L-type calcium antagonists to have neuroprotective activity in animal models of stroke (Ovbiagele *et al.*, 2003; O'Neill *et al.*, 1997). Interestingly, it appears that NGP1-01 "saved/protected" the cortical area of the brain slices, more so than the core of the stroke area. This is a good indication that NGP1-01 might attenuate some of the clinical problems associated with stroke in humans, where higher brain functions are located in the cortex.

Although a variety of compounds have shown very promising activity as neuroprotective agents in animal models, they have not proven effective in human trials (Green *et al.*, 2003; Gulyaeva *et al.*, 2003). One of the reasons proposed for the failure of these compounds in clinical trials, is the fact that animal studies generally do not answer the questions normally addressed in human trials, and are therefore most often not predictive in the clinical situation. Human studies largely monitor behavioural parameters, in contrast to animal studies which largely rely on histological outcomes to define stroke damage. It is evident that in order to make future animal studies more clinically relevant, increasing attention should be paid to behavioural measures of stroke damage (Green *et al.*, 2003; Gulyaeva *et al.*, 2003). Further studies with NGP1-01's effect on stroke, should therefore be directed at

elucidating the effects of NGP1-01 on attenuating behavioural deficits seen in animal stroke models.

7.5 Acknowledgements

The help of Dr. Alexander Mdzinarishvili (Texas Tech University HSC, Amarillo, TX USA) for performing the surgeries is gratefully acknowledged.

8.1 Introduction

Parkinson's disease (PD) is a debilitating age-related neurodegenerative disease. The symptoms of PD are due to the selective loss of dopaminergic neurons in the substantia nigra (Przedborski & Vila, 2002). Currently, this disease is treated only symptomatically with no drugs available that are used clinically to prevent neurodegenerative diseases. Few compounds have been shown to exhibit neuroprotective activity, in addition to their provision of symptomatic relief. These include pramipexole (Bennet & Piercy, 1999), and memantine (Wenk *et al.*, 1995).

Memantine is a low affinity uncompetitive NMDA receptor antagonist and is used clinically to treat Alzheimer's disease (Riesberg *et al.*, 2003; Jain, 2000; Vedantam, 2003). Memantine prevents the excessive influx of Ca^{2+} into neuronal cells, associated with neuronal cell death. The important role of Ca^{2+} in neuronal cell death led us to consider the L-type calcium channel antagonist NGP1-01 (1; Van der Schyf *et al.*, 1986) as a lead compound with the expectation to discover new drugs to treat and/or prevent neurodegenerative diseases such as PD. In addition, there exists structural similarity between NGP1-01 and memantine. These include an amino functionality and a polycyclic carbon cage moiety.

In our approach, three targets were identified in PD where drugs may be used as symptomatic and/or preventative treatment. These include (but are not restricted to) the dopamine transporter (DAT), the monoamine oxidase (MAO) B enzyme and the NMDA receptor/ion channel complex. The polycyclic cage compounds described in this study were evaluated for their effects on these targets *in vitro*. One compound

was then selected to evaluate its efficacy in ameliorating behavioural deficits in the MPTP parkinsonian mouse model as well as in a murine stroke model, *in vivo*.

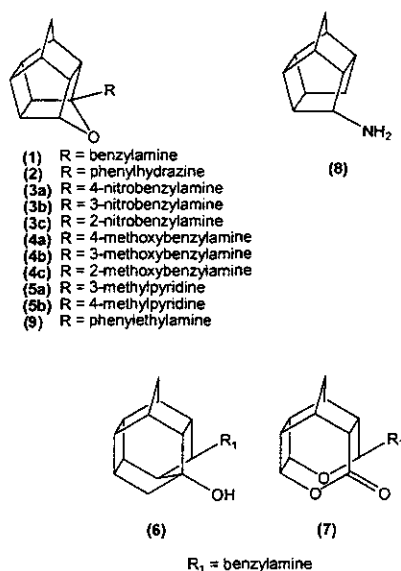


Figure 8.1. Pentacycloundecylamines used in this study.

8.2 The dopamine transporter (DAT) and MAO-B enzyme

In previous studies, compound **9** showed positive effects in neuroprotection studies with MPTP, *in vivo* (Geldenhuys *et al.*, 2003). In view of these findings, we examined additional compounds in this series for their effects on [³H]dopamine ([³H]DA) release and uptake inhibition in murine striatal synaptosomes, as well as for inhibition of baboon liver monoamine oxidase (MAO) B activity. In order to focus on specificity, initial experiments were performed on compounds that blocked dopamine (DA) uptake without causing appreciable release (< 40% at 100 μM) of the transmitter. NGP1-01 (**1**) blocked the uptake of [³H]DA with an IC₅₀ of 57 μM, while another compound, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**9**), blocked uptake at an IC₅₀ value of 23 μM. These values were comparable to that of another polycyclic cage amine, amantadine (IC₅₀; 82 μM), that is used in parkinsonian therapy. Structure-activity relationships in this series of compounds support the importance of geometric and steric, rather than electronic effects, in determining biological activity. MAO-B inhibition for this group was weak, with less

than 50% inhibition at 300 μM measured for any of the compounds in the series. The present study thus suggests that blockage of the DAT may underlie, at least in part, the neuroprotective effects of these polycyclic amines against MPTP-induced parkinsonism.

