Selective Retention of β-Carbolines and 7,12-Dimethylbenz[a]anthracene in the Brain

Role of Neuromelanin and Cytochrome P450 for Toxicity

ANNA ÖSTERGREN
Dissertation presented at Uppsala University to be publicly examined in C4:301, BMC, Uppsala, Friday, October 14, 2005 at 09:30 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in Swedish.

Abstract

The β-carbolines norharman and harman structurally resemble the synthetic compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) that is known for its ability to damage neuromelanin-containing dopaminergic neurons of the substantia nigra and thereby induce parkinsonism. MPTP is, however, not normally present in the environment whereas the β-carbolines are present in cooked food and tobacco smoke.

In this thesis it was demonstrated that norharman and harman had affinity to melanin and were retained in neuromelanin-containing neurons of frogs up to 30 days post-injection (the longest survival time examined). It was also demonstrated that norharman induced neurodegeneration, activation of glia cells and motor impairment in mice. Furthermore, this compound induced ER stress and cell death in PC12 cells. An in vitro model of dopamine melanin-loaded PC12 cells was developed in order to study the effect of melanin on norharman-induced toxicity. In this model, melanin seemed to attenuate toxicity induced by low concentrations of norharman. After exposure to the highest concentration of norharman, melanin clusters were disaggregated and there was an increased expression of stress proteins and caspases-3, known to be involved in apoptosis.

The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene was demonstrated to have a CYP1A1-dependent localization in endothelial cells in the choroid plexus, in the veins in the leptomeninges and in the cerebral veins of mice pre-treated with CYP1-inducers.

These results demonstrate that the distribution of environmental compounds could be influenced by the presence of neuromelanin and expression of CYP enzymes in the brain and that norharman may induce neurotoxic effects in vivo and in vitro.

Keywords: β-carboline, neuromelanin, norharman, harman, parkinsonism, Parkinson’s disease, motoric impairment, behaviour, glia cells, substantia nigra, dopamine melanin, PC12 cells, ER stress, grp78, bsp90, cytochrome P450, CYP1A1, CYP1B1, blood-brain interfaces, 7,12-dimethylbenz[a]anthracene, polycyclic aromatic hydrocarbon, endothelial cell, smooth muscle cell, bioactivation

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Two roads diverged in a wood, and I -
I took the one less travelled by,
And that has made all the difference

-Robert Frost-

To those who love me
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Professor Eva B Brittebo

Co-supervisor
Professor Nils Gunnar Lindquist
Doctor Anne-Lie Svensson

Faculty opponent
Professor Hans Tjälve

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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>B_max</td>
<td>Binding maximum</td>
</tr>
<tr>
<td>BNF</td>
<td>β-Naphthoflavone</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-Dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>Grp78</td>
<td>Glucose regulating protein 78</td>
</tr>
<tr>
<td>Harman</td>
<td>1-Methyl-9H-pyrido[3,4-b]-indole</td>
</tr>
<tr>
<td>HCA</td>
<td>Heterocyclic amines</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>K_d</td>
<td>Equilibrium constant of dissociation</td>
</tr>
<tr>
<td>MPP-</td>
<td>1-Methyl-4-phenylpyridinium ion</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTT</td>
<td>3-((4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>Norharman</td>
<td>9H-Pyrido[3,4-b]-indole</td>
</tr>
<tr>
<td>n.s.</td>
<td>No statistical difference</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>PC12</td>
<td>Cell line from rat pheochromocytoma</td>
</tr>
<tr>
<td>PCB126</td>
<td>3,3',4,4',5-Pentachlorobiphenyl</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotide transferase mediated deoxyuridine triphosphate nick end labelling</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
</tbody>
</table>
INTRODUCTION

It is likely that several disorders of the brain such as parkinsonism and stroke are results from multiple events and interactive mechanisms. These may include genetic predisposition as well as environmental factors. Pathological mutations have been identified in some genes (Ross and Farrer 2005). Nevertheless, twin studies provide only a limited support for a genetic aetiology, but demonstrate a substantial environmental component in most late onset cases of Parkinson’s disease (PD) (Tanner et al. 1999). The risk factors for parkinsonism that have been identified in epidemiological studies include well-water drinking, rural living and exposure to pesticides, herbicides, industrial chemicals and metals such as manganese (as reviewed by Di Monte et al. 2002).

One of the most commonly used model neurotoxicant is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) known for its ability to induce parkinsonism in humans, primates and some laboratory animals (Langston et al. 1983; Heikkila and Sonsalla 1992). Since MPTP is not normally present in the environment, other compounds with structural resemblance to MPTP have been suggested to be contributing factors for development of PD, among these compounds are some heterocyclic amines (HCA) including the subgroup of β-carbolines. Other HCA (i.e. γ-carbolines) and some polycyclic aromatic hydrocarbons (PAHs) have been identified for their ability to form adducts in blood vessels after bioactivation by xenobiotic-metabolizing cytochrome P450 (CYP) enzymes (Annas and Brittebo 1998; Zhang et al. 1998; Granberg et al. 2000). Therefore it has been suggested that blood vessels may be a target of PAH-induced toxicity, which is supported by epidemiological studies indicating that exposure to PAHs could be of importance for development of atherosclerosis and stroke (Hansen 1990; Bonita et al. 1999). Despite that, there is limited knowledge about the uptake, biotransformation and effects of HCA and PAHs in the brain.
MPTP is one of the most commonly used model neurotoxicants for induction of parkinsonism. In humans and primates low doses of MPTP cause irreversible parkinsonism (Langston et al. 1983; D’Amato et al. 1987), whereas higher doses are needed in rodents where the effects are often reversible (Petroske et al. 2001).

MPTP was first synthesized by Ziering and co-workers in 1947. It was ironically examined clinically as a treatment for PD and depression (Lewin 1984). It was not until 1982 when a large group of students in California developed symptoms of PD within days after administration of MPTP-contaminated drugs that MPTP was identified as a parkinsonism-inducing agent (Langston et al. 1983). Neuropathological studies of some of the students revealed depletion of neuromelanin-containing neurons in the substantia nigra and in one case large amounts of extraneuronal neuromelanin was found (Langston et al. 1999). Gliosis and clustering of microglia around neurons were also observed but no Lewy bodies.

After systemic administration, MPTP readily crosses the blood-brain barrier, and is converted to a 1-methyl-4-phenylpyridinium ion (MPP+) by monoamine oxidase B in astrocytes. MPP+ is then selectively accumulated in neuromelanin-containing dopaminergic neurons of substantia nigra, where it inhibits the complex I (ubiquinone oxidoreductase) of the mitochondrial respiratory chain. Cell death results from a complex interplay among various phenomena such as impairment of adenosine 5’-triphosphate (ATP)-production and oxidative stress (as reviewed by Przedborski and Jackson-Lewis 1998). The neurons that are affected by MPTP are preferentially dopaminergic neurons that contain neuromelanin (Herrero et al. 1993). MPTP has also been shown to have affinity for melanin and this has been proposed to be a mechanism behind the selective vulnerability of the neuromelanin-containing neurons (Lydén et al. 1983; Sokolowski et al. 1990). Pre-treatment with chloroquine, that could block binding sites for MPTP on the neuromelanin, inhibits MPTP-induced toxicity in primates, suggesting that the MPTP binding to melanin is of importance for the ability of MPTP to cause neurotoxicity (D’Amato et al. 1987).

MPTP is not normally present in the environment but it has been suggested that other compounds that are present in our environment may act in a way similar to MPTP and thereby contribute to the development of PD. Particular interest has been drawn to compounds that structurally resemble MPTP, such as the β-carbolines.
The β-carbolines norharman (9H-pyrido[3,4-b]-indole) and harman (1-methyl-9H-pyrido[3,4-b]-indole) are formed by heating of amino acids such as tryptophan and tryptamine during cooking of proteinaceous materials (Johansson et al. 1995; Felton et al. 2000).

The β-carbolines are present in coffee, tobacco smoke and alcoholic beverages. For an overview of the levels of norharman and harman in food products, see table 1. Certain plants can also biosynthesize norharman and harman (as reviewed by Rommelspacher and Susilo 1985; Bourke et al. 1992).

### Table 1

<table>
<thead>
<tr>
<th>Product</th>
<th>Levels of norharman (ng/g or ng/ml)</th>
<th>Levels of harman (ng/g or ng/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>1.2-795.0</td>
<td>3.6-169.0</td>
<td>Totsuka et al. 1999</td>
</tr>
<tr>
<td>Pork</td>
<td>2.4-59.6</td>
<td>0.6-32.5</td>
<td>Totsuka et al. 1999</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.1-6.9</td>
<td>0.3-7.5</td>
<td>Solyakov and Skog 2002</td>
</tr>
<tr>
<td>Fish</td>
<td>1.3-40.7</td>
<td>2.0-8.4</td>
<td>Herraiz 2000</td>
</tr>
<tr>
<td>Pan residue</td>
<td>52.6</td>
<td>14.0</td>
<td>Solyakov et al. 1999</td>
</tr>
<tr>
<td>Wine</td>
<td>0.3-0.7</td>
<td>0.2-2.5</td>
<td>Adachi et al. 2000</td>
</tr>
<tr>
<td>Beer</td>
<td>2.7-22.7</td>
<td>0.7-5.4</td>
<td>Rommelspacher et al. 1994</td>
</tr>
<tr>
<td>Coffee</td>
<td>450-91400</td>
<td>340-22380</td>
<td>Herraiz 2002</td>
</tr>
<tr>
<td>Cigarette smoke condensate</td>
<td>133000-623000</td>
<td>478000-849000</td>
<td>Totsuka et al. 1999</td>
</tr>
</tbody>
</table>
In mammals norharman can also be biosynthesized endogenously with tryptamine or tryptophan as precursors (Rommelspacher 1981; Fekkes et al. 2001a). Fekkes and co-workers (2001a) reported that ingestion of tryptophan resulted in a low increase of the plasma concentration of norharman, but concluded that the main norharman content in the body came from exogenous sources.

