Aus der Klinik und Poliklinik für Psychiatrie und Psychotherapie
der Universität Würzburg

Direcktor: Professor Dr. med. Dr. h. c. Helmut Beckmann

Differential effects of neuromelanin and synthetic dopamine melanin on cell lines

Inaugural – Dissertation
zur Erlangung der Doktorwürde der
Medizinischen Fakultät
der

Bayerischen Julius-Maximilians-Unversität zu Würzburg

vorgelegt von
Jie Li
aus Tianjin, China

Würzburg, November 2004

Referent: Prof. Dipl.-Ing. Dr. techn. Peter Riederer

Koreferent: Prof. Dr. W. Roggendorf

Dekan: Prof. Dr. med. Georg Ertl

Tag der mündlichen Prüfung: 03, 06, 2005

Die Promovendin ist Ärztin

Content

CNS-central nervous system; COMT- catechol-O-methyl-transferase; CSF-cerebrospinal fluid; DA-dopamine; DAM- dopamine melanin; GABA-gamma amino-butyric acid; GPx- glutathione peroxidase; GSH-glutathione; H2O2- hydrogen peroxide; MAO-A - monoamine oxidase A; MAO-B- monoamine oxidase B; MPP+- 1-methyl-4-phenylpyridinium; MPTP- 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine; mtDNA-mitochondrial DNA; MAO-monoamine oxidase; •OH- hydroxyl radical; 6-OHDA- 6-hydroxydopamine; PD-Parkinson's disease; ROS- reactive oxygen species; SOD-superoxide dismutase; TBA- thiobarbituric acid; TNF- tumor necrosis factor

1. Introduction

1.1 About Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease affecting 1 to 2% of individuals over the age of 65 (de Rijk et al. 1997). It was first described in 1817 by James Parkinson in his "Essay on the shaking palsy". The main pathological features of the disease are the progressive death of the dopaminergic neurons in the substantia nigra and the appearance of abnormal protein aggregates called Lewy bodies in the cytoplasm of some surviving neurons. Three types of cellular dysfunction that may be important in the pathogenesis of PD: oxidative stress, mitochondrial respiration defect, and abnormal protein aggregation (Dauer and Przedborski 2003).

1.1.1 Clinical manifestation of PD

The hallmark physical signs of PD are rigidity, tremor and bradykinesia (slowness of movement). Poor postural reflexes are sometimes included as the fourth hallmark sign. This combination of symptoms is known as "parkinsonism", a clinical profile resembling PD. Other characteristic symptoms are hypokinesia (reduction in movement amplitude), akinesia (absence of normal unconscious movements, such as arm swing in walking), poor balance and an abnormal, often shuffling, gait. These motor symptoms result from a lack of the neurotransmitter dopamine in the caudate nucleus and putamen, which is normally produced by the degenerating dopaminergic substantia nigra neurons (Maret et al. 1990). Non-motor symptoms may also be present and include depression, dementia, confusion, sleep disorders, speech and swallowing difficulties, urinary problems, constipation, skin problems, sexual difficulties and communication problems (Litvan 1998). The degeneration of specific subcortical regions in PD results in the disruption of a number of neurotransmitter systems, some of which may also influence higher mental functions. Reduced cholinergic, noradrenergic, and serotonergic activity may contribute to cognitive or psychiatric manifestations, and most striking - dementia. Most symptoms, motor or otherwise, are subtle at first and worsen over time. PD also affects individuals differently, thus patients experience a range and severity of symptoms, which may differ greatly compared to other patients.

1.1.2 Epidemiology of PD

The incidence of PD increases with age. Patients aged 60-70 years are most common and a diagnosis of PD is rare before the age of 30 years. The frequency of PD is predicted to triple over the next 50 years as the average age of the population increases (Goldman and Tanner 1998). The crude prevalence of PD has been reported to vary from 15 (per 100,000 population) in China to 657 in Argentina in door-to-door surveys (Wang 1991; Melcon et al. 1997) and estimates in the North America and Europe populations range from 100 to 250 per 100, 000 population (Mitchell et al. 1996). These

variations in prevalence may partly result from study design differences, such as diagnostic criteria and methods of case ascertainment. PD is also more common among men than women, for reasons, which are unknown (Wooten et al. 2004). Early reports suggested that white people in Europe and North America have a prevalence of the disease four to ten times of that in Asians or black Africans. More recent studies however, reported that the incidence PD in African-American and Asian Americans were similar to rates for Americans of European origin (Morens et al. 1995). These data suggest that environmental factors are more important than racial factors in the development of PD.

1.2 Etiopathogenesis of PD

Although the causes of PD remain unknown, a number of factors are thought to increase or decrease the risk of PD. The only clear delineating factor among Parkinson's sufferers is age: The average age of onset is 60, although some authorities estimate 5% to 10% of patients experience symptoms before the age of 40 (Le Couteur et al. 2002). Chemicals such as carbon monoxide, certain diuretics (e.g. reserpine), antipsychotics (e.g. chlorpromazine), calcium channel blockers (e.g. verapamil) and neurotoxins (e.g. MTPT) have all been implicated to cause or worsen PD symptoms (Pedrell et al. 1995; Playfer 1997). Occupational use of herbicides and pesticides, the high intake of animal fats and sugars, the high aluminum content of drinking water may also involved in the pathogenesis of this disease (Ebadi and Srinivasan 1996; Hellenbrand and al 1996). Other metals, notably iron, manganese, cadmium, copper and mercury have also been linked to a significantly higher risk of developing PD (Playfer 1997) (Shukla et al. 1996) (Pall et al. 1987) (Reinhardt 1992; Gorell et al. 1997) (Ngim and Devathasan 1989). Cerebrovascular disease (e.g. multiple lacunar strokes) and head trauma may also

contribute to development of the disease. A number of studies reported that some infectious agents or diseases, such as HIV, Japanese B encephalitis, Coxsackie's B, influenza B, herpes simplex, measles, mumps, diphtheria, croup, or rheumatic fever, might be linked to post-infectious Parkinsonism either acutely or as a long-term complication. PD may also develop later in life as a result of a neurotoxic event that occurred at an early age, e.g. a whooping cough (pertussis)(de Pedro-Cuesta et al. 1996). Genes linked to PD have opened up new and exciting areas of research in PD. At least 10 distinct loci are responsible for rare Mendelian forms of PD, such as α-synuclein, Parkin, Tau, and etc (Dawson and Dawson 2003). Family history is implicated as a risk factor by a large body of reports, the estimated prevalence of positive family history ranges from 5% to 40% (Tanner et al. 1997). Recent reports suggested that α-synuclein gene might be an important genetic etiology for sporadic PD, although other events also have to occur in these vulnerable people for them to get PD (Singleton et al. 2003). None of above has been proved to be a definite causative factor and most patients do not have a clear risk factor. In the absence of a clear causative factor, for example a known genetic link, PD is referred to as "idiopathic". Idiopathic PD is thought to be due to a combination of both genetic and environmental "influences" or "causes" which might differ from individual to individual.