8.3 The NMDA receptor

NGP1-01 (**1**) and its derivatives were evaluated further in a functional assay to assess N-methyl-D-aspartate (NMDA) antagonism and therefore possible neuroprotection. The IC_{50} values for the reference compounds MK-801 and memantine in this experiment were found to be 1.23 μM and 3.05 μM , respectively, for blocking NMDA-mediated $^{45}\text{Ca}^{2+}$ influx into whole brain murine synaptoneurosomes. NGP1-01 proved to be the most potent experimental compound with an IC_{50} of 2.98 μM , while 8-amino-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (**8**) had a comparable IC_{50} of 4.06 μM . The Hill-slopes of most compounds tested in dose-response studies were near unity, suggesting that a one-to-one stoichiometry was operative to induce antagonism. Increasing the polycyclic cage size to the larger tridecane moiety decreased potency 10 fold, indicating a limitation on the volume of the cage for the presumed channel binding site. NMDA increased $^{45}\text{Ca}^{2+}$ influx with a maximal effect measured at 1 mM, and an EC_{50} value of 49 μM . In the presence of NGP1-01 maximal uptake was inhibited by 34% with little effect on agonist potency (EC_{50} = 68 μM). These results are consistent with the noncompetitive antagonism described for this group of compounds.

8.4 Predictive modelling of NMDA receptor binding

After confirming that NGP1-01 acts as an NMDA receptor antagonist, comparative molecular field analysis (CoMFA) and comparative molecular similarity index analysis (CoMSIA) techniques were used to provide models to predict the PCP binding affinity of NGP1-01 and other polycyclic cage amines. The resulting r^2 values found for the CoMFA and CoMSIA models were 0.976 and 0.883 respectively. The resulting model predicted the binding affinity of selected compounds to be in the low μM range. Radioligand binding studies with [^3H]MK-801 and [^3H]TCP,

however, showed little or no displacement by pentacycloundecylamines. This may indicate that these compounds bind to another site on the NMDA receptor. Further studies are therefore required to elucidate the mechanism by which NGP1-01 and its derivatives block the NMDA receptor.

8.5 Effect of NGP1-01 on restoring normal behaviour in the MPTP mouse model

Glutamate antagonists also have a role in the symptomatic treatment of PD (Lange *et al.*, 1997). In PD, there exists an overactive indirect (glutamate) pathway via the subthalamic nucleus. The balance between the indirect and direct pathways may be restored by glutamate antagonists. Since NGP1-01 (**1**) was shown to have NMDA receptor antagonism activity, this compound was tested in a battery of behavioural tests in the MPTP-parkinsonian mouse model using retired breeder C57BL/6 male mice to determine whether it would alleviate behavioural deficits in this model. MPTP was injected (ip) at 30 mg/kg two weeks before NGP1-01 (ip 20 mg/kg). The behavioural tests included fore-paw stride length, pole test (bradykinesia), catalepsy, rearing, and movement. Biochemical confirmation of MPTP-induced toxicity was done by measuring striatal DA levels using HPLC with electrochemical detection. The amount of dopamine transporter (DAT) present was determined by Western Blot. NGP1-01 (**1**) was found to alleviate bradykinesia and catalepsy to a small extent.

8.6 Effect of NGP1-01 in the murine MCAO stroke model

NGP1-01 (**1**) was also evaluated in the middle cerebral artery occlusion (MCAO) induced focal ischemia model in mice to evaluate its neuroprotective potential in stroke. In this study, NGP1-01 (**1**), at a dose of 20 mg/kg ip, injected 30 min before MCAO, significantly reduced the edema volume by 13% and the infarct volume by 42%, compared to vehicle (DMSO) control. These results show great promise for stroke therapy, but functional behavioural studies will be required to determine if NGP1-01 (**1**) protects the brain's higher functions, a very important issue with human patients.

8.7 Conclusion

The pentacycloundecylamines tested represent a novel group of compounds that have potential as therapeutic agents for Parkinson's or other neurodegenerative diseases such as Alzheimer's disease and stroke. The dual NMDA receptor antagonism and L-type calcium channel antagonism exhibited by these compounds make them ideal candidates to be used as preventative therapy in neurodegenerative disorders associated with excess accumulation of Ca^{2+} in neuronal cells. In addition, these compounds were shown to inhibit the DAT; thereby possibly offering symptomatic relief in addition to neuroprotection capabilities. Furthermore, the PD *in vivo* study showed NGP1-01 to alleviate bradykinesia and catalepsy to a small extent in the MPTP mouse model. The *in vivo* MCAO stroke model also showed that NGP1-01 protected the murine brain to an extent from ischemia. These results warrant the further investigation of the pentacycloundecylamines as possible symptomatic and/or neuroprotective drugs in the treatment of neurodegenerative diseases.

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Synthesis and biological evaluation of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane derivatives as potential therapeutic agents in Parkinson's disease

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Abstract—In previous studies, the polycyclic cage amine 8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (NGP1-01) and a number of its derivatives showed positive effects in neuroprotection studies with MPTP, *in vivo*. In view of these findings, we examined these compounds for their effects on [³H]dopamine ([³H]DA) release and uptake inhibition in murine striatal synaptosomes, as well as for inhibition of baboon liver monoamine oxidase (MAO) B. In order to assess specificity, initial experiments focused on compounds that blocked dopamine uptake without causing appreciable release (<40% at 100 μM) of the transmitter. NGP1-01 blocked the uptake of [³H]DA with an IC₅₀ of 57 μM, while another compound, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, blocked uptake at an IC₅₀ value of 23 μM. These values were comparable to that of another polycyclic cage amine, amantadine (IC₅₀; 82 μM), that is used in parkinsonian therapy. Structure–activity relationships of this series of compounds support the importance of geometric and steric, rather than electronic effects, in determining biological activity. MAO-B inhibition for this group was weak, with less than 50% inhibition at 300 μM for any of the compounds in the series. The present study suggests that blockage of the dopamine transporter may underlie, at least in part, their neuroprotective effects against MPTP-induced parkinsonism. These compounds may be considered as potential lead compounds for Parkinson's Disease therapy. © 2004 Elsevier Ltd. All rights reserved.