**Tissue distribution and levels**

In rats the normal concentration of norharman is reported to be 0.0011 ± 0.0003 µM in plasma and 12.3-22.5 ng/g tissue in heart, brain, liver, lung, spleen and kidney (Fekkes and Bode 1993). After an intraperitoneal (i.p.) injection of norharman (2 mg/kg) the concentrations are increased in all the above tissues with the highest level in spleen, followed by liver, brain, kidney, lung and heart. The level in the brain after administration of norharman was more than four times higher than that in plasma (Fekkes and Bode 1993).

Several binding sites for norharman have been identified in rat brain, including substantia nigra (Pawlik et al. 1990). Since norharman also bound to nucleus accumbens it was suggested that norharman was able to influence the dopamine reward system and Baum and co-workers (1995) reported that injections of norharman in rat caused an increased concentrations of dopamine in this brain region. Norharman is also a potent ligand for benzodiazepine binding sites (Müller et al. 1981; Rommelspacher et al. 1981).

In post-mortem brain from humans without signs of neurodegeneration, the concentration of norharman and harman is significantly higher in the substantia nigra (3.28 ± 1.64 respectively 0.21 ± 0.02 ng/g tissue) than in cortex (0.12 ± 0.02 respectively 0.05 ± 0.01 ng/g tissue) (Matsubara et al. 1993).

The levels of norharman and/or harman in humans are increased in some disorders. For example chronic alcoholics have increased levels of norharman in plasma and urine (Spies et al. 1995), although the results from other studies indicate that smoking might be the main cause of the increased levels of β-carbolines in alcoholics (Breyer-Pfaff et al. 1996; Spijkerman et al. 2002). The levels of norharman and harman have also been reported to be increased in plasma and lumbar cerebrospinal fluid (CSF) from individuals with PD (Kuhn et al. 1995; Matsubara et al. 1995) and patients with essential tremors have increased plasma levels of harman (Louis et al. 2002).

High affinity binding sites for norharman and harman have also been identified in the liver where norharman binds to the microsomal membrane fraction of rat liver membranes while harman binds to mitochondrial membranes (May et al. 1994; Stawowy et al. 1999).

**Metabolism and bioactivation**

Norharman is mainly metabolized in the liver, where its half-life is 20 minutes (Fekkes and Bode 1993). It has also been reported that norharman binding in rat liver microsomes can be inhibited by CYP2E1 ligands and indole-3-carbinol.
Role of Neuromelanin and Cytochrome P450 for Toxicity

(Stawowy et al. 1999). Furthermore, norharman has been demonstrated to inhibit the metabolism of the CYP1 substrate benzo[α]pyrene in rat liver microsomes (Fujino et al. 1978). Norharman and harman are also reported to be methylated by S-adenosyl-L-methionine-dependent N-methyltransferase on both the pyridyl (2N-) and indole (9N-) nitrogens, forming a metabolite that resembles MPP⁺, as shown in figure 2 (Gearhart et al. 1997). In the brain the N-methyltransferase activity is normally highest in substantia nigra and this enzyme is increased in post-mortem brains from PD-patients (Gearhart et al. 2000). As for the parental compound norharman, the concentrations of the 2,9N-methylated norharman ions are higher in substantia nigra than in cortex (Matsubara et al. 1993) and the levels are also elevated in CSF of PD-patients (Matsubara et al. 1995).

In vivo effects

Humans
Plants containing harmala alkaloids such as β-carbolines have been used as spices and hallucinogens (reviewed by Rommelspacher 1981). After ingestion of these plants humans fall into a contemplative mood accompanied with vivid fantasies. Intake of higher doses leads to the sensations of flying or being transformed into a bird (reviewed by Rommelspacher 1981). The doses of norharman in these reports are, however, difficult to evaluate. In the pharmacokinetic study by Fekkes and co-workers (2001b) neither a peroral administration of 7 or 65 µg/kg nor a sublingual administration of 6.5 µg/kg caused any behavioural alterations.

Experimental animals
Several studies have reported that norharman and harman alter the behaviour in rodents. As presented in table 2, the initial effects of norharman are mainly convulsions and impairment of motor activity. Similar effects have also been reported after administration of harman (Rommelspacher et al. 1981) and after administration of 2N- and 9N-methylated norharman (Matsubara et al. 1998a). Furthermore, norharman and harman (up to 54 mg/kg) are reported to induce behavioural changes such as hypomotility, head tremor, limb paresis and mild sedation in sheep (Bourke et al. 1992). All these behavioural studies have been performed shortly after administration. In behavioural studies performed soon after
the last treatment it may be difficult to distinguish between persistent changes in motor activity and temporal inactivity resulting from acute effects due to receptor interactions. Additionally, the handling during administration of the neurotoxicant may be stressful to the animal and influence the motor performance.

### Table 2

<table>
<thead>
<tr>
<th>Effects</th>
<th>Dose (mg/kg)</th>
<th>Time after administration</th>
<th>Administration route</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Convulsions</td>
<td>24.5</td>
<td>0-1 hours</td>
<td>i.v.</td>
<td>Rommelspacher et al. 1981</td>
</tr>
<tr>
<td>Dishminished movements, exploratory behaviour,</td>
<td>100</td>
<td>0-1 hours</td>
<td>i.p.</td>
<td>Fekkes and Bode 1993</td>
</tr>
<tr>
<td>loss of rightening reflexes, total muscle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>relaxation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradykinesia</td>
<td>84x2 for 7 days</td>
<td>2 days</td>
<td>i.p.</td>
<td>Matsubara et al. 1998a</td>
</tr>
</tbody>
</table>

Norharman, 2N-, 2,9N-methylated norharman and 2,9N-methylated harman have furthermore been reported to reduce the striatal levels of dopamine after direct injection into substantia nigra in rats. Nigral cell loss was also observed after the intranigral administration (Neafsey et al. 1995). Reduction of dopamine in striatum is also reported in mice after i.p. injections of norharman and its 2N- and 9N-methylated metabolites where the dopamine decrease was combined with loss of the number of tyrosine hydroxylase (TH)-positive neurons in substantia nigra (Matsubara et al. 1998a).

Selective uptake by dopamine transporters (DAT) has been suggested as a mechanism behind selective toxicity on dopaminergic neurons. Norharman and harman are, however, unfavourable substrates for the DAT whereas their N-methylated metabolites could use DAT for uptake in dopaminergic neurons (Drucker et al. 1990; Matsubara et al. 1998b).

### In vitro effects

Exposure to norharman or harman (250-500 µM) for three hours has been reported to cause DNA damage in a cell line from human neuroblastoma (SH-SY5Y cells) (Uezono et al. 2001).
The 2,9N-methylated metabolites of norharman (500 µM) and both 2N- and 2,9N-methylated metabolites of harman (500 µM) have been reported to cause increased lactate dehydrogenase release in a cell line from rat pheochromocytoma (PC12 cells) after 48 hours of exposure (Collins et al. 1992; Cobuzzi et al. 1994). The 2,9N-methylated norharman metabolite has also been reported to have a selective effect on dopaminergic neurons in primary mesencephalic cell cultures from rat. After incubation with norharman (1 µM) for two days there was a 80% decrease in the number of TH-positive neurons, whereas the concentration had to be increased to more than 10 µM to affect TH-negative neurons (Matsubara et al. 1998a).

Metabolites of norharman and harman have been suggested to cause their effects by inhibition of the mitochondrial respiratory chain, although only the 2,9N-methylated norharman and harman are mitochondrial inhibitors whereas the parent compounds and the 2N-methylated metabolites only display weak effects (Albores et al. 1990; Collins et al. 1992).

**NEUROMELANIN**

“The neuromelanin granule may be the secret key to the understanding of Parkinsonism. I don't believe God put the melanin in the central nervous system for nothing. It must be doing something. Something big…”

G C Cotzias

**Melanogenesis and occurrence of melanin**

The origin of the name melanin, from the Greek word *melanos* (dark), is usually attributed to the Swedish chemist Berzelius (reviewed by Fedorow et al. 2005).

Melanin is an extremely dense and insoluble chromophore of high molecular mass (reviewed by Sarna 1992; Usunoff et al. 2002). The melanin granules are mixtures of more or less similar polymers linked through heterogenous non-hydrolysable bonds where covalent bonds are the most common (Bridelli et al. 1999).