Idiopathic PD is the most common type of Parkinsonism and is thought to account for more than two thirds of the cases (Stacy and Jankovic 1992). Parkinsonism can also result from many other disorders such as: hereditary diseases (e.g. Wilson's disease), multiple system atrophies (e.g. Shy-Drager syndrome), other degenerative diseases (e.g. Creutzfeldt-Jakob disease), other central nervous system disorders (e.g. tumor), infections (e.g. viral encephalitis), exposure to toxins (e.g. MPTP, carbon monoxide), metabolic disturbances (e.g. hepatocerebral encephalopathy), and the use of medications (e.g. narcoleptics) (Stacy and Jankovic 1992; Pahwa and Koller 1995; Zack and Langston 1995). PD is generally accepted as a clinicopathologic entity that is different

from other causes of extra pyramidal signs (Zack and Langston 1995). Currently, a clinical diagnosis of idiopathic PD is based on the history and clinical signs typical of this disorder and there are no biological markers for the disease. As the causes of Parkinsonism can differ widely, the use of only clinical data for diagnosis is relatively insensitive and the differential diagnosis of Parkinsonism can be difficult (Zack and Langston 1995). Indeed, 35% of diagnoses made at the onset of symptoms that may or may not result from the loss of dopamine neurons are false (Meara et al. 1999). Accuracy of diagnoses for disorders characterised by dopamine cell loss has thus been an acknowledged problem for many years and has very important implications for the correct treatment and prognosis for the patient.

1.3 Pathological anatomy of PD

The predominant neuropathology features of PD are the progressive death of the neuromelanin-containing dopaminergic cells of the substantia nigra and locus ceruleus, and the appearance of intraneuronal inclusions called Lewy bodies in some surviving dopaminergic neurons in the substantia nigra. Although Lewy bodies can also develop in other brain regions, for example, in the cortex as well as the substantia nigra, this pattern of Lewy body development is diagnostic for a separate disorder called Dementia with Lewy bodies (Lennox et al. 1989).

1.3.1 Gross pathology

Macroscopic sections through the substantia nigra exhibit a marked loss of pigment from the substantia nigra (Figure 1) and locus ceruleus. The other part of basal ganglia: caudate, putamen, globus pallidus, thalamus, and other brainstem structures appear normal.

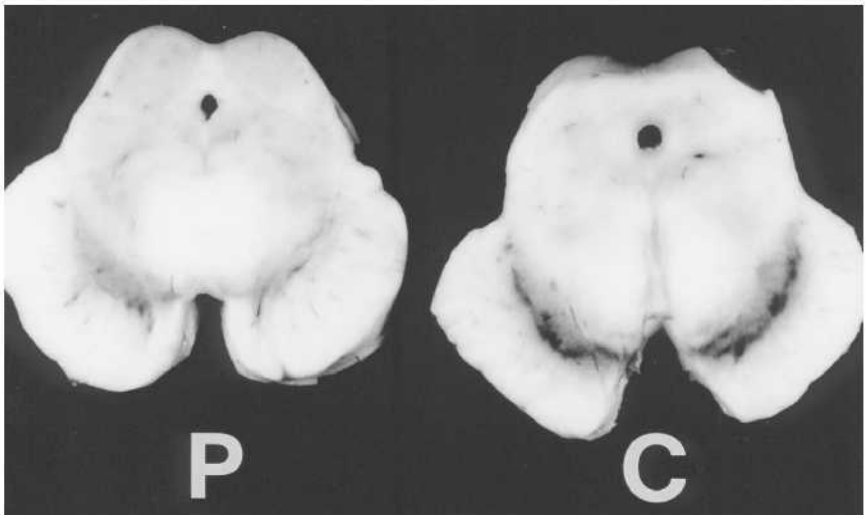

Figure 1 Horizontal sections of midbrain from a patient with PD (P) and a normal control (C), showing loss of pigmentation of the substantia nigra in PD.

1.3.2 Microscopic findings

The human substantia nigra is composed of eleven different cell groups or nuclei but cell death in PD does not occur uniformly in all regions but is concentrated primarily in the ventral region. This pattern of cell loss differs to that occurring in post-encephalitic Parkinsonism where cell loss is uniform throughout the SN (Gibb and Lees 1987). It is estimated that at least 50% of the nigra neurons must degenerated to produce symptoms and, at autopsy, most cases show more than 80% reduction (Halliday and McRitchie 1996). Significant neuronal loss also occurs in the locus ceruleus, dorsal motor nucleus of the vagus, raphe nuclei, and nucleus basalis (Halliday and McRitchie 1996). Lewy bodies may be found in all of these locations, as well as in numerous other subcortical structures. In pigmented nuclei, neuromelanin is released from dying neurons and may lie free within the neuropil (the complex, felt-like net of axonal, dendritic, and glial arborisation that forms the bulk of the central nervous system's grey matter) or be taken up by macrophages.

1.3.3 Lewy bodies

Lewy bodies in the substantia nigra are the traditional pathological hallmark of idiopathic PD. In other types of Parkinsonism, they can also appear in locus ceruleus, dorsal motor nucleus of the vagus, raphe nuclei, and nucleus basalis and in numerous other subcortical structures (Ian and Mackenzie 2001). The major components of Lewy bodies are abnormally phosphorylated neurofilaments, ubiquitin and a protein that normally occurs in the presynaptic membrane, α-synuclein. Neurofilament is a major component of the neuronal cytoskeleton, suggesting the 'building blokes' of the cytoskeleton is altered. The presence of ubiquitin suggests that Lewy bodies may represent a structural manifestation of a cytoprotective response designed to eliminate damaged cellular elements. α - synuclein is a presynaptic nerve terminal protein of unknown function. Antibodies against this protein are proving to be highly sensitive and specific markers of Lewy bodies. In PD, the Lewy bodies appear as concentric hyaline cytoplasmic inclusions within the cytoplasm of the pigmented nigral cells, but are not present not in all cases of parkinsonism (e.g., encephalitis lethargic and juvenile PD) (Marsden 1989) and are also not present in some genetic forms of PD (Hattori et al. 2000).

1.4 Pathogenesis of PD

1.4.1 Oxidative stress and free radicals

A large number of evidence suggests that oxidative stress-mediated mechanisms may be at least partially responsible for the progressive and selective neuronal degeneration observed in PD. The term "oxidative stress" refers to cytotoxic consequences of hydrogen peroxide and oxygen-derived free radicals generated as by-products of normal and aberrant metabolic processes that utilized molecular oxygen. These potentially damaging molecules are referred to as 'reactive oxygen species" or ROS. Oxidative

stress occurs when the production of ROS increases and/or the activity of cellular defense systems that exist within the cell to counteract ROS decrease.

In dopaminergic cells ROS is generated by the deamination of dopamine by monoamine oxidase (MAO), which yields significant amounts of hydrogen peroxide, as well as the non-enzymatic autoxidation of dopamine. Hydrogen peroxide interacts with the reduced forms of transitional metal ions, such as iron, and decomposes to the highly reactive hydroxyl radical (•OH), a process known as the Fenton reaction (Equation 1).

$$\bullet\ H2O2 + Fe^{2+} \rightarrow \bullet HO + OH^{-} + Fe^{3+} \qquad (1)$$

The hydroxyl radical can interact with, and thus damage, essential neuronal components including DNA, proteins, cell membranes and mitochondria which can result in functional disruption and ultimately cell death. Since the concentration of transitional metal ions, especially iron, are significantly and selectively higher in the substantia nigra of PD patients at post-mortem compared with controls (Dexter et al. 1989; Gerlach et al. 1994) this suggests that the environment of the parkinsonian nigra favours the production of ROS, and thus may result in a situation of oxidative stress in this brain region.

ROS levels cannot be measured in the living patient, nor can the ROS themselves be directly measured because of their short half-lives. Indirect indices of ROS activity in the parkinsonian brain post-mortem however support the hypothesis that this tissue exists in a state of oxidative stress. This evidence includes the following factors:

1) Increased membrane peroxidation in the substantia nigra and widespread ROS-induced protein modifications, with protein carbonyl levels elevated in all regions of the brain (Foley and Riederer 2000) (Dexter et al. 1989).

2) Elevated TBA-reactive substance levels (a measure of the secondary products of lipid peroxidation) accompanied by decreased polyunsaturated fatty acids levels (the peroxidation substrate) (Dexter et al. 1989).