1. Introduction

Parkinson's disease (PD) is a terminal, late-onset neurodegenerative disorder characterized by a progressive and relatively selective degeneration of dopaminergic neurons in the substantia nigra.¹ To date, most therapies for PD are symptomatic in nature, with no neuroprotective therapies currently approved for prevention of this chronic disease. Some therapeutic agents used in the early treatment of PD have, however, been shown to have neuroprotective activity in addition to their symptomatic effects, such as the dopamine agonist, pramipexole.²

Amongst several drug therapies used in the symptomatic treatment of PD patients is the polycyclic cage amine, amantadine (Fig. 1). Amantadine expresses its antiparkinsonian activity, at least in part, by increasing extracellular dopamine levels via re-uptake inhibition³ or dopamine (DA) release.⁴ Electrophysiological studies further indicated that amantadine binds to the phencyclidine (PCP) or MK-801 (dizocilpine) binding site located within the NMDA receptor/ion channel complex.⁵ In addition, amantadine and its dimethyl derivative memantine have been shown to be neuroprotective.^{5,6}

Early interest in the *in vivo* biological activities of polycyclic cage amines was raised when Oliver et al.^{7,8} reported antagonism of reserpine-induced catatonia as well as reduction of oxotremorine-induced tremor and salivation in rats by novel pentacycloundecylamines and trishomocubanes, at doses comparable to those of

Keywords: Pentacycloundecylamines; Neuroprotection; Dopamine transporter; Monoamine oxidase B; MK-801; Amantadine; Memantine.

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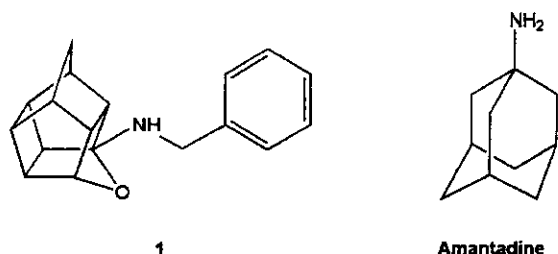


Figure 1. Structures of the prototypical cage amines NGP1-01 (1) and amantadine.

amantadine. These authors suggested that the polycyclic cage amines might be a new class of possible anti-parkinsonian agents due to anticataleptic and mild to weak anticholinergic activities.

L-type calcium channel antagonism has been reported by our laboratory for pentacycloundecylamines, in particular for the prototypical compound 8-benzyl-amino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (Fig. 1, 1, NGP1-01).^{9–13} Recently, we reported an in vivo pilot evaluation of a small series of pentacycloundecylamines in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) parkinsonian mouse model and found that one of the compounds, 8-phenylethyl-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane, tested positively as a neuroprotective agent.¹⁴ MPTP is a protoxin that requires activation by means of monoamine oxidase (MAO) B to form the neurotoxic pyridinium species, 1-methyl-4-phenyl-pyridinium (MPP⁺).¹⁵ MPP⁺ is then transported into dopaminergic neurons via the dopamine transporter (DAT), where it inhibits mitochondrial complex I.^{16,17} Excitotoxicity is also implicated in the MPTP model of neuronal cell death, and involves the over-stimulation of the *N*-methyl-D-aspartate (NMDA) receptor by glutamate, ultimately resulting in excess intracellular calcium and cell death.^{18,19}

On the basis of this information, we have chosen to evaluate a series of polycyclic cage amines for activity in the dopamine system as part of an ongoing investigation into the therapeutic potential of novel pentacycloundecylamines. We are hoping to develop drugs that are dual neuroprotective and symptomatic therapy for neurodegenerative diseases such as PD. Since the DAT and MAO-B are key elements in the DA depletion seen in the MPTP mouse model, we investigated the pharmacology of the compounds in this study for interaction with the DAT and MAO-B in vitro, aiming to select compounds for future in vivo studies. Moreover, because our future plans include analysis of effects on the NMDA receptor and MPTP/C57 mouse model, we included MK-801 as an established NMDA antagonist in the present study.

2. Chemistry

Van der Schyf et al.^{9–12} postulated that the cage-like pentacycloundecane skeleton mostly served as a bulk contributor to the calcium channel blocking activity of the parent compound, 1. Structure–activity studies were

combined with synthetic efforts to investigate the effects of different aromatic amine side chains as well as gaining insight into the effects of the polycyclic skeleton.

Synthesis of compounds 1–7 and 9 was conducted as previously described^{12,13} and is shown in Scheme 1. The common starting diketone (a), was prepared according to the method of Cookson et al.²⁰ Reductive amination of a followed by Dean–Stark dehydration and reduction with NaBH₄ furnished 1–5, and 9. Syntheses of 6 and 7 were achieved following our previously reported procedure.¹³ Transformation of a by a boron trifluoride etherate reaction with ethyl diacetate, followed by subsequent decarboxylation, yielded 11, the starting ketone for 6. Similarly, the starting ketone (12) of 7 was prepared by Baeyer–Villiger oxidation of the diketone (a). Final compounds 6 and 7 were prepared by reductive amination. Compound 8 was prepared by lithium aluminum hydride reduction of the oxime, which was obtained by condensation of the mono ketone 13 with hydroxyl amine.⁷ Compound 8 was purified as the HCl salt.