In humans, melanin is formed in melanocytes, pigment epithelial cells and in neurons of substantia nigra and locus coeruleus. The melanin in melanocytes and epithelial cells is produced from L-tyrosine under the influence of tyrosinase in melanosomes in the basal layer of the epidermis of the skin, the retinal pigment epithelium and uveal tract of the eye, the hair matrix, the inner ear, in mucus membranes and in leptomeninges of the central nervous system (as reviewed by Sarna 1992; Fedorow et al. 2005).

In general, melanin has been suggested to protect tissues and cells. For example, the main action of melanin in human skin is to absorb UV-light and attenuate UV-penetration (reviewed by Fedorow et al. 2005). Melanin can also scavenge reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide, formed during biochemical and photochemical reactions (Sarna 1992). Morphologically, the
distribution of melanin gives further support for a protective role. Melanin has been suggested to act as a filter that prevents noxious chemicals from reaching receptor cells. This is supported by the localization of melanocytes in close proximity around capillaries and that nutrients for the receptor cells in the eye and in the inner ear are passing through the pigmented epithelia (Lindquist and Ullberg 1975).

In contrast to melanin in melanocytes and epithelia, neuromelanin is thought to be formed by oxidative polymerization of dopamine or noradrenaline, with the possible involvement of cysteinyldervatives (as reviewed by Usunoff et al. 2002; Zucca et al. 2004). Neuromelanin is a granular, dark brown pigment, which is almost exclusively found in dopaminergic neurons of substantia nigra and noradrenergic neurons of locus coeruleus (Bazelon et al. 1967; Bogerts 1981). In heavily pigmented neurons, neuromelanin granules are closely packed in clusters that are mainly found in the axon hillock and initial portion of the axon (figure 3). Neuromelanin is less abundant in the dendrites and is absent in the neuronal processes remote from the cell bodies, the synaptic nerve endings and the glial cells (Fedorow et al. 2005).

Also the distribution of neuromelanin suggests that it has a protective role, since it could protect the nucleus from catecholamines and their metabolites. The synthesis of neuromelanin itself has been proposed to represent a mechanism where highly reactive compounds such as quinones and semiquinones originating from excess of cytosolic catecholamines are incorporated into inert neuromelanin polymers (Sulzer et al. 2000; Zecca et al. 2003).

Figure 3. Schematic drawing of a neuromelanin-containing neuron with nucleus (n) and neuromelanin (nm) in the axon hillock and in the initial portion of the axon.

During life there is a gradual accumulation of neuromelanin in the dopaminergic neurons with a maximum around 60 years of age, followed by a decline (Mann and Yates 1983; Zecca et al. 2002a). The reason for the decrease in neuromelanin with age is not clear although it could depend on a selective loss of dopaminergic neurons.

A more pronounced selective loss of neuromelanin has been observed in parkinsonism, as best described in patients with PD where the level of neuromelanin is less than 50% of that seen in age matched controls (Zecca et al. 2002a). The general view is that neuromelanin-containing neurons are the most vulnerable to degeneration in parkinsonism (Mann and Yates 1983; Hirsch et al. 1988). Some studies have indicated that the level of neuromelanin may also decrease in surviving neurons (Mann and Yates 1983; Kastner et al. 1992).

After a cell loss of 80% of the dopaminergic neurons, the nigrostriatal pathway
is disrupted and symptoms of parkinsonism become apparent. The most noticeable symptom in parkinsonism is impaired motor performance with symptoms such as bradykinesia, akinesia, loss of postural reflexes, tremor at rest, rigidity and involuntary movements (Fahn 2003). The diagnosis parkinsonism is defined by the presence of two of the above motor symptoms, and at least one of them has to be tremor at rest or bradykinesia (Fahn 2003). The most common form of parkinsonism is PD which aetiology is incompletely understood.

**Melanin affinity**

Melanin contains a large number of free carboxylic acid residues and semiquinones, which are responsible for the cation exchange properties observed for melanin and are thought to provide most of the ionic binding sites. There are also other types of melanin binding including hydrogen bonds and van der Waal’s attractions (Larsson and Tjälve 1979).

In vivo there is an accumulation of different compounds to melanin. In studies where neuromelanin has been analysed, several metals such as iron, zinc, copper, manganese, chromium, cobalt, mercury, lead and cadmium were detected in the polymer (Zecca et al. 2002b). Other compounds that has been reported to bind to melanin include calcium (Liu et al. 2004), PAHs such as 7,12-dimethylbenz[a]-anthracene (DMBA) and benzo[a]pyrene (Roberto et al. 1996), neurotoxicants such as MPTP/MPP+ (Lyden et al. 1985; D’Amato et al. 1986), pesticides like paraquat (Larsson and Tjälve 1979; Lindquist et al. 1988) and drugs such as chlorpromazine and chloroquine (Lindquist 1973; Larsson and Tjälve 1979).

The binding of toxicants to melanin can probably protect tissues against toxic insults after occasional exposures. The binding is, however, normally slowly reversible and can furthermore be inhibited by pre-exposure of other compounds with higher melanin affinity (Larsson and Tjälve 1979). Thereby melanin may serve as a depot from which stored toxicants may be slowly released into the cytosol and trigger oxidative stress or induce cell death. For example, long-term treatment with chloroquine has been reported to cause oculotoxicity (Lindquist 1973; Dayhaw-Barker 2002).

Neuromelanin can also act as a pro-oxidant, stimulating the production of ROS due to its loading of metals and interaction with the cellular environment. For example, when high concentrations of iron are bound to neuromelanin its high-affinity binding sites are saturated. The excess metal binds to low-affinity iron binding sites where ferrous iron may remain redox-active or alternatively be released and stimulate hydroxyl radical production through the Fenton reaction (Youdim et al. 1989; Faucheux et al. 2003). This is supported by results demonstrating that in vitro incubations of iron with human neuromelanin or dopamine melanin stimulate rather than decrease oxidative tissue damage (Mochizuki et al. 1993; Double et al. 1999).

On the other hand the melanin binding could also scavenge hydroxyl radicals (Korytowski et al. 1995) and cells with melanin are protected against hydrogen
peroxide-induced toxicity (Hoogduijn et al. 2004). This protection may not only be limited to an antioxidant defence but may also be connected to the ability of melanin to bind to calcium since exposure to hydrogen peroxide and other ROS has been shown to increase the levels of intracellular calcium (Hoogduijn et al. 2004).

**NEUROTOXIC EVENTS PROCEEDING PARKINSONISM**

**Mitochondrial dysfunction and oxidative stress**
Several neurotoxicants including MPTP and 6-hydroxidopamine (6-OHDA) induce mitochondrial impairment by inhibiting mitochondrial complex I (as reviewed by Schober 2004). These findings imply that inhibition of the mitochondrial respiratory chain may cause a selective loss of dopaminergic neurons in the substantia nigra. A selective decrease (30-40%) in activity of the mitochondrial respiratory chains from substantia nigra of post-mortem brains from PD-patients compared to matched patients without neurological or psychiatric diseases has also been reported (Schapira et al. 1990).

Mitochondrial dysfunction often results in oxidative stress and MPTP, 6-OHDA and rotenone produce ROS by inhibiting the mitochondrial respiratory chain. The oxidative stress can damage the mitochondria and thereby induce a release of proapoptotic factors such as cytochrome c that triggers caspase-9 (Liang et al. 2004). Oxidative stress has also been reported to activate the Fas ligand, which in turn can activate the caspase-8/caspase-3 pathway. Furthermore, mitochondrial ROS generation may promote calcium release from the endoplasmic reticulum (ER), resulting in disturbed calcium homeostasis that may further promote production of ROS (Jacobson and Duchen 2002; Gibson and Huang 2004).

Dopaminergic neurons have been reported to be particularly sensitive to oxidative stress and some reports have suggested that this sensitivity is due to the fact that dopamine easily transforms into dopamine adducts and quinones (Weingarten and Zhou 2001). Others have suggested that neuromelanin has a prominent role for the sensitivity since the melanin has an iron-content, which is suggested to promote oxidative stress through the Fenton reaction (Youdim et al., 1989). However, in humans it has been shown that when the neuromelanin content is reduced, the cells become more susceptible to oxidative damages. Increased redox activity has for example been detected in PD-patients (+69%) and was highest in patients with the most severe neuronal loss (Faucheux et al. 2003).

**ER stress**
ER stress is triggered by the loss of calcium homeostasis or an accumulation of unfolded/misfolded proteins in the ER lumen. ER stress induces a highly specific “unfolded protein response” (UPR) (Kaufman 1999). Glucose regulating protein
Role of Neuromelanin and Cytochrome P450 for Toxicity

78 (grp78) is considered to be a key regulator of ER stress transducers due to its binding to the endoribonuclease, the serine/threonine kinase and the basic leucine-zipper transcription factor in unstressed cells. During ER stress all three of these transducers are released from grp78 and UPR target genes are activated (Lee 2005). These reactions are attempts to reduce the load on the ER and prevent a subsequent cellular damage. However, when ER stress is severe or long lasting there is an induction of apoptosis.