3) Increased 8-hydroxy-2 deoxyguanosine levels in the substantia nigra and other brain regions, indicative of ROS-mediated DNA damage (Foley and Riederer 2000) (Sanchez-Ramos et al. 1994).

Furthermore, the measured activities of a number of phospholipid-catabolising enzymes in the normal substantia nigra have been found to be low compared with other regions of the human brain, suggesting slow phospholipid turnover in the nigra, which would restrict the capacity for rapid repair of oxidative membrane damage (Ross et al. 1998).

Cellular systems for the detoxification of ROS, even in normal individuals, are especially important for the substantia nigra as these cells are particularly vulnerable to oxidative damage because of the presence of dopamine and high levels of iron. In PD these systems appear to be overwhelmed by the increased production of ROS and the majority seem to be incapable of increasing their activity to correspond with the increased production of ROS. It is also relevant that PD develops in general in older individuals as some of the most important cellular radical detoxification systems, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), decline with normal aging. Increased levels of Mn-dependent SOD in substantia nigra and cerebrospinal fluid (CSF) have been reported, whereas there was no change in the levels of CuZn-dependent cytosolic form of the enzyme (Saggu et al. 1989). Levels of catalase and glutathione peroxidase in substantia nigra are either unaltered or mildly decreased (Ambani et al. 1975; Kish et al. 1985). Further, a decline in the reduced form of glutathione peroxidase, glutathione (GSH) levels without an increase in the oxidized form, GSSG, which suggests a decrease in GPx activity in the nigra. Of the other enzymes that modulate GSH levels, only the levels of γ-glutamyl transpeptidase, which is associated with GSH translocation and degradation, are significantly increased in the

nigra in PD (Perry et al. 1982). The decline in GSH appears to occur early in the course of the disorder, as a result it has been suggested that GSH depletion renders the cell vulnerable to neurodegeneration possibly induced by other factors (Perry et al. 1982). Considered together, these changes suggest that the activity of the cellular defences against ROS are inadequate in PD and support the hypothesis that this brain region exists in a state of oxidative stress in this disorder.

1.4.2 Mitochondrial dysfunction

Although the nature of neuronal death in Parkinson's disease is still undetermined, there are indications that it involves a combination of apoptotic processes and necrotic degeneration, processes which have both been associated with mitochondrial dysfunction. There is significant evidence supporting the hypothesis that progressive reduction in mitochondrial respiration (a feature of the aging brain) is involved in a number of neurodegenerative diseases, including PD. In idiopathic PD, there is a 30-40% decreasing in complex I activity in the SN without detectable structural or mitochondrial DNA changes (Schapira 1996). The reduced activity is not secondary to cell death (Schapira et al. 1990) and is generally restricted to the substantia nigra pars compacta (Gu et al. 1998). This nigral complex I deficit is one of the best described biochemical changes in PD, and the question of whether the defect is primary or secondary need intensive research. The precise relationship between reduced transport chain function and neurodegeneration thus remains to be established.

1.4.3 The role of glial cells

Glial cells may also be involved in the pathophysiology of PD. Recent evidence supports the possibility that glial cells may secrete diffusible factors capable of either protecting, or exerting a deleterious effect on, dopaminergic neurons in PD.

The protective role of glial cells in PD is supported by the fact that glial cell distribution is not uniform across the brainstem: it is lowest in the areas of control brains where dopaminergic neurons degenerate in PD and highest in the areas where dopaminergic neurons are preserved (Damier et al. 1993). This suggests that dopaminergic neurons surrounded by a low density of glial cells are particularly prone to degeneration in PD. The mechanisms of neuroprotection by the glial cells could consist of secretion of trophic factors or capacity to counteract oxidative stress (Hirsch 2000). Glial cells may play a protective role against oxygen free radicals. GPx, an enzyme that prevents the transformation of hydrogen peroxide to highly toxic hydroxyl radicals, is expressed by glial cells in the human mesencephalon especially those associated with the catecholaminergic cell groups least affected in PD (Damier et al. 1993). Astrocytes may also protect neurons by preventing dopamine-induced free radical formation in the vicinity of dopaminergic neurons. Astrocytes express enzymes, such as MAO-B and COMT, involved in the catabolism of dopamine, so dopamine may be metabolised intracellularly. The oxygen free radicals produced by enzymatic degradation of dopamine may be prevented from entering the neuronal environment, because it occurs within glial cells. Furthermore, as the glial cells contain high levels of GPx, they may be protected against free radical generation, thus explaining the survival of astrocytes in such an environment.

However, it was suggested recently that glial cells might damage dopaminergic neurons by secreting factors that might diffuse into neurons or act on membrane receptors. The density of glial cells expressing pro-inflammatory cytokines such as TNF-α, interferon-γ and interleukine1ß is increased in the SN in PD (Boka et al. 1994). Cytokines were found to induce the expression of a molecule called CD23, a low-affinity immunoglobulin E receptor. Given the presence of appropriate ligands, CD23 induces the expression of the enzyme NOS II that produces nitric oxide in the central nervous system. NO is diffusible and may penetrate into neurons, combine with superoxide

radicals and form peroxinitrates that are extremely toxic; indeed, the concentration of nitrites is increased in the CSF of PD patients (Qureshi et al. 1995), and 3-nitrotyrosine, an index of protein nitrosation induced by peroxynitrate, was detected in nigral dopaminergic neurons in PD (Good et al. 1998). Alternatively, NO can release iron from ferritin, and iron is extremely toxic and potential the formation of hydroxyl radicals.

In brief the cytokines produced by glial cells may reinforce oxidative stress by producing nitric oxide and increasing the levels of free iron. Cytokines may also participate more directly in the molecular events leading to neural cell death. Some cytokines, such as TNF- α, may bind to specific receptors located in the cytoplasmic membrane of the dopaminergic neurons and activate pro-apoptotic pathways. Dopaminergic neurons have indeed been shown to possess TNF- α receptors (Boka et al. 1994). Along with TNF-a, interleukin 1ß was also elevated in CSF in PD (Le et al. 1999).

1.4.4 Dopamine metabolism

Dopamine is oxidized in the brain *via* two pathways.

a) Auto-oxidation of dopamine leads to the production of toxic semiquinone species (SQ•), which can be polymerized to neuromelanin *via* a metabolic cascade.

b) In biochemical catalysis, primary, secondary and tertiary amines, including the neuronal transmitters or hormones dopamine, noradrenaline, adrenaline and serotonin, are broken down into their respective metabolites in a two-step enzymatic conversion.

The enzymatic metabolism of dopamine by MAO generates H2O2 that is normally inactivated by GPx and its cofactor GSH. During the preclinical phase of PD, if dopamine turnover were increased as part of a compensation effort, and if the GSH system itself were deficient, excessive H2O2 production and its conversion to •OH *via*

the iron-mediated Fenton reaction could generate increased local oxidative stress (Foley and Riederer 2000).

Also, increased MAO-B level (approximately 25% higher) in the SN of PD patient points to the role of MAO-B in accelerating dopamine breakdown in PD pathogenesis. *In vitro*, H2O2 induces the activity of MAO-B (Riederer et al. 1989a). If the same action would be confirmed *in vivo*, then it would be worth considering a feedback-positive mechanism in which dopamine oxidation, catalyzed by MAO-B, produces H2O2 that would only further enhance the activity of MAO-B.