3. Pharmacology

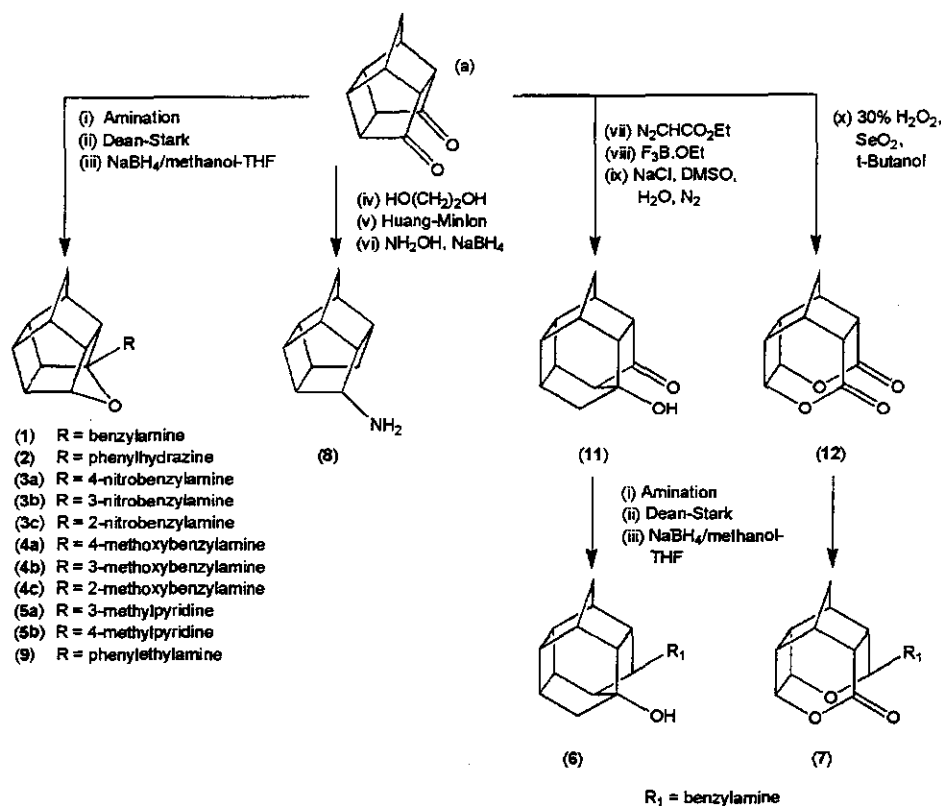
Release and uptake inhibition of [³H]DA was carried out in murine striatal synaptosomes as described earlier.²¹ Compounds were screened at 100 μM, except for GBR-12909 ([³H]DA release, 10 μM; inhibition of [³H]DA uptake, 2 μM). Initial experiments identified compounds that blocked [³H]DA uptake without causing appreciable release (≤40% at 100 μM), in order to ensure specificity. IC₅₀ values for inhibiting [³H]DA uptake were then determined for selected compounds. Inhibition of MAO-B was carried out in baboon liver MAO-B. The baboon liver contains MAO-B almost exclusively.²² All compounds were screened initially in this assay at 100 μg/mL.

4. Molecular modeling

Molecular modelling to obtain a reasonable minimum energy conformation for each structure was performed by optimizing the structures with the ChemplusTM extension of Hyperchem[®] modeling software (Release 4.5 for Windows 1994, Hypercube Inc. Ontario, Canada) using MM⁺ and AM1 with the Polak–Ribière minimization procedure.²³ The lowest energy conformer found for compounds 3b and 4b was used in superpositioning and 9 was used to calculate its physicochemical properties, and to assist in estimating log BB (eq 1), the concentration of 9 in the central nervous system (CNS) following intraperitoneal treatment.

$$\begin{aligned} \text{Log BB} = & 4.56796 - 0.52226(\log \text{Poct}) \\ & + 0.0052(\text{Energy}) + 0.05559(\text{SV}) \\ & - 0.33873(\text{MR}) \end{aligned} \quad (1)$$

where log BB = log (Concentration_{brain}/Concentration_{serum}); Energy = minimum energy conformation; SV = solvent accessible molecular volume; and MR = molar refractivity.



Scheme 1. Synthesis of pentacycloundecylamine analogues.

5. Results and discussion

Initial screening studies determined the compounds' ability to release DA, and compounds with low activity were selected for further evaluation (Table 1). The compounds fell into three groups with respect to causing DA release; those that were able to elicit no more than 0–20% release of label (1, 2, 3a, 6, and 7), those that elicited 20–40% release of label (3c, 4a, c, 5a, b, 8, 9, memantine, and MK-801), and those that released > 60% of label (3b and 4b). Because these studies served as a screen only to eliminate potentially toxic compounds, each assay was replicated only twice.

For [^3H]DA release (Fig. 2, Table 1) it was apparent that replacement of the benzylamine moiety of 1 by phenylhydrazine (2) did not alter the release of [^3H]DA significantly, suggesting the importance of a linker atom spaced between the nitrogen and phenyl moieties in both these compounds. In addition, substitution on the aromatic ring seemed to influence the degree of [^3H]DA release. Introduction of a nitro group (3a–c) on the phenyl ring of 1 resulted in varying levels of [^3H]DA retention, with the rank order of release activity as *meta* > *ortho* > *para*. The *meta* nitro compound (3b) showed the least intracellular retention of [^3H]DA while the *para* substituted nitro compound showed at least twice the intracellular retention of [^3H]DA compared to *ortho*. For the methoxy compounds (4a–c), *meta* substitution yielded the most active compound in the whole

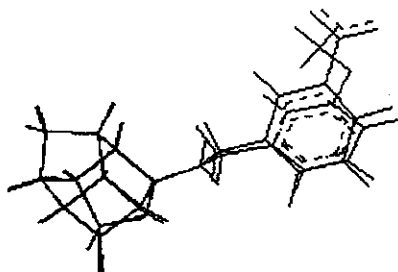
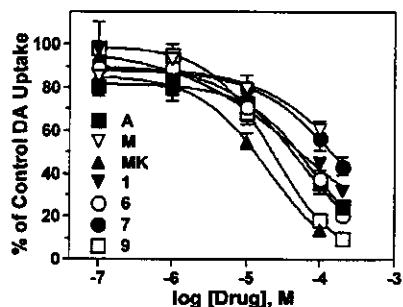
series, while *para* and *ortho* compounds elicited lower, but equipotent release of [^3H]DA.