ER stress is suggested to be a common denominator for neurodegenerative diseases like PD and model neurotoxics like MPTP/MPP+, 6-OHDA and rotenone are reported cause ER stress by producing an increased amount of oxidized proteins (Ryu et al. 2002; De Iuliis et al. 2005). ER stress may also contribute to the inhibition of mitochondrial respiration by promoting cytochrome c release (Hori et al. 2002). This indicates a mechanistic commonality of parkinsonism-inducing neurotoxicants that is beyond their effect on the mitochondria.

Activation of glia cells

Astrocytes have important roles such as maintenance of ion homeostasis, provision of energy substrate to neurons and uptake of neurotransmitters (Kandel et al. 1991). Several studies have reported that astrocytes can confer neuronal protection through the synthesis and release of glutathione (as reviewed by Teismann et al. 2003).

In response to any kind of injury in the central nervous system the astrocytes become activated, proliferate and increase in size. The astrocytic processes also become larger and more numerous and there is an increase of glial fibrillary acidic protein (GFAP) content (Eng 1985; Pekny and Nilsson 2005). Activated astrocytes are reported to produce inflammatory mediators such as tumour necrosis factor (TNF)-α (Johnstone et al. 1999), that can stimulate activation of astrocytes and other glia cells and thereby amplify and promote the glial response and consequently the glia-related injury to neurons. TNF-α may also bind its receptor on dopaminergic neurons and thereby trigger apoptotic signals (as reviewed by Teismann et al. 2003).

Furthermore, it has been proposed that astrocytes also can produce ROS and nitric oxide and also thereby promote neuronal degeneration (Johnstone et al. 1999).

Necrosis versus apoptosis

Necrosis and apoptosis are two distinct forms of cell deaths. Necrosis is always a pathological process where a large number of cells die. It is characterized by cellular swelling due to loss of ionic homeostasis, damage to organelles and cell lysis.

The term apoptosis from the Greek term for “dropping off” of leaves from a healthy plant, was coined by Kerr and co-workers (Kerr et al. 1972). In apoptosis, the plasma membrane stays intact while the nucleus fragmentizes, the chromatin condensates and the cytoplasm shrink. Subsequently fragments of the cells are packed into apoptotic bodies, which on their surface membranes express ligands
that are recognized by phagocytes (Kerr et al. 1972). Thereby apoptosis may affect only selective cells.

There are two pathways through which apoptosis is induced. One is the extrinsic pathway that involves death receptors and is exemplified by Fas-mediated caspase-8 activation. The other is the intrinsic pathways that involves the mitochondrial and/or the ER pathway that activate caspase-9 and caspase-12 respectively (Ferri and Kroemer 2001; Ueda et al. 2002). As shown in the schematic overview of apoptosis in figure 4, these pathways converge on caspase-3 activation resulting in nuclear degradation and cellular morphological changes (Ferri and Kroemer 2001; Ueda et al. 2002).

There are several reports of apoptosis in parkinsonism. In post-mortem brains of PD-patients as well as in brains of MPTP- or 6-OHDA-treated rats the levels of pro-apoptotic cytokines such as TNF-α are increased while the levels of anti-apoptotic neurotrophins are decreased (reviewed by Teismann et al. 2003; Nagatsu and Sawada 2005).

Figure 4. Schematic overview of some apoptotic pathways.

Apoptosis induced by MPTP/MPP⁺ is considered to mainly be induced by events in the mitochondria, including release of cytochrome c, caspase-9 and caspase-3 (Kaul et al. 2003). Apoptosis can also be preceded by ER stress that can be induced by 6-OHDA (Ryu et al. 2002). ER stress can activate caspase-12, which in turn can activate caspase-3 and/or caspase-9 (as reviewed by Groenendyk and Michalak 2005).
Although apoptosis is the preferential cell death after exposure to MPTP and 6-OHDA, high concentrations of these neurotoxicants induce necrosis (Hartley et al. 1994). Since apoptosis is an energy dependent (ATP-requiring) process, the deviation from apoptosis to necrosis may depend upon the lack of ATP (Eguchi et al. 1999). This may explain why high concentrations of neurotoxicants preferably induce necrosis.

**Blood-brain interfaces**

*Endothelial cells*

Xenobiotics are transported via the blood and have to pass the blood-brain barrier to reach the brain. The blood-brain barrier consists of capillary endothelial cells (EC) that are tightly joined together and glia cells that provide a mechanical and physical support (Kandel et al. 1991).

Toxic damage to EC and to the vascular smooth muscle cells (SMC) surrounding the blood vessels have been proposed as key events in the development of atherosclerosis and stroke (Ross 1993).

Similar to other cell types, both cerebral EC and choroidal epithelial cells contain a variety of drug metabolizing enzymes, including CYP enzymes. Many are inducible and capable of converting xenobiotics to more water-soluble species, thereby preventing them from entering the brain. The role of CYP1A1 in endothelial linings of the brain has, however, not been clarified although studies have shown a strong correlation between the induction of CYP1A1 activities in EC and endothelial dysfunction such as impaired barrier function, oedema and cardiovascular dysfunction (Guiney et al. 1997).

*7,12-Dimethylbenz[a]anthracene*

**Formation and occurrence**

DMBA is a synthetic compound that often is used as model compound for PAHs. PAHs are formed by the incomplete combustion or pyrolysis of organic material during certain industrial processes such as work with machines that produce sparks, combustion in engines and oil heating. Occupational exposures also occur during work with coal tar, asphalt and chimney sweeping (as reviewed by Boffetta et al. 1997). Another major source for PAH exposure is tobacco smoke (Lodovici et al. 2004).

**Tissue distribution**

DMBA has been demonstrated to bind to the liver and to melanin in pigmented mice (Roberto et al. 1996). In albino mice, DMBA binding was primarily observed
in the liver (Granberg et al. 2000). After pre-treatment with the CYP1-inducer β-naphthoflavone (BNF) or 3,3',4,4',5-pentachlorobiphenyl (PCB126) there was, however, a selective irreversible binding of radioactivity in EC of arteries and veins in albino mice (Granberg et al. 2000).

**Metabolism and bioactivation by CYP1 enzymes**

CYP enzymes are heme-containing proteins that oxidize, hydrolyze or reduce compounds. Based on the sequence similarity, CYP enzymes are categorized into families and subfamilies. The mammalian CYP1 family consists of CYP1A1, CYP1A2 and CYP1B1. CYP1A1 and CYP1B1 have been detected in various extrahepatic tissues, whereas CYP1A2 is mainly localized in the liver (as reviewed by Nebert et al. 2004). The CYP1 enzymes are regulated by the aryl hydrocarbon receptor. Although the purpose of the enzymatic activity is to facilitate the elimination of xenobiotics, CYP1 enzymes have also been reported to bioactivate numerous PAHs including DMBA into reactive metabolites such as epoxides and diolepoxides (figure 5), metabolites that can form DNA-, RNA- or protein adducts. For DMBA, this bioactivation is primarily performed by CYP1A1/2 enzymes, but also by CYP1B1 (Shimada et al. 1996).

![Figure 5. Metabolic bioactivation of DMBA leading to the ultimate toxicants (+)-DMBA-4R,3S-diol-2S,1R-epoxide.](image)

**Vascular effects**

Epidemiological studies have reported an increased risk for stroke and atherosclerosis among persons exposed to PAHs through direct and indirect tobacco smoking (Bonita et al. 1999). There is also evidence that PAHs can induce atherosclerotic lesions in experimental animals (Penn 1990; Wakabayashi 1990) and induce apoptosis in human umbilical venous endothelial cells (HUVEC) (Wang et al. 2001). The mechanism behind these events are complex and not completely understood (Powell 1998). In concordance with results from benzo[a]pyrene (Ramos 1999) it is likely that the electrophilic dihydrodiols of DMBA bind covalently to bases of DNA, while mono-hydroxylated metabolites of DMBA form quinones that can participate in redox cycles and thereby generate ROS.
AIMS

The overall objective of this thesis has been to study the uptake and distribution of β-carbolines and DMBA in the brain and to identify mechanisms for selective retention and toxicity. Furthermore the potential of β-carbolines for induction of parkinsonism was examined.

The specific aims of the present thesis were:

* **Paper I**
  To investigate the role of melanin for uptake and distribution of the β-carbolines norharman and harman in the brain of mice and frogs, and to study melanin-binding of these β-carbolines in vitro.

* **Paper II**
  To investigate if norharman can induce neurodegeneration in substantia nigra and/or induce behavioural impairment in C57BL/6 mice.

* **Paper III**
  To develop an in vitro system of PC12 cells with and without melanin that could be used for studies of the role of melanin in chemical-induced toxicity.

* **Paper IV**
  To compare the effects of norharman in conventional PC12 cells without melanin and in melanin-loaded PC12 cells.

* **Paper V**
  To examine the expression of CYP1A1 and CYP1B1 in the brain and to investigate the role of these enzymes for the distribution of DMBA in the brain.
METHODS

The methods that have been used in this thesis are listed in table 3. The reader is referred to “Material and Methods” in the specific papers for details.