1.4.5 Increased free iron levels

The brain structures comprising the basal ganglia are rich in iron in the normal brain with highest levels found in the substantia nigra, globus pallidus and putamen. Iron levels in substantia nigra are 20ng/mg in the first year of life and increase to about 200ng/mg at the fourth decade of life then remain atable until 90 years of age (Zecca et al. 2001b). Absolute iron levels are increased in PD by about 35%, specifically in the substantia nigra pars compacta (Gerlach et al. 1994). Iron is a pro-oxidant that can donate an electron to enhance redox reactions through conversion from the ferrous (FeII) to the ferric (FeIII) form (Gutteridge et al. 1983). Of particular significance in PD is the shift in the Fe (II)/Fe (III) ratio from almost 2:1 to 1:2 (Gerlach et al. 1994). Iron-induced oxidative stress has been widely implicated in the pathogenesis of PD (Olanow 1992; Gerlach et al. 1994; Youdim and Riederer 1994). Dopamine can be either oxidized by MAO or converted by auto-oxidation to generate H2O2. Reactive iron (FeII) can catalyze DA auto-oxidation and convert H2O2 to the highly active •OH via the Fenton reaction described in equation 1 (Ross et al. 1998). •OH, in turn, can damage proteins, nucleic acid, and membrane phospholipids, eventually leading to cellular degeneration (Beal 1992). Iron can also selectively bind to neuromelanin, producing a ferric (FeIII)-melanin complex, which will be discussed below.

Increased concentrations of iron may also contribute to progressive nigrostriatal degeneration in PD (Gerlach et al. 1994; Gassen and Youdim 1997; Jenner and Olanow 1998). Mice that were fed a high-iron diet resulting in increased iron in the brain, display a substantial reduction in amounts of the antioxidant GSH in the SN along with a corresponding increase iron in •OH levels(Lan and Jiang 1997). If L-DOPA or DA is present, they form semiquiniones that can deplete GSH and cysteine very quickly. If H2O2 is present, Fe (III) promotes this reaction (Spencer et al. 1998). Alterations of iron concentration have also been reported in other disorders of the basal ganglia such as Huntington's disease and progressive supranuclear palsy.

Iron participates in free radical-generating reactions only in the free, ferrous form. Ferric iron in the nigra is normally bound either by ferritin (about 90%) or by neuromelanin (10%). Ferritin is a 450kDa protein with 24 subunits forming a cavity, which can store up to 4500 atoms of ferric iron. A variety of molecules with an *o*-dihydroxy- phenyl structure (including 6-OHDA and dopamine) effectively release ferritin-bound iron in vitro and thereby stimulate lipid peroxidation (Foley and Riederer 2000) (Double et al. 1997); the iron chelator desferrioxamine has antagonistic actions, protecting rats against 6-OHDA induced loss of striatal dopamine (Ben-Shachar et al. 1991b). In animal models, infusion of iron into the substantia nigra induced neurodegeneration and a dose-related decline in striatal dopamine.

In PD, it is not known whether the increased iron levels are due to reduced ferritin levels or increased cellular uptake of iron. The role of ferritin in PD is controversial. Different groups have reported ferritin to be either increased, unchanged, or decreased in the SN of PD brains (Riederer et al. 1989b) (Dexter et al. 1990) (Mann et al. 1994). Recently, a study using subunit-specific antibodies suggests that ferritin concentrations do not compensate for increased iron content in PD (Faucheux et al. 1998). Whether increased amounts of ferritin can protect the neurons from iron-induced free radicals is not an easy question to answer.

It is considered unlikely that iron is involved early in the pathogenesis of PD, since laboratory tests have not indicated elevated levels of iron in the stage without clinical symptoms of the disease, however, its potent ability to generate ROS means that it may play a significant role in the progression of PD (Foley and Riederer 2000), especially as it is accompanied by a dramatic rise in aluminum levels (Gutteridge et al. 1985) which would exacerbate iron-related oxidative damage; aluminum salts accelerate lipid peroxidation induced by iron salts (Gutteridge et al. 1985).

1.4.6 The function of neuromelanin

Neuromelanin (NM) is an insoluble black–brown granular pigment that accumulates in the cytoplasm of neurons of the substantia nigra and locus ceruleus. According to X-ray diffraction studies, NM has a multi-layer, graphite-like, three-dimensional structure (Zecca et al. 2001a). NM has similar properties to the melanin found in the eye and in hair; for example it exhibits the presence of stable free radical centres, low solubility and an affinity for transition metals (Barden 1969; Zecca and Swartz 1993). Neuromelanin accumulates normally with age in human, rat, canine and primate substantiae nigrae (DeMattei et al. 1986). In humans, the first NM granules appear around the third year of life (Fenichel and Bazelon 1968) and NM concentrations in the substantia nigra increase linearly over aging, reaching values as high as 4.0 mg g^{-1} in those aged in their eighties. By contrast, in PD patients, NM concentrations drop to <50% of those in age-matched controls (Zecca et al. 2002a).

The process of NM formation is obscure. Evidence suggests that NM is not synthesized by a tyrosine-tyrosinase system as in skin melanocytes, but by a different pathway involving spontaneous autoxidation of brain catecholamines, in particular dopamine. The fact that the distribution of NM and that of the brain catecholamines - noradrenaline and dopamine is strikingly similar (Saggu et al. 1989) leads to the conclusion that melanin in neurons is intimately related to the ability of these cells to synthesize

dopamine or noradrenaline. While tyrosinase does not appear to have a role in the synthesis of NM no other enzyme system has been demonstrated to be involved in neuromelanogenesis, although studies have investigated enzymes as various as macrophage inhibitory factor, prostaglandin H synthase and peroxidase.

Melanin in neurons containing catecholamines confined to primates and carnivores suggest that NM may serve some positive function. It is proposed that the formation of NM from the metabolic products of dopamine protects the cell against toxic quinone and semiquinone species produced during the metabolism of dopamine (Toshihide et al. 1997).

In PD, there is a preferential loss of pigmented neurons. Anatomical studies in PD's SN demonstrate that the dorsal tier, containing high levels of neuromelanin, is less vulnerable than the ventral tier, which contains less neuromelanin and is more affected by the degenerative process (Mann and Yates 1983). In contrast, the high correlation observed between the percentages of surviving neurons in PD patients and their neuromelanin content, indicates that the heavily melanized neurons are more vulnerable (D'Mello et al. 1993) (Youdim et al. 1994). Therefore, the question of whether neuromelanin plays a role in the pathogenesis of the dopaminergic neuronal loss remains undetermined.

One of the interesting questions concerning neuromelanin in the normal SN is whether it has physiological importance or it is a toxic by-product. Neuromelanin is not conserved during evolution: it appears only in humans and few other species (i.e. primates and dogs) (Agid et al. 1993) (Bridelli et al. 1982). And neuromelanin accumulates in adults, and is not found in infants (Swan 1963; Bridelli et al. 1982; Porebska-Budny et al. 1992). Several in-vitro experiments demonstrate possible protective features of neuromelanin (Brayda-Bruno and Levi 1979) (Kastner et al. 1992), however, many more observations indicate that it is a toxic product of the DA metabolism. Overload of neuromelanin is neurotoxic both, in vitro and in vivo (Offen et

al. 1997a) (von Baumgarten et al. 1980). Furthermore, NM is possibly associated with the vulnerability of dopaminergic neurons in SN (Cotzias et al, 1964, Hornykiewicz et al, 1987, Ben-Shachar et al, 1991). In PD the melanized neurons of SN are more vulnerable than the non-melanized ones. However in PD such vulnerability does not correlate with the amount of NM since the highest pigmented neurons are spared (Kastner et al, 1992, Gibb et al. 1992). It was reported (Gibb, 1992) that the more vulnerable nigral ventral tier cells contain less neuromelanin than the more heavily pigmented cells in the dorsal tier, suggesting that neuromelanin may confer an advantage upon the cells in which it is found.