It would therefore seem that from the perspective of [^3H]DA release activity, addition of electron donating groups (e.g., methoxy) results in more active compounds when compared to electron withdrawing groups (e.g., nitro). However, electron donation seems to play only a small additive role, with steric considerations more important, since it was not the *para* compounds (which would be expected to elicit a stronger electron effect, than either of the other two positions on the phenyl ring), but the *meta* compounds that showed the highest activity (i.e., caused more release), when comparing the nitro and methoxy compounds. Both the nitro and the methoxy groups occupy the same spatial area, when 3b and 4b are superimposed. In addition, the oxygen of the methoxy group and one on the nitro group, are orientated in the same direction away from the cage (Fig. 3). The distance between these two oxygens is only 1.77 Å, which suggests that they are important for release of [^3H]DA, and that the orientation of the methoxy oxygen of 4b may be more preferable than for either of the two oxygens of the nitro group of 3b.

Further structure–activity relationships for [^3H]DA release are evident from results with additional molecules. When a nitrogen is included in the aromatic ring of 1 to produce the methylpyridine compounds (5a and b), release of [^3H]DA was only slightly less than that

Table 1. Summary of effects of cage amines and related compounds on dopamine release (at 100 μ M), inhibition of dopamine transport (at 100 μ M, with selected IC_{50} values), and inhibition of monoamine oxidase B activity (at 300 μ M)

Compd	DA Release, % Control $\pm$ S.E.M. ($n=2$)	DA Uptake, % Control $\pm$ S.E.M. ($n=3$)	IC_{50} , DA Uptake, μ M, $\pm$ S.E.M.	MAO-B Activity, % Control $\pm$ S.E.M. ($n=2$)
1	88.00 $\pm$ 23.00	42.66 $\pm$ 1.76*	57 $\pm$ 1.6	71.77 $\pm$ 0.002*
2	79.50 $\pm$ 16.50	54.66 $\pm$ 9.17	n.d.	29.58 $\pm$ 0.005*
3a	81.50 $\pm$ 0.50	86.00 $\pm$ 2.00	n.d.	70.16 $\pm$ 0.016*
3b	30.00 $\pm$ 12.00	25.66 $\pm$ 6.69	n.d.	24.62 $\pm$ 0.005*
3c	55.00 $\pm$ 6.00	41.00 $\pm$ 4.61	n.d.	34.95 $\pm$ 0.009*
4a	61.00 $\pm$ 1.00	43.00 $\pm$ 7.09	n.d.	33.12 $\pm$ 0.010*
4b	22.00 $\pm$ 2.00*	7.00 $\pm$ 1.73*	n.d.	35.74 $\pm$ 0.004*
4c	57.50 $\pm$ 3.50	33.00 $\pm$ 2.30*	n.d.	77.97 $\pm$ 0.021*
5a	71.50 $\pm$ 8.50	66.00 $\pm$ 1.00	n.d.	83.67 $\pm$ 0.010*
5b	62.50 $\pm$ 12.50	64.33 $\pm$ 5.84	n.d.	88.82 $\pm$ 0.003*
6	99.00 $\pm$ 4.00	33.33 $\pm$ 0.88*	55 $\pm$ 1.2	88.33 $\pm$ 0.011*
7	96.50 $\pm$ 6.50	51.33 $\pm$ 6.69*	199 $\pm$ 1.1	97.64 $\pm$ 0.016*
8	67.00 $\pm$ 14.00	40.33 $\pm$ 2.02*	n.d.	91.73 $\pm$ 0.023*
9	76.00 $\pm$ 3.00	18.66 $\pm$ 0.88*	23 $\pm$ 1.0	56.09 $\pm$ 0.013*
Amantadine	72.50 $\pm$ 3.50	30.00 $\pm$ 6.55*	82 $\pm$ 1.1	86.09 $\pm$ 0.005*
Memantine	74.50 $\pm$ 7.50	49.66 $\pm$ 7.33*	> 100	75.98 $\pm$ 0.041*
MK-801	81.50 $\pm$ 5.50	13.33 $\pm$ 0.66*	18 $\pm$ 1.1	92.64 $\pm$ 0.017*
GBR 12909	69.57 $\pm$ 0.32	6.00 $\pm$ 2.51*	0.007 $\pm$ 0.4*	n.d.
Deprenyl	n.d.	n.d.	n.d.	0.82 $\pm$ 0.0002*
TMN	n.d.	n.d.	n.d.	4.40 $\pm$ 0.002*

Significantly different from control, $p < 0.05$; n.d. = not determined. Reported IC_{50} .²⁴**Figure 2.** Superpositioning of 3b and 4b through the Chemplus extension of Hyperchem[®].**Figure 3.** Dose–response relationships for the inhibition of [^{3} H]DA transport by selected compounds. Each data point represents the mean ($\pm$ S.E.M.) of 3 determinations, each performed in triplicate. Lack of error bars indicates that the S.E.M. resides within the size of the symbol. Abbreviations are: Amantadine (A), Memantine (M) and MK-801 (MK).

found with 1. This finding emphasizes the importance of aromaticity. Enlargement of the polycyclic cage (6 and 7) resulted in decreased release of [^{3} H]DA. In contrast, the primary amine 8, afforded release of DA similar to that found with 1. The three established NMDA receptor

antagonists exhibited similar potency in releasing [^{3} H]DA, with amantadine and memantine slightly more active than MK-801. As was expected, the reference compound GBR-12909, a potent DAT inhibitor²⁴ was not very active in this assay.