Table 3
Methods used in this thesis

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<td>Light-microscopic autoradiography</td>
<td>I and V</td>
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<td>I, II and V</td>
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<tr>
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<td>V</td>
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RESULTS AND DISCUSSION

MELANIN-DEPENDENT LOCALIZATION OF β-CARBOLINES

In parkinsonism there is a selective degeneration of neuromelanin-containing dopaminergic neurons. Earlier studies have demonstrated that norharman and harman are unfavourable substrates for the DAT (Drucker et al. 1990; Matsubara et al. 1998b), therefore it was of interest to study if other factors such as melanin-binding could be of importance for a selective distribution of norharman and harman in the brain.

As presented in paper I there was a selective localization of radioactivity in pigmented tissues and in the liver of C57BL/6 mice injected with ^3^H-norharman or ^3^H-harman (figure 6).

**Figure 6.** Autoradiograms of pigmented C57BL/6 mice 30 minutes (A), four hours (B), 24 hours (C) and five days (D) after an i.v. injection with ^3^H-harman. A selective localization of radioactivity (as demonstrated by white areas) was observed in melanin-containing tissues.
The high level of radioactivity remained in the melanin-containing tissues up to five days after injections (the longest survival time examined), indicating both a selective distribution and a long-time retention of radioactivity in melanin-containing tissues such as the skin and the eyes. In albino NMRI mice no such distribution or retention of β-carbolines was observed.

Both pigmented and albino mice are known to lack neuromelanin. In order to examine whether neuromelanin can mediate an uptake in discrete brain regions, the distribution of radioactivity after injection of 3H-norharman and 3H-harman was also examined in frogs. Amphibians such as frogs are known to accommodate neuromelanin. In this species there was a selective localization and retention of radioactivity in pigmented parts of the brain still after 30 days post injection (the longest survival time examined). The radioactivity in the pigmented brain regions could not be removed by extensive extractions with organic solvents suggesting that the β-carbolines were not bound to the lipid component of neuromelanin but to the actual melanin. These results were confirmed by light-microscopic autoradiography, which revealed that there was a distinct and selective distribution of silver grains in pigmented neurons (figure 7).

![Figure 7](image)

**Figure 7.** Light-microscopic autoradiograms of frogs 30 days after an i.p. injection with 3H-norharman (A) and 3H-harman (B). A selective binding of radioactivity (as demonstrated by black silver grains) was observed in melanin-containing neurons.

Neuromelanin in the mammalian brain is strictly accumulated in the catecholaminergic neurons, whilst the neuromelanin in frogs has a more general distribution (reviewed by Usunoff et al. 2002). In paper I the frog melanin-containing neurons with silver grains were also TH-positive in an immunohistochemical staining. These results demonstrate that norharman and harman were bound to dopaminergic melanin-containing neurons.

In post-mortem brains of humans the levels of norharman and harman are reported to be higher in substantia nigra than in cortex (Matsubara et al. 1993). The reason for this selective distribution may be melanin-binding, as demonstrated in paper I.

In paper I both norharman and harman were also demonstrated to bind to dopamine melanin in vitro. Scatchard plots revealed two binding sites for norharman respectively harman, with binding parameters presented in table 4.
Table 4

Binding of β-carbolines to dopamine melanin in vitro

<table>
<thead>
<tr>
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<th>Norharman</th>
<th>Harman</th>
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<tr>
<td>$K_d$ high affinity site (µM)</td>
<td>5.0</td>
<td>3.2</td>
</tr>
<tr>
<td>$K_d$ low affinity site (µM)</td>
<td>1057.0</td>
<td>2364.0</td>
</tr>
<tr>
<td>$B_{ma}$ high affinity site (µmol/mg melanin)</td>
<td>1.6</td>
<td>0.8</td>
</tr>
<tr>
<td>$B_{ma}$ low affinity site (µmol/mg melanin)</td>
<td>51.3</td>
<td>130.7</td>
</tr>
</tbody>
</table>

Equilibrium constant of dissociation ($K_d$), binding maximum ($B_{ma}$)

Dopamine melanin is often used as a model for neuromelanin since it exhibits several structural similarities to this pigment (Youdim et al. 1994; Bridelli et al. 1999; Double et al. 2000), but although dopamine melanin is the main precursor of neuromelanin the latter also contains a peptide component (eg cystein) and metals such as iron (Zecca et al. 1992, Zecca et al. 1994; Zucca et al. 2004). The binding of norharman and harman to dopamine melanin was in the same range as MPTP, which is reported to have three binding sites with $K_d$-values of 1.7 µM, 82.0 µM and 1302.1 µM (Lydén et al. 1983).

Norharman and harman were also demonstrated to bind to melanin granules from Sepia officinalis. The binding to the melanin granules was in the same range as other compounds with well-known melanin affinity, for example chloroquine (Larsson and Tjälve 1979).

The present results suggest that neuromelanin is an intracellular binder of norharman and harman. The binding of toxicants to neuromelanin has usually been considered as a way of protection. A progressive release of toxicants that have been accumulated on the neuromelanin granules may, however, lead to a sustained exposure of these compounds at this site.

**Norharman-induced changes in the brain**

Neuromelanin-containing neurons of the substantia nigra are selective targets for neurotoxicants like MPTP (Herrero et al. 1993). High doses of norharman (>100 mg/kg twice daily for 7 days) have been reported to induce neurodegeneration in this specific cell population (Matsubara et al. 1998a). In paper II lower doses (3 mg/kg or 10 mg/kg) were injected subcutaneously (s.c.) twice daily for five days. Eighteen hours after the last injection the animals were killed and the brains were subjected to immunohistochemical staining against TH (figure 8).
Figure 8. Immunohistochemical staining against TH in sections of substantia nigra from C57BL/6 mice injected s.c. with vehicle (A), 3 mg/kg norharman (B) or 10 mg/kg norharman (C) twice daily for five consecutive days and thereafter killed 18 hours after the last injection.

The number of TH-positive neurons was 95.6 ± 28.3% (3 mg/kg), and 99.0 ± 42.3% (10 mg/kg) of the vehicle-treated control. The lack of norharman-induced effect on the number of TH-positive neurons is in concordance with some reports on subacute and acute treatment with MPTP (Petroske et al. 2001). Although several reports demonstrate an acute loss of TH-positive neurons in substantia nigra pars compacta after MPTP-treatment (as reviewed by Schober 2004), others report that the number of TH-positive neurons do not change during the first month post MPTP lesioning (Jakowec et al. 2004).

Fluoro-jade was used as another histological marker for neurotoxicity. Fluoro-jade is an anionic fluorescein derivative that can be used for localization of neuronal degeneration in brain tissue sections. Detection of neuronal damage using fluoro-jade has been validated by a variety of well-characterized neurotoxicants, such as MPTP (Schmued et al. 1997; Schmued and Hopkins 2000). Degenerating neurons stained with fluoro-jade appear bright yellow-green against a dark background (Schmued et al. 1997; Schmued and Hopkins 2000).

In paper II the fluoro-jade staining revealed that repeated norharman treatment induced degenerative changes in the substantia nigra (figure 9).

Since fluoro-jade stains all types of degenerating neurons and glial cells (Schmued and Hopkins 2000), the norharman-induced degeneration in substantia nigra could be related to changes both in neurons and glia cells. Glia cells are activated during all types of damage in the central nervous system and activated astrocytes synthesize increased amount of GFAP (Eng 1985; Pekny and Nilsson 2005). In paper II norharman induced increased number of GFAP-stained astrocytes in substantia nigra of C57BL/6 mice (figure 10).
Figure 9. Degeneration as demonstrated by fluoro-jade staining (green) in substantia nigra of C57BL/6 mice injected s.c. with vehicle (A), 3 mg/kg norharman (B) or 10 mg/kg norharman (C) twice daily for five consecutive days and thereafter killed 18 hours after the last injection. A semi-quantitative measurement of fluoro-jade stained area (D) where each point represent three sections from one animal, n=5-6 animals/treatment, and the straight line indicates the mean value. Statistical analysis (ANOVA) indicated a significant difference between the groups (p<0.05). Post-hoc testing (GraphPad QuickCalcs) indicated a higher value for animals receiving norharman (10 mg/kg) compared to vehicle-treated animals, * p<0.05.
Figure 10. Immunohistochemical staining against GFAP in sections of substantia nigra from C57BL/6 mice injected s.c. with vehicle (A), 3 mg/kg norharman (B) or 10 mg/kg norharman (C) twice daily for five consecutive days and thereafter killed 18 hours after the last injection. A semi-quantitative measurement of GFAP stained area (D) where each value represent two to six sections from one animal, n=2-3 animals/treatment, and the straight line indicates the mean value. Statistical analysis (ANOVA) indicated a significant difference between the groups, p<0.05. Post-hoc testing (GraphPad QuickCalcs) indicated a higher value for the mice treated with 10 mg/kg norharman compared to vehicle, *p<0.05.
Astrocytes are considered to be able to protect neurons against fluctuations in ion homeostasis and excess of released neurotransmitters under normal conditions (Kandel et al. 1991). Nevertheless, all forms of brain injury induce activation of astrocytes. When the astrocytes become reactive, their ability to carry out normal support functions is reduced (Pekny and Nilsson 2005). Activation of astrocytes is a common feature of both acute and chronic neurodegenerative conditions (as reviewed by Teismann et al. 2003). For example can astrocyte activation be observed after exposure to neurotoxicants such as MPTP and 6-OHDA (Strömberg et al. 1986). However, neither the process by which astrocytes become activated nor the functional consequences of this activated phenotype are well understood, though it has been proposed that activated astrocytes may be involved in oxidative stress and apoptosis (as reviewed by Teismann et al. 2003).