NM interacts with organic molecules including lipids, pesticides, and toxic compounds and this interaction may have important implications for the cell. Some of these interactions have been suggested to be of possible benefit to the cell, for example it has been suggested that NM might reduce the efficacy of the neurotoxin MPTP by accumulating its toxic metabolite MPP^+ in vivo (D'Amato et al. 1986). NM also interacts with many heavy metal ions, such as zinc, copper, manganese, chromium, cobalt, mercury, lead, cadmium and iron. Indeed the accumulation of iron in the substantia nigra seems, at least in part, to occur within the NM granules (Dexter et al. 1989; Sofic et al. 1991). Several studies have demonstrated iron is directly bound to NM granules in the substantia nigra (Zecca et al. 1996; Gerlach et al. 1997) and that the iron is bound in the ferric, rather than ferrous state as polynuclear oxy-hydroxyl aggregates in a manner similar to that in ferritin and haemosiderin (Jellinger et al. 1992; Gerlach et al. 1995). This suggests that NM may be a high capacity storage system for iron.

Several reports suggest that at physiological concentration of iron, most iron ions are bound to NM or other iron-binding molecules within the substantia nigra, inhibiting free radical generation (Zareba et al. 1995; Double et al. 1999). On the other end, when dopamine-melanin (DAM), a synthetic form of this pigment produced by the

autoxidation of DA used to model NM, is saturated with ferric ions the formation of free hydroxyl radicals by redox activation of the ions is increased. Such an event may also occur in vivo, thus NM has been described as a "two-edged sword", with both positive and negative effects on the cell, depending upon the concentration of iron (Youdim et al. 1994). At normal concentrations of iron, NM attenuates free radical production, which protects neurons from radical-induced damage. But in the presence of high iron concentrations, NM may act as an effective pro-oxidant, resulting increased production of •OH. When ferric ion is predominant, the production of •OH in the presence of melanin is significantly greater (Pilas et al. 1988) and increased lipid peroxidation of rat cerebral cortex is stimulated in the presence of ferric iron and DAM (Ben-Shachar and Youdim 1991). In contrast Double et al showed that the addition of isolated NM pigment significantly attenuates iron-induced lipid peroxidation in the rat brain homogenate measured in vitro (Double et al. 1999), suggesting that the effect of NM reflects not so much the pigment itself, but also the environment which it is found, in agreement with suggestions by other authors (Krol and Liebler 1998) (Youdim and Riederer 1994) (Zecca et al. 2002b). The mechanism of this possible protective effect of NM is obscure. NM might either inactivate free radicals directly or bind potential damaging species, such as transition metals in low concentrations, or both.

The mechanism of a possible toxic effect of NM is also unclear. Double et al. suggested that the damage is iron-mediated (Double et al. 1999). Iron bound to many compounds is not redox-active, for example ferritin. It is possible that within the dopaminergic neurons in the parkinsonian substantia nigra a) the production of free radicals is mediated by the pool of mobile iron with the cell (Halliwell 1992) b) iron can be released from the iron-NM complex or c) the iron-NM complex may remain redox-active.

1.5 Hypothesis and aims of this project

The overall aim of this project is to investigate the comparative effects of NM and DAM on the two primary cell types within the brain, neurons and glia, and to investigate if this effect is changed under an oxidative stimulus.

The specific aims of this work are:

1. To establish a method for the quantification of ·OH in a cell culture system

2. To quantify the effects of NM and DAM on indices of cell damage in neuronal and glial cell lines

3. To quantify the effects of NM and DAM on cell survival in neuronal and glial cell lines.

4. To quantify ·OH production in NM-treated neurons under conditions of normal and increased iron loads

The hypotheses to be tested in this work are:

1. Synthetic "neuromelanin" (DAM), but not native NM, will be toxic to both neuronal and glial cell lines.

2. Toxicity stimulated by oxidative stress will be attenuated by NM, but not by the synthetic melanin DAM.

3. Neuronal cells will be relatively more vulnerable to cell damage induced by DAM and an oxidative stimulus compared to glial cells.

2. Materials and Methods

2.1 Cell culture as a model of nigral pigmented neurons

NM-containing neurons of the human substantia nigra cannot be grown in culture as adult brain cells do not divide and while human fetal neurons can be cultured NM is not present prenatally. The use of animal models for this work is also inappropriate, as the midbrains of commonly used laboratory animals do not contain NM pigments in the central nervous system (Marsden 1961). NM first appears in the human brain at 2-3 years of age and accumulates with aging (Cowen 1986). Therefore the culture of human brain cells to which NM is added is the only model available for these cells in the adult human brain in vivo. It is an ideal method for research as the cells grow in an easily manipulated environment and both in situ and in vitro measurements of cell responses can be made. Human NM and synthetic melanin are incorporated into both human-derived neuronal and glial cells in *vitro* via a phagocytosis-like mechansim (Dr Kay Double, Sydney, personal communication), providing a convenient model for NM/cell interactions in the human brain.

Two different types of cell cultures were used: a human-derived neuroblastoma cell line, which exhibits dopaminergic characteristics (SK-N-SH) and a human-derived gliablastoma cell line (U373), which exhibits glial characteristics. These two cell lines model the major cell types within this region.

It is significant that human NM was used in these experiments; this was the first time that isolated human NM has been investigated in a cell culture system. Double et al have shown that human NM differs significantly in its structure compared with the synthetic pigment, suggesting that the synthetic pigment commonly used in published work pertaining to NM to date is an inappropriate model of NM (Double et al. 2000).

As oxidative stress is believed to be aetiologically important for PD, Fenton reagent was added to the culture medium to induce an environment of oxidative stress.

2.2 General cell culture methods

2.2.1 Cells used

Cell name: SK-N-SH

Provided by: European Collection of Cell Cultures (ECACC), Salisbury, UK

ECACC No: 86012802

Description: Human Caucasian neuroblastoma, established from a bone marrow metastasis from a 4-year-old Caucasian female suffering with neuroblastoma. It exhibits dopaminergic characteristics.

Cell name: U373 MG

Provided by: European Collection of Cell Cultures (ECACC), Salisbury, UK

ECACC No: 89081403

Description: Human glioblastoma astrocytoma, derived from a malignant tumour by explant technique. It exhibits glial cell's characterisitics

2.2.2 Culture Medium used and preparation

The cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM). A stock media was prepared including 100IU/ml Penicillin, 100µg/ml Streptomycin and 1mM Hepes buffer and stored at 4°C. Complete (incubation) media was freshly prepared from the stock solution with the addition of 4mM L-glutamine and 10% Fetal Bovine Serum (FBS and used at 37°C) and it could be safely stored at -4°C for up to 5 days. Prior to use, FBS was inactivated by heating to 56 °C for 30 minutes. All solutions were prepared in sterile conditions in a laminar flow hood.

2.2.3 Defrosting cells

Cells were shipped frozen and stored in liquid nitrogen. After defrosting by placing the ampoule in a water bath at 37°C until a small amount of ice remained, cells were rinsed with serum-free medium (4°C) to expel DSMO. The cells were resuspended in warm complete medium after centrifugation at 4°C, 170 g for 5 minutes and added with freshly prepared complete medium to a culture flask and incubated in a humid 37°C, 5%CO2-95% air incubator.

2.2.4 Feeding cells

Medium was replaced by fresh medium every 3 days. After the spent medium was removed, the cells were rinsed with PBS without Ca^{2+}/Mg^{2+} using a volume equivalent to half the volume of culture medium, and an equivalent volume of fresh warm complete medium was added to the flask, care being taken as not to disturb the cells.