With some exceptions, structure–activity relationships similar to those for the release of [^{3} H]DA were observed when the compounds were screened for inhibition of [^{3} H]DA uptake (Table 1). Substitution of the aromatic ring of 1 resulted in a more pronounced influence on [^{3} H]DA uptake inhibition than was seen for release of [^{3} H]DA. Substitution of the aromatic ring with nitro groups (3a–c) resulted in the *meta* compound 3b being the most active uptake inhibitor in this group. The inhibition of [^{3} H]DA uptake by 3b is over three times greater than that of its *para* analogue and one third more than that of the *ortho* compound 3c. Substitution with methoxy groups on 1 (4a–c) resulted in the most active inhibitor of [^{3} H]DA uptake in this series, which was the *meta* compound, 4b. Thus, as was seen for [^{3} H]DA release, the rank order of activity for both the nitro and methoxy compounds was found to be *meta* > *ortho* > *para*. We conclude similarly that the *meta* position was preferred for inhibition of [^{3} H]DA uptake, rather than the *ortho* and *para* positions, because of steric rather than electronic effects. The greater activity of *meta*-substituted compounds suggest an optimal binding pocket where this substituent fits into the DAT, compared to 1 with no substitution on the phenyl ring. The *para* position seems to be less favorable in this regard, especially with the nitro group. This finding might indicate a steric interaction that prevents the phenyl ring from forming a bond with the DAT. The greater activity of methoxy group substitution on the phenyl ring of 1 suggests an additional bond formation with the DAT, where a small electron donating effect of

the methoxy group increases hydrophobic/ π - π bonding stabilization with **3b**, compared to **4b**.

Differences in inhibition of dopamine uptake were seen for the 'enlarged cages' in that compound **6** was more active than **7** (Table 1). These differences could be attributed to the hydroxyl group in **6** and the carbonyl group and ether ring linkages in **7**, suggesting that different associations are relevant for the two compounds in their interactions with the DAT. Removal of the side chain to yield the primary amine (**8**) resulted in slightly more activity for this compound than found for **1**, but less activity than found for amantadine. Increasing the distance between the polycyclic cage and the aromatic ring seemed to increase activity for compound **9**. The increased chain length instills more flexibility, which might improve fit at the site of interaction. Memantine exhibited less DA uptake inhibition than did amantadine and **8**. It was surprising that MK-801 exhibited such a potent inhibition of [^{3} H]DA uptake. The reference compound GBR-12909, a potent DAT inhibitor²⁴ was most active in this assay (Table 1). Binding studies done by us using the cocaine analogue WIN 35,248 suggested that these compounds bind to a different site on the DAT than WIN 35,248 (data not shown).

Initial experiments identified compounds that blocked DA uptake without causing appreciable release (<40% at 100 μ M) of the neurotransmitter, in order to assess specificity. We are looking to develop neuroprotective drugs for PD, to be used as a daily preventative or as additional therapy with other compounds that treat PD symptomatically. Selectivity for DA uptake inhibition is desirable since excessive release of DA is thought to be neurotoxic.²⁵ DA uptake inhibition arguably may also be beneficial to patients in the early stages of PD, because decreasing DA uptake might not only result in increased synaptic and decreased intracellular DA concentrations, but also may help slow the progression of the disease. Moreover, in cases where endogenous or exogenous toxins use the DAT as a gateway (e.g., MPP⁺), uptake inhibitors would be ideal neuroprotective compounds.

To this end, we undertook further dose-response studies (Fig. 3) with compounds **1**, **6**, **7**, **9**, as well as the reference compounds amantadine, memantine, and MK-801. Compounds **3b** and **4b** were excluded, because although potent in inhibition uptake assays, they are also potent releasers of DA. We focused on compounds that would be neuroprotective against endogenous or exogenous toxins, by selectively inhibiting DAT. The IC₅₀ values ($\pm$ SEM) of compounds **1**, **6**, and **9** were similar in magnitude to those measured for the reference compounds amantadine and MK-801 (Table 1). In contrast, a higher IC₅₀ value was observed for **7** (Table 1), and memantine was similarly active (IC₅₀ > 100 μ M, Fig. 3). The shallower dose-response slopes of **1**, **6**, **7**, and memantine (Fig. 3) suggest that these compounds might be therapeutically more acceptable.²⁶ Steeper dose-response curves such as those found with **9** (Fig. 3) indicate a narrow index of therapeutic activity that might be compromised in vivo by individual differences

in pharmacokinetics. It is therefore likely that compounds such as **1**, **6**, and **7** may be more viable for future in vivo studies. The slight difference in IC₅₀ values between MK-801 and **9** may be explained by the steeper slope of the dose-response curve of MK-801 compared with **9** (Fig. 3).

The dose-response curves generated for **1**, **6**, **7**, amantadine, and memantine (Fig. 3) show incomplete blockade of [^{3} H]DA uptake ($\leq$ 80%). Possible explanations for this may be low drug penetration to the DAT, affinity differences, or enantiomeric preference for DAT binding with these compounds. In addition, multiple ligand acceptor sites have been reported to exist on the DAT,²⁶ and these may account for the differences in blocking activity. In contrast, **9** and MK-801 exhibited complete inhibition of [^{3} H]DA uptake.

The activity of **9** as an uptake inhibitor of DA in these experiments might offer an explanation for its neuroprotective activity as seen in the MPTP parkinsonian mouse model.¹⁴ The degeneration of dopaminergic neurons by MPTP is initiated by accumulation, via the DAT, of the MAO-B generated pyridinium metabolite of MPTP, MPP⁺. Blocking the DAT might underlie this neuroprotection in striatal neurons by preventing the action of agents or toxins similar to MPTP/MPP⁺ that require access to dopaminergic cells through the DAT.^{25,27} Based on this hypothesis, compounds **1** and **6** would additionally be ideal candidate compounds for screening in the MPTP parkinsonian mouse model.