**Norharman-induced impairment of motor activity in mice**

Earlier studies have shown that norharman causes motor impairments in rodents and sheep (Bourke et al. 1992; Fekkes and Bode 1993; Matsubara et al. 1998a). All these studies were, however, performed shortly after the administration of norharman. Considering that β-carbolines such as norharman have been proposed as neurotoxicants contributing to parkinsonism it was of interest to examine if norharman could cause sustained behavioural impairments such as hypoactivity that is a noticeable finding in parkinsonism.

In paper II the behavioural study demonstrated that repeated injections of norharman (10 mg/kg; s.c.) in C57BL/6 mice during five days caused a significant decrease in motor activity two weeks later. This study included three different parameters for measuring motor activity: total activity, locomotion and rearing. As shown in figure 11 A, the highest dose of norharman (10 mg/kg) caused a reduction of total activity in all measured time periods. Locomotion and rearing were decreased in the first and second period, but not during the last 20 minutes (figure 11 B and C).

A seeking behaviour is normally induced when mice are put into a new environment. When the mice have examined the cage, a reduced motor activity can be observed. Mice treated with norharman (10 mg/kg) were hypoactive compared to control mice at all time points. However, in the group of norharman-treated mice (10 mg/kg) no reduction of total activity and locomotion was observed between the first and second 20-minute time period indicating that these mice might also have reduced acclimatisation behaviour.

The behavioural study in paper II also included measurements on spatial learning and memory, as evaluated by the radial arm maze and Morris swim maze. Spatial learning and memory have been associated with the cholinergic system especially in hippocampus. The key feature of spatial learning may be the need to integrate information from multiple stimuli. Lesions of the hippocampal region disrupt
Figure 11. Spontaneous motor activity represented by total activity (A), locomotion (B) and rearing (C) in C57BL/6 mice two weeks after s.c. injections of norharman or vehicle twice daily for five consecutive days. The values represent mean ± SD, n=12 animals/treatment. Statistical analysis (ANOVA) indicated significant difference between the groups, p<0.001 for total activity and locomotion, p<0.01 for rearing. Post-hoc testing (Tukey) indicated a reduced level of activity in norharman-treated animals compared to vehicle-treated animals, **p<0.01, *p<0.05.
memories of visited arms in a radial arm maze. Mice could still learn to avoid arms that were never baited. No norharman-induced changes were observed in these mazes.

Since norharman only caused impairment of motor activity, but not of altered learning or memory capacities, it is tempting to suggest that norharman could cause a selective damage to brain areas of importance for motor activity.

In vitro models of neuromelanin-containing neurons
A major problem for the study of the significance of neuromelanin in development of parkinsonism is that common experimental animals such as mouse, rat and guinea pig lack neuromelanin (Marsden 1961; Barden and Levine 1983). Primates and amphibians do have neuromelanin (Marsden 1961), but these species lack available non-pigmented variants making comparative studies impossible.

PC12 cells are commonly used for studies of neurotoxicity in vitro. These cells are comparable to other cells derived from pheochromocytomas in that they synthesize, store and secrete large quantities of catecholamines (Greene and Tischler 1982). In PC12 cells these catecholamines predominantly consist of dopamine and noradrenaline (Greene and Tischler 1982).

PC12 cells do not normally contain melanin. Nevertheless, long-time exposure to D- or L-dihydroxyphenylalanine has been reported to induce biosynthesis of neuromelanin in PC12 cells and in primary mesencephalic cells (Sulzer et al. 2000). Furthermore, Offen and co-workers (1997, 1999) have reported that PC12 cells are able to phagocytize dopamine melanin, although the internalized melanin decreased the cell viability.

In paper III, PC12 cells were cultured in presence of L-dihydroxyphenylalanine for 54 days, as a pilot study. No melanin was, however, observed by light-microscopic examination or after staining against melanin with the Fontana-Masson silver method. PC12 cells were therefore instead exposed to dopamine melanin. In contrast to the results from Offen and co-workers (1997, 1999) exposure to dopamine melanin up to 0.5 mg/ml for 48 hours did not cause any marked cytotoxic effect. There were, however, several differences between the dopamine melanin used in these studies. The dopamine melanin used in paper III was synthesized by a longer oxidation step and the melanin suspension was heated in an autoclave (121°C for 45 minutes) before used in cell cultures. It was also observed that a four-week storage period of the dopamine melanin suspension after synthesis could increase the number of melanin-loaded cells (figure 12).
Figure 12. Number of melanin-loaded PC12 cells after 48 hours of incubation with dopamine melanin, that been freshly synthesized or stored for four weeks. The values represent mean ± SD, n=4 for freshly synthesized dopamine melanin and n=3 for stored dopamine melanin. Statistical analysis (Student’s t-test) indicated that there was a higher number of melanin-loaded cells after incubation with 0.25 and 0.5 mg/ml stored dopamine melanin compared to cells exposed to freshly synthesized dopamine melanin, *p<0.05.

After exposure to dopamine melanin (0.1-0.5 mg/ml) for 48 hours black dopamine melanin granules were preferentially localized in the cytoplasm close to the neurite hillock and into the proximal part of the neurites (figure 13). This corresponds to the in vivo localization of neuromelanin granules in pigmented neurons, suggesting that the intracellular handling of the synthetic dopamine melanin in PC12 cells is similar to that of neuromelanin in neurons.

Figure 13. Representative light-microscopic image of undifferentiated PC12 cells incubated with dopamine melanin for 48 hours.
The effects of dopamine melanin (0.5 mg/ml) in PC12 cells were studied by transmission electron microscopy, trypanblue exclusion assay, immunocytochemistry against grp78 and cleaved caspase-3, and Western immunoblot against cleaved caspase-3. Neither of these assays demonstrated any cytotoxic effect of dopamine melanin. When the concentration of dopamine melanin was increased to 1.0 mg/ml there was, however, a statistically significant increase of necrotic cells as demonstrated by the trypanblue exclusion assay. Also several morphological changes were observed, including marked cell membrane blebbings, swelling and disappearance of neurites.

When grown in the presence of nerve growth factor (NGF), PC12 cells extend neurites, become electrically excitable, have increased numbers of calcium channels and increase their synthesis of several neurotransmitters (Greene and Tischler 1982). Generally NGF-differentiated PC12 cells are less sensitive to toxicants (Ohyashiki et al. 2002; Salinas et al. 2003), but the opposite has also been reported (Chung and Hong 1998). In this study, the effects of dopamine melanin were similar in NGF-differentiated and undifferentiated cells. In addition, cleaved caspase-3 could not be detected in either undifferentiated or NGF-differentiated PC12 cells after exposure to dopamine melanin (0.25, 0.5 or 1.0 mg/ml) for 48 hours. In cells exposed to staurosporine (positive control) cleaved caspase-3 was present (as shown in figure 14).

There was furthermore no difference between undifferentiated and NGF-differentiated cells in their response to the cytotoxic peptide amyloid-β.

The results from paper III demonstrated that dopamine melanin (≤ 0.5 mg/ml) did not induce cytotoxicity and suggest that dopamine melanin-loaded PC12 cells may be used as an in vitro model for neuromelanin-containing neurons.
Norharman-induced cytotoxicity in conventional and melanin-loaded PC12 cells

Morphological changes

In paper IV conventional PC12 cells without melanin and melanin-loaded PC12 cells were cultured on collagen-coated glass slides and exposed to norharman (50 and 500 µM) or vehicle for 24 hours. In conventional cells without melanin there was a decreased number of neurites after exposure to norharman and the neurite terminals became swollen (figure 15 B, C).

There was also a decreased number of neurites and appearance of swollen neurite terminals after exposure to norharman in melanin-loaded cells (figure 15 E, F), although these changes were less severe than those in conventional cells. Furthermore, the melanin did not only appear as large clusters in the neurite hillocks (15 D), but also as uniformly dispersed granules throughout the cell body (15 E, F). In 75% of the cells dispersed melanin granules were also detected in swollen neurite terminals.
Mitochondrial activity could be measured by adding yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; MTT) to cells. MTT is taken up by cells and yield a purple formazan product when reduced by a mitochondrial succinate dehydrogenase. Accumulation of formazan thereby reflects the activity of mitochondria directly and the cell viability or cell number indirectly.