2.2.5 Subculturing cells

Cells grew to confluence in 4-5 days and were then sub-cultured. The medium was removed and pre-warmed trypsin–EDTA was pipetted onto the washed cells monolayer using 1ml per 25cm ^{2} of surface area. After centrifugation at 170g, 25°C for 5 minutes, the cells were resuspended into a small amount of warm complete medium to inactive the trypsin. Aliquots of dissociated cells were transferred to new flasks with pre-warmed complete medium at a concentration of 1:3.

2.2.6 Harvesting treated cells for analyses

The medium was removed into a centrifuge tube to capture any dead cells released from the cell monolayer. The remaining cells were removed from the culture flask using pre-

warmed trypsin-EDTA and added to the tube. The tube was centrifuged at 170g, 4°C for 5 minutes. After the supernatant was completely removed, the cell pellet was rinsed twice with cold PBS without Ca^{2+}/Mg^{2+} (4°C), and the pellet was snap frozen on dry ice and stored at -70°C.

2.2.7 Freezing cells for storage

Pre-warmed trypsin–EDTA was pipeted onto the washed cells monolayer to suspend the cells. After centrifugation at 170g, 25 °C for 5 minutes, the cells were resuspended in fresh cold medium and transferred to a cryotube with an equivalent volume of ice-cold cryoprotection medium. Cryoprotection medium contained 10% DMSO and 30% FCS. The cryotube was kept at 4 °C for 30 minutes before being placed at -70 °C overnight; the frozen ampoule was transferred to the vapor phase of a liquid nitrogen storage vessel for long-term storage.

2.3 Preparation of melanin pigments

2.3.1 Neuromelanin isolation from the human brain

NM was isolated by Double according to her published protocol (Double 2000a). Five grams of fresh frozen human substantia nigra tissue was weighed and homogenized in 20ml double-stilled water using a Potter at 1000rpm until a uniform homogenate formed. Aliquots of the resultant homogenate were divided into tubes and centrifuged at 12,000g, 4°C for 10 minutes. After being rinsed twice with 0.05M phosphate buffer, pH=7.4, the tissue was combined into one pellet be centrifugation and incubated in 0.05M SDS in Tris buffer, pH=7.4 and then in 0.2mg/ml fresh prepared proteinase K in 0.05M SDS in Tris buffer. Both the incubations were carried out at 37°C for 3 hours under conditions of shaking. After these incubations, the tissue was again centrifuged and the pellet

rinsed four times with NaCl, water, HPLC grade methanol and HPLC grade hexane respectively. The resultant neuromelanin (NM) pellet was dried under a gentle flow of argon. A 0.15M solution of EDTA was then added to the tube and incubated at room temperature to remove any loosely bound metals. This incubation step was repeated, and then the NM was finally retrieved by centrifugation and dried by lypholisation.

FeNM was prepared by Double as previously described (Double et al. 2003). A sample of NM was incubated with 10 mM $Fe_2(SO_4)_3$ for 2 hrs to load the NM with iron and subsequently washed in citrate buffer to remove excess iron, washed twice in water and dried to a powder. Iron content in this sample, designated FeNM, was measured by atomic absorption spectroscopy to be 100.23μg iron/mg NM. The iron content of a sample of NM treated with EDTA as described (EDTA-NM), was 2.84μg iron/mg NM.

2.3.2 Synthesis of synthetic dopamine melanin (DAM)

Synthetic DAM was prepared by Double according to Ben-Shachar et al (Ben-Shachar et al. 1991a). 10ml of 10mM CuSO4 · 5H2O and 100ml of 10mM of dopamine hydrochloride in 1×PBS were combined and made up to 1L with 1×PBS. This solution was protected from light and incubated at room temperature for two weeks. An oxidized product was formed, and after removal of the top layer, it was separated from supernatant by centrifugation at 2500 rpm for 15 minutes. After being washed four times with water, the DAM pellet was dried by lyophilization.

2.4 Treatment of cells

2.4.1 DAM and NM

Neuromelanin and synthetic dopamine melanin were sonified in sterile double distilled H2O at 50% amplitude and pulse on 0.5 second and pulse off 0.5 seconds the until DAM and NM formed even suspensions. Different concentration of DAM was added

into culture from 0. 05mg/ml to 0.5mg/ml, and 0.1mg/ml was chosen as the optimal concentration for the following experiments.

2.4.2 Fenton reagent

Fenton reagent (FR) consisted of 400μM Iron (II) – sulfate Heptahydrate ($FeSO4 \cdot 7 H2O$) and 200μM hydrogen peroxide ($H2O2$) in sterile distilled $H2O$. Cultures were incubated with FR for different time periods and 24 hours was chosen as the optimal concentration and used in the following experiments.

2.4.3 Iron chelator

Desferrioxamine mesylate salt (DFOM) was added to the culture prior to the addition FR to chelate iron. Different concentration of DFOM was added at different time points in pilot experiments, and 100μM DFOM pre-incubation for 4 hours was chosen as the optimal condition to attenuate the effects of the FR

2.5 Electron photography

Treated cells seeded into a 6-well plate were fixed in 2.5% glutaraldehyde in 01. M Phosphate buffer (pH 7.3) for 60 min, then washed three times with 5% sucrose in 0.1M Phosphate buffer (pH 7.3) and post-fixed with 2% Osmium tetroxide for 60 min. After 3 further washes in 5% sucrose in 0.1M Phosphate buffer the cells were dehydrated in graded ethanol washes and flat embedded in Epon. Blocks were thin-sectioned at 90 nm and viewed on a Zeiss 10CR transmission electron microscope. (This work was done by Dr. Double in Prince of Wales Medical Research Institute, Australia.)

2.6 Measurement of experimental endpoints

2.6.1 Production of hydroxyl radicals

As the production of hydroxyl radical was of primary interest in this work, a novel method of measurement of these molecules was developed which is described in detail in Chapter 3.

2.6.2 Lipid peroxidation

2.6.2.1 Principle

Lipid peroxidation is a major indicator of oxidative stress. This process leads to the distruction of membrane lipids and production of lipid peroxides and their by-products such as aldehydes. Malonadehyde (MDA) and 4-hydroxyalkenals, such as 4-hydroxy-2 (E)-nonenal (4-HNE), are end products derived from the breakdown of polyunsaturated fatty acids and related esters. Lipid hydroperoxides degrade to MDA and 4-HNE in the presence of transition metals when heated. Measurement of such aldehydes provides a convenient index of lipid peroxidation.

2.6.2.2 Method

Calbiochem Lipid peroxidation assay kit (Cat. No. 437634, Merck KGaA, Darmstadt, Germany) were adopted. Cells were passaged into 12-well-plates and kept growing until confluent. After feeding with fresh medium, cultures were treated with Fenton reagent and different concentration of DAM or NM. After incubation at 37°C, 5% CO2 for different times, cells were trypsinised and harvested. Both the medium and cells were centrifuged 200 × g for 10 minutes at 4°C. After rinsing twice with PBS, the cell pellets

were resuspended by 50µl dd H2O with 5mM butylated hydroxyl tobuene (BHT) to prevent sample oxidation. The cells were lysed by repetitive freeze/thawing until homogenates were formed. 40µl homogenate were taken out for the assay and the rest for protein measurement.

Next steps were performed following the directions of the kit. The standards were prepared according to Table 1 in clean glass test tubes. The MDA standard stock solution (S2) was diluted 500-fold in water just prior being used and a 20µM stock solution was yielded.

Table 1: Standard Curve Dilution Volumes

Target concentration of standard in reaction mixture, µM	0	0.50	1.00	2.00	3.00	4.00
Volume of 20µM standard to add	0	5	10	20	30	40
Volume of water or buffer to add	40	35	30	20	10	0

Diluted R1 reagent was prepared by adding one volume of Diluent (ferric iron in methanol) to three volumes reagent R1 (N-methyl-2-phonylindole in acetonitrile). 40µl unknown sample or standards and 130µl of Diluted R1 reagent were added to a clean glass test tube. 30µl R2 reagent (methanesulfonic acid) was added into the mixture. The tubes were stoppered after vortexing and incubated at 45°C for 45 minutes. After centrifuged, the supernatant was transferred to a plate and measured absorbance at 586nm. Both the standards and unknowns were run in triplicate.