Monoamine oxidases (MAOs) are thought to be involved in the etiology of PD.²⁸ For example, MPTP is converted by MAO-B to MPP⁺, the active neurotoxin. Inhibition of this enzyme prevents the neurotoxicity of MPTP and increases the amount of free DA that is still present in the synaptic cleft.²⁹ We therefore screened the pentacycloundecylamines, the three NMDA receptor antagonists amantadine, memantine, and MK-801, and the two MAO-B inhibitors deprenyl (a mechanism-based irreversible inhibitor)³⁰ and 2,3,6-trimethyl-1,4-naphthoquinone (TMN, a reversible inhibitor)¹⁵ in a robust MAO-B inhibition assay (Table 1). Although some of the compounds elicited marginal inhibition, none of the pentacycloundecylamines that were found to be selective DAT inhibitors, inhibited MAO-B in any significant way (inhibition > 50%) at therapeutically relevant concentrations. As expected, the known MAO-B inhibitors deprenyl and TMN resulted in complete (95%+) inhibition of the enzyme (Table 1).

During the drug discovery process it is important to consider the pharmacokinetic properties of the drugs evaluated.³¹ Due to the central nervous system site of action of the drugs screened in this study, it is important to mention that the pentacycloundecylamines are capable of crossing the blood-brain barrier. Permeation into the brain was observed in a study done by Zah et al.³² These researchers developed a physicochemical prediction model from in vivo studies, to predict blood-brain distribution of pentacycloundecylamines. Using this predictive equation of Zah et al., and assuming ther-

apeutic serum levels are similar to that of amantadine (9 μM),⁴ Eq 1 gives a predicted CNS concentration of 17 μM for **9**, which is higher than the therapeutic concentration range for amantadine (4–9.5 μM). This predicted concentration (17 μM) is near the IC_{50} value (23 μM) for DAT inhibition by compound **9**, and suggests that compound **9** is a good lead for further drug development as potential neuroprotective therapy in PD.

6. Conclusions

In this report, we describe the syntheses and in vitro biological studies (interaction with the DAT and the enzyme MAO-B) of a novel group of polycyclic cage amines, in order to identify promising compounds for future neuroprotection studies in the well known MPTP parkinsonian mouse model. The cage compounds proved to be effective inhibitors of dopamine uptake, with IC_{50} values comparable to that of amantadine. The most active compound (**9**) had an IC_{50} value of 23 μM . In structure–activity studies, we investigated both aromatic substitution as well as increased size of the cage moiety. All of the polycyclic cage amines showed greater activity for blocking DA uptake than for causing release of DA. For cage compounds with aromatic substituents, no selectivity between uptake inhibition and release of DA was observed. Increasing the cage moiety size enhanced selectivity for DA uptake inhibition. The distance between the aromatic moiety and the cage appears to play a vital role since **9** (phenylethylamine), the compound with the largest distance, was also the most active inhibitor in this series. Structure–activity relationships for this group of compounds therefore appear to be influenced by geometric and steric considerations rather than by electronic characteristics. Results from the in vitro assays suggest that inhibition of the DAT might underlie the neuroprotective effect of **9** in the MPTP parkinsonian mouse model seen in earlier work.¹⁴ Inhibition of MAO-B activity by these compounds occurred in the high (several hundred) micromolar range and likely would not contribute any protection against MPTP. A primary goal of future PD therapy is the development of neuroprotective strategies that will retard or prevent neuronal death.²⁷ The spectrum of activity within the pentacyclicundecylamines makes them candidates for future exploration as possible lead compounds for dual symptomatic and neuroprotective therapies against PD.

7. Experimental

7.1. Chemistry

All melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. The ^1H and ^{13}C spectra were recorded on a Varian VXR 300 spectrometer. Samples were dissolved in a deuterated solvent (CDCl_3) with tetramethylsilane (Me_4Si) as an internal standard. Infrared spectra were recorded as a neat film on KBr plates with a Shimadzu FT IR 4200 spectrophotometer. Mass spectra and HR-MS were recorded at 70 eV (EI) on a VG 7070E spectrometer. Elemental analyses were performed on a Perkin–Elmer model 240 instrument and data were all within accep-

table limits ($\pm 0.04\%$). Amantadine (hydrochloride salt) and memantine (hydrochloride salt) were purchased from Sigma Chemical Co. (St. Louis, MO). GBR-12909 (hydrochloride salt) and (+)MK-801 (maleate) were purchased from Research Biochemicals (Natick, MA, USA). [^3H]Dopamine (20.3 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). *R*-deprenyl and TMN were gifts from Dr. Neal Castagnoli Jr. at the Harvey W. Peters Center, Department of Chemistry, Virginia Tech. Buffer constituents were obtained from other commercial sources.

7.1.1. General synthetic method for benzylamine phenylhydrazine, nitrobenzylamine, methoxybenzylamine, amino-methylpyridine, and phenylethylamine derivatives of pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane. Pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane-8,11-dione (5 g, prepared according to the method of Cookson et al.)²⁰ was dissolved in tetrahydrofuran (THF, 50 mL) and cooled to $\pm 5^\circ\text{C}$ in ice. An equimolar quantity of the desired amine was slowly added under stirring. The white precipitate that formed after about 10 min was filtered and washed with cold THF to render the hydroxylamine. This product was dehydrated in dry benzene under Dean–Stark conditions for 1 h or until no more water collected in the container. Evaporation yielded the Schiff base as a yellowish oil and reduction of this imine was accomplished with sodium borohydride in dry methanol (30 mL) and dry THF (150 mL) for 24 h at room temperature. The solvent was removed under reduced pressure and water (100 mL) was added. The mixture was extracted with dichloromethane (4 $\times$ 50 mL) and the combined organic fractions washed with water before being dried over magnesium sulfate. Evaporation yielded the desired products. Spectral characteristics for the compounds used in this study were previously reported.^{13,14,32} Spectral parameters for the recently published³² compound **9** are given below, with elemental analysis performed by Atlantic Microlab Inc. (Norcross, Georgia, USA).