In paper II and IV the MTT-assay was used to study the ability of norharman to cause a decrease of mitochondrial activity. Conventional PC12 cells without melanin and melanin-loaded PC12 cells were exposed to norharman (50, 100, 250 and 500 µM) for 24 or 96 hours. In the conventional cells there was a concentration-dependent decrease of mitochondrial activity. In melanin-loaded cells there was a similar concentration-dependent decrease. The melanin-loaded cells were, however, more sensitive to 50 and 100 µM norharman after 96 hours of exposure, although melanin was observed to protect against 500 µM norharman after both 24 and 96 hours of exposure (table 5). The shift of melanin-induced sensitivity/protection has not been fully understood.

Table 5

<table>
<thead>
<tr>
<th>Concentration of norharman</th>
<th>Conventional cells without melanin (% of control without treatment)</th>
<th>Melanin-loaded Cells (% of control without treatment)</th>
<th>Statistics (Student’s t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hours exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.1 ± 0.1</td>
<td>99.9 ± 0.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>Vehicle</td>
<td>100.5 ± 3.2</td>
<td>103.3 ± 1.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>50 µM</td>
<td>98.9 ± 10.1</td>
<td>102.6 ± 0.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>100 µM</td>
<td>97.1 ± 5.8</td>
<td>100.8 ± 1.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>250 µM</td>
<td>84.9 ± 11.4</td>
<td>90.6 ± 2.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>500 µM</td>
<td>84.0 ± 6.9</td>
<td>94.3 ± 6.9</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>96 hours exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.0 ± 6.7</td>
<td>100.0 ± 6.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>Vehicle</td>
<td>107.0 ± 10.6</td>
<td>104.6 ± 10.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>50 µM</td>
<td>94.4 ± 6.2</td>
<td>85.7 ± 7.0</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>100 µM</td>
<td>79.9 ± 4.3</td>
<td>73.2 ± 7.4</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>250 µM</td>
<td>46.1 ± 5.4</td>
<td>47.7 ± 4.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>500 µM</td>
<td>29.9 ± 3.3</td>
<td>34.6 ± 3.9</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

no statistical difference (n.s.)
Oxidative stress

In paper IV the ability of norharman to induce oxidative stress was determined in conventional PC12 cells without melanin and melanin-loaded PC12 cells.

The cells were preincubated with carboxy H$_2$DCFDA, a probe that becomes fluorescent in the presence of ROS. Thereafter the cells were exposed to norharman (5, 50 and 500 µM) for up to 24 hours.

The results indicated, nevertheless, that norharman does not induce ROS. This result is consistent with studies on other β-carbolines such as tetrahydrocarbolines and γ-carbolines, which are not considered as pro-oxidants but have been reported to be efficient antioxidants (Stolc 1999). When PC12 cells were exposed to the positive control (hydrogen peroxide, 4 mM) there was a rapid increase of oxidative stress. The induction of ROS was lower in melanin-loaded cells. These results are consistent with previous reports where Hoogduijn and co-workers (2004) demonstrated that highly melanized melanocytes are protected against hydrogen peroxide-induced DNA damage. This protection was suggested to be dependent on the ability of melanin to bind calcium. Increased intracellular levels of calcium are known to activate calcium-dependent nucleases. Both natural and synthetic dopamine melanin can interact with ROS and scavenge superoxide radical anions and hydroxyl radicals (Korytowski et al. 1986). The degree of pigment aggregation has been suggested to influence the interaction of melanin with radicals and it has been suggested that intracellular aggregates of melanin granules might be less effective than a suspension of melanin particles in test tubes (Korytowski et al. 1995). Natural neuromelanin appears in highly aggregated form and clusters in vivo. In melanin-loaded PC12 cells most of the aggregates were localized in the neurite hillocks and showed a similar intracellular localization as neuromelanin granules in dopaminergic neurons in vivo. Korytowski and co-workers (1995) have suggested that the aggregation of natural melanins may affect the radical scavenging efficiency and that the effective units of melanin decrease with increasing aggregation. They propose that the scavenging of radicals plays a minor role in vivo for the protection of pigmented cells against oxidative stress and damage. Since melanin-loaded PC12 cells were protected against hydrogen peroxide, it seems reasonable that also melanin aggregates protect cells against oxidative stress induced by hydrogen peroxide.

ER stress

In paper IV the ability of norharman to induce ER stress was determined. Conventional PC12 cells without melanin and melanin-loaded PC12 cells were exposed to norharman (5, 50 or 500 µM) for 24 hours. In the conventional cells without melanin, norharman (up to 50 µM) induced an increased number of grp78-positive cells. Melanin-loaded cells were less sensitive to 5 µM norharman than the conventional cells, equally sensitive to 50 µM and more sensitive to be grp78-positive after 500 µM norharman (figure 16).
These results indicate that melanin may have protective effects after exposure to lower concentrations of norharman. After exposure to high concentrations of norharman the melanin may become saturated and does not protect against ER stress.

A large amount of work has established that specific induction of grp78 is indicative of ER stress and that UPR is triggered (Lee 2005). These events are probably induced to promote cell survival. The ER has, however, been pointed out as a site of convergence of both pro- and anti-apoptotic molecules and may therefore represent a novel focal point for the regulation of apoptosis (Lee 2005).

**Apoptosis**

In paper II conventional PC12 cells without melanin were exposed to norharman (5, 50 and 500 µM) for 24 hours. Thereafter the cells were stained against cleaved caspase-3 or terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL). The results demonstrated that norharman (5 and 50 µM) caused an increased number of both caspase-3- and TUNEL-positive cells (figure 17), although the number of caspase-3- and TUNEL-positive cells decreased

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**Figure 16.** Immunocytochemical staining against grp78 in conventional PC12 cells without melanin and in melanin-loaded PC12 cells exposed to norharman for 24 hours. Values represent mean ± SD, n=5-6 sections, including ≥150 cells/concentration. Statistical analysis (Student’s t-test) indicated difference between cells without melanin compared to melanin-loaded cells, ***p<0.001, **p<0.01.
after exposure to 500 µM norharman. Similar results have been reported for other neurotoxicants such as MPTP and 6-OHDA, and it has been proposed that high concentrations of these neurotoxicants may severely decrease rates of ATP synthesis and result in an inability of the cells to maintain normal cellular function and so result in necrosis (Hartley et al. 1994).

In paper IV, melanin-loaded PC12 cells were also stained against cleaved caspase-3 after exposure to norharman (5, 50 and 500 µM) for 24 hours. The results from this study demonstrated that melanin could protect against caspase-3 activation after exposure to 5 and 50 µM. It was also demonstrated that there was an increased number of caspase-3-positive melanin-loaded cells after exposure of 500 µM norharman. These results may be interpreted as a protective effect of melanin against necrosis, since melanin-loaded cells were able to undergo apoptosis even after exposure to 500 µM norharman while cells without melanin lost this ability. However, the number of cells stained against cleaved caspase-3 increased in the melanin-loaded cells after exposure to 500 µM norharman, demonstrating that melanin does not protect against apoptosis (figure 18).
Role of Neuromelanin and Cytochrome P450 for Toxicity

Figure 18. Immunocytochemical staining against cleaved caspase-3 in conventional PC12 cells without melanin and in melanin-loaded PC12 cells exposed to norharman for 24 hours. Values represent mean ± SD, n=6 sections, including ≥750 cells/concentration. Statistical analysis (Student’s t-test) indicated a significant difference in the number of caspase-3 positive cells when comparing melanin-loaded cells and conventional cells, ***p<0.001.

Necrosis

In paper II conventional PC12 cells without melanin were exposed to norharman (5, 50 or 500 µM). After 24 or 96 hours the cells were trypsinated, resuspended in medium and the number of necrotic cells was determined by the trypan blue exclusion assay. The results demonstrated that there was a low but statistically significant increase of necrotic conventional cells without melanin after exposure to 500 µM norharman for 24 hours and after exposure for 50 µM and 500 µM for 96 hours.

In paper IV also melanin-loaded PC12 cells were exposed to norharman. The melanin had a protective effect on induction of necrosis after exposure to all concentrations of norharman for 24 hours and to 5 µM norharman for 96 hours, as shown in figure 19. These results further support that melanin could protect PC12 cells against norharman-induced toxicity.
Figure 19. Trypanblue staining of conventional PC12 cells without melanin and melanin-loaded PC12 cells exposed to norharman for 24 or 96 hours. The values represent mean ± SD, n=5, including ≥1300 cells/concentration exposed for 24 hours, and n=4, including ≥100 cells/concentration. Statistical analysis (Student’s t-test) indicated a significantly lower level of norharman-induced necrosis in melanin-loaded cells as compared to cells without melanin, ** p<0.01, *p<0.05.

Altogether, the results from paper I-IV demonstrate that neuromelanin may be of importance for a selective retention of the β-carbolines norharman and harman. Furthermore, norharman was demonstrated to induce degenerative effects in vivo and in vitro and to cause motor impairment in C57BL/6 mice. The in vitro effects could be attenuated by the presence of melanin. This is in concordance with earlier suggestions that melanin has protective effect during short-term exposures. Melanin binding is, however, slowly reversible, therefore the long-term effects of melanin binding may be a continuous release of the neurotoxicant. This may possibly promote rather than protect against toxicity.