The kinetics of color development on the sample was tested followed in comparison with MDA standard by measuring absorbance at different wavelength. The A586 of the sample reach a plateau and then remain stable.

2.6.3 Calculations

The net A586 for each standard is calculated by subtracting the blank value from each of the standard A586 values. Net A586 vs [MDA] was plotted and a linear regression analysis was performed on [MDA]: [MDA] = a [MDA] + b.

The concentration of analyte in each unknown is calculated from the net A586 of the sample.

2.6.4 Optimize incubation time

Fenton reagent (400μM Fe^{2+} + 200μM H_2O_2) and DAM 0.1mg/ml were added into confluent culture respectively and incubated for different time. The MDA and HNE productions were increased in a time-dependent manner. 24 hours was found to be the optimal incubation time and was thus used in the following experiments (Figure 2).

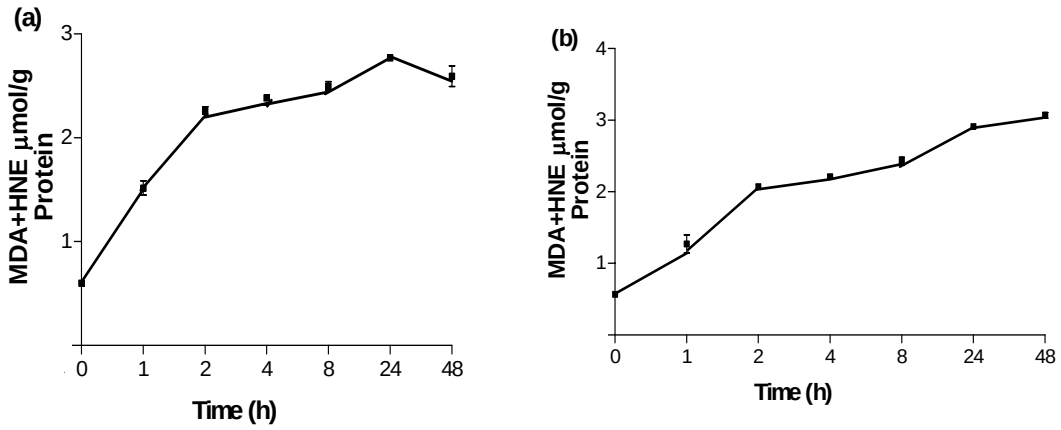

Figure 2. Lipid peroxidation in SK-N-SH cultures treated by 0.1mg/ml DAM (a) and Fenton reagent (400μM Fe^{2+} + 200μM H_2O_2,) (b) was in a time-depend manner. At 48hours, the production was not increased significantly compared with 24 hours.

2.7 Apoptosis detection by FACscan

Treated SK-N-SH cells were trysinized and transferred into the FACS tubes. After centrifugate, the cells were saved and 50μl of the Annexin-V-FITC staining solution

(yielded by 1µl of 7-AAD dissolved in 49 µl of medium) was added into tubes and incubated at room temperature for 20 minutes. Immediately prior to FACS measurement, 50 µl of the 7-AAD-staining solution (yielded by dissolving 1 µl of 7-AAD in 49 µl of medium) was added into each tube and incubated for 10 seconds. Cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany), and the acquisistion software LysisII (Becton Dickinson). Cells treated with the apoptosis-inducing topoisomerase inhibitor camptothecin (2 µM, Alexis Biochemicals, Germany) served as a positive control for apoptototic cells. Markers were set according to untreated cells. Annexin-V-positive/7-AAD-negtive events were scored as apoptotic cells; double-positive events were scored as late apoptotic or necrotic cells. Double negative events were scored as living cells. This method was established by Dr. Scheller and Dr. Koutsilieri, Virology Institute, Wuerzburg University.

2.8 Statistical analyses

All experiments were performed three times in triplicate. Data were pooled and analysed using regression analysis and one- or two-way Analysis of Variance followed by Bonferroni's t test corrected for multiple comparisons as appropriate.

2.9 Chemical sources

Dulbecco's Modification of Eagle's Medium (DMEM), without L- Glutamine, with 4,5g/L Dextrose, Cat No. 1233254, ICN, Ohio, USA

L-Glutamine solution, 200mM, Cat No. G7513, Sigma-Aldrich, USA

Fetal Bovine Serum (FBS), Biochrom KG, Cat No. S0115, Berlin, Germany.

Penicillin-Streptomycin (500×), Cat No. 92253300, Roche, Mannheim, Germany.

Hepes Buffer 1M, Cat No. H0887, Sigma-Aldrich, USA

1× Dulbecco's Phosphate Buffer Saline (PBS), without Ca^{2+}/Mg^{2+}, Cat No. 1860454, ICN, Ohio, USA

Trypsin – EDTA solution 1×, from bovine pancreas, in PBS Ca^{2+}/Mg^{2+} free, Cat No. 210242, Roche, Mannheim, Germany

3, 4-dihydroxybenzoic acid (3, 4-DHBA), Sigma-Aldrich, USA

Dimethyl Sulphoxide (DMSO) Hybrid-Max, C2H6OS, Cat. No. D2650, Sigma Chemical Co. St. Louis, MO, USA

SDS (Laurysulfat), Cat. No. L-4390, Sigma Chemical Co. St. Louis, MO, USA

Tris, C4H11NO3, Cat. No. 77-86-1, Applichem GmbH, Darmstadt, Germany

Proteinase K, Cat No. 745723, Boehringer Mannhaim GmbH, Mannheim, Germany

Nacl (Sodium Chloride), Cat. No. 7647-14-5, Applichem GmbH, Darmstadt, Germany

Methanol, CH3OH, Cat. No. 106018, Merck KGaA, Darmstadt, Germany

n-Hexane, C6H14, Cat. No. 104391, Merck KGaA, Darmstadt, Germany

EDTA, $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$, Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Cupric Sulphate Pentahydrate ($CuSO_4 \cdot 5H_2O$), Cat. No. C-6283, Sigma-Aldrich, USA

Dopamine Hydrochloride ($C_8H_{11}NO_2 \cdot HCL$), Cat. No. H-8502, Sigma-Aldrich, USA

Hydrogen Peroxide Solution (H_2O_2), 30%, Cat. No. 904 K11321197, Merck KGaA, Darmstadt, Germany

Eisen (II) – sulfate Heptahydrat ($FeSO_4 \cdot 7H_2O$), Cat. No. 005 TA 660165, Merck, KGaA, Darmstadt, Germany

Desferrioxamine Mesylate Salt (DFOM), Cat. No. D9533, Sigma-Aldrich, USA

Salicylic acid (2-Hydroxybenzoic acid) $C_7H_5O_3Na$, Cat No. S3007, Sigma-Aldrich, USA

Phosphoric acid 85%, M=98.00g/mol, Cat No. 1.00552, Merck, KGaA, Darmstadt, Germany

Diethylenetriaminepentaacetic acid (DTPA), M=497.36g/mol, Cat. No. 35904-1, Aldrich-Chemie, Steinheim, Germany

2, 6-Di-tert-butyl-4-methylphenol (BHT), Cat No. B1378, Sigma-Aldrich, USA

Lipid Peroxidation Assay Kit, Cat. No. 437634, Calbiochem, USA

Annexin V-FITC, Cat. No. 556419, BD Pharmingen, Heidelberg, Germany

7-Amino-Actinomycin D (7-AAD), Cat. Nr. 559925 (was: 68981E), BD Pharmingen, Heidelberg, Germany