7.1.2. 8-Phenylethyl-8,11-pentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane (9**).** Off white needles obtained by recrystallization from ethanol (yield 54.0%). $\text{C}_{19}\text{H}_{21}\text{NO}$; mp 49°C ; IR ν_{max} 3311, 2974, 1602, 1495; MS m/z 279 (M^+), 188, 131, 105, 91, 28; ^1H NMR (CDCl_3) δ 1.26 (t, 2H, $J=7.9$, H-14a,4b), 1.70 (AB-q, 2H, $J=10.44$, H-4a,4b), 2.36 (2 $\times$ m, 6H, H-1,2,3,4,5,6), 2.7 (m, 2H, H-15), 4.59 (t, 1H, $J=5.3$, H-11), 7.13–7.28 (m, 5H, H-16,17,18,19,20); ^{13}C NMR (CDCl_3) δ 37.46 (d, 1C), 41.46 (d, 1C), 41.86 (d, 1C), 43.20 (t, C-4), 44.43 (d, 1C), 44.75 (d, 1C), 44.90 (d, 1C), 54.70 (d, C-7 or 9), 55.33 (d, C-7 or 9), 82.43 (d, C-11), 126.14 (d, C-18), 128.44 (d, C-17,19), 128.83 (d, C-16,20), 140.01 (s, C-15). Anal. calcd for $\text{C}_{19}\text{H}_{21}\text{NO}$: C, 81.68; H, 7.58; N, 5.01. Found: C, 81.54; H, 7.55; N, 5.06.

8. Biological methods

8.1. Animals

ICR male mice (20–26 g, 6–8 weeks old) were obtained from Harlan, Dublin, VA. All animal care and experimental

protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee in accordance with guidelines established by the United States Public Health Service and Virginia Tech.

8.2. Preparation of synaptosomes

Male ICR mice were sacrificed by cervical dislocation and the striata were rapidly removed. The striatal tissue was homogenized in 4 mL of sucrose buffer (0.32 M sucrose, 2 mM HEPES, pH 7.4). The homogenate was then centrifuged for 10 min at 1000×g. The supernatant was removed and centrifuged for 15 min at 10,000×g. The resulting pellets were washed once and resuspended in incubation buffer (125 mM NaCl, 5 mM KCl, 1 mM MgCl₂, CaCl₂·2H₂O, 10 mM sucrose and 50 mM Tris-HCl, pargyline 0.05 mM, ascorbate 0.1 mM, pH 7.4).

8.3. Dopamine release studies

After preparation, the final pellet of synaptosomes was resuspended in incubation buffer containing 100 nM [³H]DA and incubated for 5 min at 37 °C, and then re-centrifuged at 10,000×g for 10 min. The supernatant was discarded and the pellets washed once with cold incubation buffer and resuspended. The synaptosomes were aliquoted into tubes, treated with drug, and incubated for 10 min at 37 °C. Test compounds (100 μM final concentration) were dissolved in DMSO, the final DMSO concentration in the incubations not to exceed 0.1%, with the controls receiving 0.1% DMSO alone. Synaptosomes then received 3 mL of wash buffer at 37 °C, filtered through 1 micron glass microfiber filters (Whatman GF/B) under vacuum and washed an additional 3 times with 37 °C buffer, followed by liquid scintillation counting to measure the radioactivity of [³H]DA within the synaptosomes. For each compound screened, fresh striatal tissue was used.

8.4. Dopamine uptake inhibition studies

Test compounds (screened at 100 μM final concentration, dissolved in DMSO as described above) were incubated with synaptosomes in a heat block for 10 min. at 37 °C. Following the incubation, the synaptosomes were incubated with [³H]DA for 2 min. Uptake was terminated by the addition of ice cold wash buffer, followed by vacuum filtration through 1 micron glass microfiber filters (Whatman GF/B). These filters were washed 4 times with 3 mL ice cold wash buffer, air dried, followed by liquid scintillation counting to measure the radioactivity of [³H]DA within the synaptosomes. For each compound screened for DA uptake inhibition and each dose–response replicate, fresh striatal tissue was used.

8.5. MAO-B Inhibition

Intact mitochondria from baboon liver served as the source for MAO-B.³³ The mitochondrial fractions were prepared as described previously.³⁴ The mitochondrial homogenate was suspended in sodium phosphate buffer (100 mM, pH 7.4) containing 50% (w/v) glycerol. Protein

was determined by the method of Bradford.³⁵ The inhibition of MAO-B by the test compounds (100 μg/mL) was evaluated by incubating the substrate (1-methyl-4-(1-methylpyrrol-2-yl)-1,2,3,6-tetrahydropyridine (MMTP; 0.25 mM)²¹ with the mitochondrial homogenate (0.15 mg/mL). Test compounds were dissolved in DMSO and added to the buffered incubation mixture so that the final DMSO concentration did not exceed 4%. The final volume of the incubation mixtures was 500 μL (in sodium phosphate buffer, pH 7.4) and the samples were incubated at 37 °C for 15 min. The reaction was terminated by the addition of 20 μL perchloric acid (70% v/v), centrifuged (16,000×g for 8 min) and the concentration of the of the enzyme-generated metabolite of the substrate in the supernatant fractions determined spectrophotometrically at 420 nm.

8.6. Statistical analyses

Statistical significance for DA uptake and release studies was analyzed using Student's *t*-test (InStatTM, GraphPad Software, San Diego, CA) to find statistically significant effects. Concentration–curve data were analyzed by non-linear least squares fit to a four parameter logistic equation, with resulting IC₅₀ values and goodness of fit statistics calculated using PrismTM (GraphPad Software, San Diego, CA).

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