**CYP1-dependent distribution**

Selective expression of CYP enzymes in certain tissues and cell types has been associated with tissue or cell specific toxicity of bioactivated toxicants. CYP1 metabolizes many toxicants, including benzo[a]pyrene and other PAHs, as well as HCA, into reactive metabolites, which become bound to protein and nucleic acids (Shimada et al. 1996). The expression of CYP1 enzymes can be induced by treatment with aryl hydrocarbon receptor agonists such as BNF and PCB126.
β-carbolines

Previous studies have demonstrated that several HCA could be metabolized by CYP enzymes (as reviewed by Yamazoe and Nagata 2000). Selective expression of CYP1A1 have also been demonstrated to be of importance for localization of some HCA (Annas and Brittebo 1998). Since norharman has been reported to inhibit the metabolism of the CYP1 substrate benzo[a]pyrene in rat liver (Fujino et al. 1978) it was of interest to study if induction of CYP1 could influence the distribution of norharman and harman in mouse brain. ³H-Norharman and ³H-harman were therefore injected i.v. in BNF-pretreated mice. The distribution was evaluated by tape-section autoradiography and light-microscopic autoradiography. The distribution of radioactivity after in the brain after injections of ³H-norharman or ³H-harman was similar in BNF-pretreated mice compared to vehicle-treated mice and the expression of CYP1 enzymes is not suggested to play a significant role for localization of norharman and harman in the brain of mice.

7,12-Dimethylbenz[a]anthracene

Paper V revealed a selective distribution of radioactivity in blood-brain interfaces after injection of ³H-DMBA in rodents pretreated with either BNF or PCB126. A cellular localization of radioactivity in endothelial cells was further demonstrated by light-microscopic autoradiography in vivo (figure 20) and in vitro with brain slices of BNF- and PCB126-pretreated rodents. In vehicle-pretreated animals no such selective distribution of radioactivity was observed in vivo or in vitro.

Figure 20. Light-microscopic autoradiography of BNF-pretreated mice 24 hours after an i.v. injection with ³H-DMBA. A selective localization of radioactivity (as demonstrated by white areas) was observed in the capillary loop EC of the choroid plexus (A) and in EC of cerebral veins (B).
Induction of CYP1 in small intact tissue preparations of leptomeninges and choroid plexus from PCB126-pretreated rats was also determined by the 7-ethoxyresorufin O-deethylase activity assay.

Since BNF and PCB126 are known for their ability to induce CYP1 enzymes an immunohistochemical study was performed to study if the selective distribution of $^3$H-DMBA could be related to the expression of CYP1 enzymes. It was found that CYP1A1 was selectively expressed in EC in cerebral veins, veins in the leptomeninges and in capillary loops of choroid plexus of BNF- and PCB 126-treated mice, whereas no similar expression of CYP1A1 was detected in vehicle-treated mice (figure 21). Based on these observations it was concluded that EC in veins and in the leptomeninges and capillaries of the choroid plexus of rodents express a constitutively low but highly inducible and catalytically active CYP1A1 enzyme.

**Figure 21.** Immunohistochemical staining against CYP1A1 in brain sections including EC in cerebral veins (A), veins in leptomeninges (B), cerebral arteries of the leptomeninges (C, D), capillary loops of the choroid plexus (E, F) and vasa vasorum (G, H) from NMRI mice after an i.p. injection with 0.25 mg/kg PCB126 (A, B, C, E, G) or vehicle (D, F, H), and a survival time of five days post-injection.
An immunohistochemical study against CYP1B1 was also performed in paper V. In contrast to CYP1A1, CYP1B1 expression was mainly found in smooth muscle cells (SMC) in arteries of the leptomeninges, cerebral arteries, arterioles and in choroid plexus arterioles, whereas the expression of CYP1B1 in EC was negligible. The CYP1B1-staining was observed in mice pretreated with BNF or PCB126 but also in mice pretreated with vehicle (figure 22).

Figure 22. Immunohistochemical staining against CYP1B1 in brain sections including arteries (A, B) and veins (C) from the leptomeninges and capillary loops of choroid plexus from NMRI mice after an i.p. injection with vehicle (A) or 0.25 mg/kg PCB126 (B, C and D) and a survival time of five days post-injection.

Although DMBA is known to be metabolized by CYP1B1 (Gonzalez 2001), selective localization of $^3$H-DMBA adducts was not observed in SMC of arteries in the leptomeninges or cerebral arteries, neither in vehicle-treated or in BNF- or PCB126-treated rodents.

There are several possible explanations for the differential bioactivation and binding of $^3$H-DMBA in EC and SMC: CYP1B1 could be less catalytically active than CYP1A1 toward DMBA or its metabolites; the two enzymes could form different metabolites; or other enzymes, differentially expressed in EC and in SMC could compete for DMBA or its reactive intermediates.

Injury to the blood vessel endothelium is often reported as an early event in cardiovascular disease. Earlier studies have shown that there is an induction of CYP1A1 and irreversible binding of $^3$H-DMBA also in HUVEC following pre-treatment with BNF (Annas et al. 2000b). Interestingly shear stress has been demonstrated to induce CYP1A1 and CYP1B1 in HUVEC (McCormick et al. 2001). The induction of these enzymes in human EC may be an important determinant.
for the susceptibility of EC towards protoxicants and procarcinogens. The formation
of reactive intermediates and subsequent reaction with vital macromolecules may
result in a changed or impaired function leading to detrimental consequences for
the cell.

Both tobacco smoking and combustion effluents are important sources for PAH
exposure. Epidemiological studies indicate exposure to PAHs to be a common risk
factor to be involved in the aetiology of stroke and atherosclerosis (Bonita et al.
1999). Smokers are also known to have an increased expression of CYP1 enzymes.
The results from the presents study demonstrating the presence of $^3$H-DMBA
adduct in EC of some blood-brain interfaces of CYP450-induced rodents combined
with results from others demonstrating PAH-adducts and benzo[a]pyrene-induced
DNA damage in HUVEC (Annas et al. 2000a, Annas et al. 2000b), suggest
that PAH exposure should be considered as a potential risk factor with regard to
cerebrovascular disease.
CONCLUDING REMARKS

β-carbolines have been suggested to be involved in parkinsonism due to their structural resemblance to MPTP. High levels of the β-carbolines norharman and harman have also been reported in human post-mortem brains (Matsubara et al. 1993) and the levels of these compounds are increased in plasma and CSF of PD-patients (Kuhn et al. 1995; Matsubara et al. 1995).

A general problem in studies of parkinsonism is that conventional model toxicants generates a rapid neurotoxicity. Although this is practical for the researcher these models may not be relevant to the slow, progressive nature of human parkinsonism or mimic the long and latent neurodegenerative symptoms associated with chronic exposure to environmental compounds. It is furthermore reasonable to believe that co-exposure to several environmental agents may lead to synergistic toxic effects.

In this thesis, norharman was demonstrated to induce neurodegeneration and sustained motor impairment in mice, although the results indicate that glia cells and not dopaminergic neurons, may have been the primary target for the neurotoxicity. It was also demonstrated that norharman could cause ER stress in PC12 cells. However, these results were achieved after rather high doses and concentrations, whereas humans normally are only exposed to low doses of these compounds. Nevertheless, humans are likely to be exposed to β-carbolines and other similar compounds over a lifetime. The results in this thesis demonstrated that there is a long-term retention of β-carbolines in melanin-containing tissues. Therefore it is likely that an accumulation of high concentrations of β-carbolines may be built up in melanin-containing tissues although the exposure level is moderate. To be able to study the effects of melanin on neurotoxicity induced by β-carbolines, an in vitro model was developed. In this model, dopamine melanin was demonstrated to be able to attenuate toxicity induced by low concentrations of norharman. This is in concordance with that melanin has a protective function for single exposure of toxicants. Melanin binding is, however, normally slowly reversible. Therefore, a melanin binding that initially protects can later lead to a situation where the melanin may function as a depot for neurotoxicants that are slowly released into the cytosol and thereby promote a continuous exposure that may induce toxicity. It is not possible to perform in vitro experiments over a long time, therefore it would be of interest in to study if pre- or co-exposure with melanin-binding compounds could reduce the protective effect of melanin on norharman-induced toxicity. Such studies may include co-exposure to β-carbolines and calcium since others have reported that melanin and calcium interact and disruption of the calcium homeostasis are reported in several neurodegenerative disorders (Hoogduijn et al. 2004; Gibson
and Huang 2004). When melanin becomes saturated it may not longer protect against cytotoxic events. Indeed, when the PC12 cells were exposed to the highest concentration of norharman melanin was still able to attenuate necrosis, but the intracellular stress or apoptosis increased.

PAHs have been suggested to contribute to disorders such as stroke and atherosclerosis that may be caused by toxic effects on the endothelial cells. Earlier studies have reported that DMBA could be metabolized and bioactivated by CYP1A1 as well as CYP1B1. In this thesis it was shown that there was a differential expression of these enzymes; CYP1A1 was inducible and expressed in some EC whereas CYP1B1 was constitutively expressed in SMC. Furthermore, the distribution of DMBA was in concordance with the expression of CYP1A1 suggesting that this enzyme may be important for bioactivation and adduct formation of DMBA in the brain.
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