2.10 Instruments

Biofuge Fresco, Heraeus Instrument, Germany

Centrifuge, ROTANTA 96RS, Germany

NUAIRE Biogogical Safty Cabinet, ZAPF Instrument, Germany

Microscope, Leica DMIL, Germany

Centrifuge, ROTANTA 96RS, Germany

BRANSON Digital Sonifier, Model 250, EDP 100-214-239, USA

Multiscan Spectrum, Thermo Life Science, Germany

HPLC, Agilent 1100 Series, Bio-Rad, USA

75cm^2 – Tissue culture flask, BD Falcon, Cat No. 353 136,USA

25cm^2 – Tissue culture flask, Becton Dickinson and company, Cat No. 353 108, USA

Serological Pipette 5ml, Sarstedt, Aktiengesellschaft and Co. Cat No. 86,1253,001, Nuembrecht, Germany

Serological Pipette 10ml, Sarstedt, Aktiengesellschaft and Co. Cat No. 86,1254,001, Nuembrecht, Germany

Serological Pipette 25ml, Sarstedt, Aktiengesellschaft and Co. Cat No. 86,1685,001, Nuembrecht, Germany

PP-Test tube, 15ml, Greiner bio-one, Cat No. 188,271,Germany

PP-Test tube, 50ml, Sarstedt Germany, Cat No. 62.547.254, Nuembrecht, Germany

TC-Plate 6-wells with lid, Greiner bio-one, Cat No. 657,160, Germany

Non-sterile, ultrafree-MC, 5000NMWL, Filter unit. Cat No. UFC 3LCCNB, Millipore,USA

Cuvettes 10×4×45mm, Cat No. 67.742, Sarstedt, Nuembrecht, Germany

FACscan flow cytometer, Beton Dickinson, Heildelberg, Germany

Sterile, RNase-free Pipet tips, 10µl, Cat. Nr. 81-1011; 100µl, Cat. Nr. 81-1030; 1000µl, Cat. Nr. 81-1050, PEQLAB Biothnology GmbH, Erlangen, Germany

Heating block, Thermomixer Comfort, Eppendorf-Netheler-Hinz GmbH, Germany

Electrophoresis power supply, CONSORT E844, Germany

Thermo-Fast 96-PCR-plates, Cat. Nr. 82-0900, PEQLAB Biothnology GmbH, Erlangen, Germany

Bio-Rad iCycle iQ^{TM} optical tape, Cat. Nr. 2239444, Bio-Rad Laboratories, Herculer, CA, USA

iCycler iQ^{TM} Real-Time PCR detection system, Cat. Nr. 170-8740, Bio-Rad Laboratories, Herculer, CA, USA

Thermo-Fast 96, PCR plates, Cat. No. 82-0900, Peqlab Biotechnology GmbH, 91052, Erlangen, Germany

iCycle iQ Optical Tape, Cat. No. 2239444, Bio-Rad Laboratory, 94547 Hercules, CA, USA

Thin-wall 8-tube strip, Cat. No. 712070, Biozym, Germany

Optical 8-Cap Strip, Cat. No. 712100, Biozym, Germany

3. Development a new method for the quantification of hydroxyl radicals in an in vitro system

3.1 Introduction

Iron-stimulated hydroxyl radical production has been suggested to play an important role in nigral neuron death in Parkinson's disease. •OH radicals may be a causative factor in the onset and/or progression of Parkinson's disease and may contribute to neuronal cell death (Dexter et al. 1989) through chain reactions of membrane lipid peroxidation and/or alterations in membrane fluidity. Such reactions include breakage of single and double-stranded DNA, chemical alterations of the deoxyribose purine and pyrimidine bases and of membrane lipids and carbohydrates, leading to a cascade of events that ultimately results in damage to the mitochondrial electrotransport system, disturbance of intracellular calcium homeostasis, induction of proteolysis by proteases, increased membrane lipid peroxidation, release of excitotoxic amino acids (glutamate, aspartate), and, finally, cell death.

It is not possible to accurately quantify levels of •OH radicals in vivo, because they have a very brief half-life (measureable in nanoseconds), due to their high reactivity. Therefore, a model system is needed to indirectly quantify •OH production. Salicylic acid (SA) can be used as a free radical trapping system to quantify levels of $^{\bullet}$OH production. It passes through the cell membrane and forms 2, 3-dihydroxybenzoic acid (2, 3-DHBA) when it reacts with •OH. This method has been previously used by two groups. Giovanni et al (Giovanni et al. 1995) systemically and intraventricularly administered salicylate in rats treated with methamphetamine to initiate neurodegeneration. Salicylate acted as an exogenous •OH trap in vivo, forming the stable and quantifiable products 2, 3-DHBA and 2, 5-DHBA. High-performance liquid

chromatography (HPLC) was used to determine the concentration of 2, 3-DHBA and 2, 5-DHBA in the homogenate of treated rats brains. Whereas 2, 5-DHBA may be formed enzymatically (Halliwell and Gutteridge 1986), the formation of 2, 3-DHBA appears to be dependent on the presence of •OH. A later report by Thome et al (Thome et al. 1997) utilized this method in a clinical setting. To detect increased oxidative stress in alcohol-dependent patients, these workers intravenously injected acetylsalicylic acid to patients, which was metabolized to salicylic acid in vivo. Then they determined 2, 3-DHBA and 2, 5-DHBA in patients' blood by HPLC and reported a higher production of 2, 3-DHBA in the alcohol-dependent patients. To date, this method has not been modified for use in an in vitro setting but we reasoned that this method would lend it self to the measurement of •OH in cell cultures as salicylic acid can pass freely through cell membranes. We thus developed this method for the estimation of •OH in our cell culture systems.

3.2 Methods and results

3.2.1 Treatment of cells

After rinsing with PBS, confluent cultures were treated with SA, and/or DFOM, FENTON REAGENT, DAM, NM and incubated for 24 hours, as described in Chapter 2. Cells were harvested by trypsin and centrifuged with spent medium at 4°C 13000 rpm for 10 minutes; the pellets were saved and rinsed with PBS. The samples were prepared for HPLC immediately or stored at -70°C.

3.2.2 Samples preparation for HPLC

50µl buffer (150mM H3PO4 with 500µM DTPA) with 2.5µM of 3, 4-DHBA (as an internal standard) was added to the cell pellet and sonicated at 50% amplitude for 1 minute at 0.5 second pulse on and 0.5 second pulse off or until a uniform homogenate

was formed. 10ul of homogenate was removed for protein measurement and the remainder transferred to a filter-cap and centrifuged at 13000rpm at 4°C. The resultant filtrate was used for HPLC measurement.

3.2.3 Quantification of hydroxyl radicals by HPLC

10µl Filtrate was injected into Agilent autosampler 1100 (Bio-Rad) and analyzed for 2, 3-DHBA by reverse-phase HPLC with electrochemical detection. The HPLC system consisted of a solvent delivery pump (Agilent pump, G1312A, Bio-Rad), an electrochemical detector (Model 1640, Bio-Rad) with a glassy carbon electrode. Peaks were recorded by means of an integrator (ChemStation Agilent, Program 2). The detector potential was set at +75mV vs Ag/Agcl, with the sensitivity setting at 10nA. A 5µm analytical column was used, operating at a flow rate of 1.0 ml/min. All separations were performed at room temperature (22-27°C). The mobile phase consisted of 0.1M sodium phosphate buffer, PH 3.9, 84% (v/v) and 16% (v/v) methanol containing 0.1mM EDTA, 0.65 mM 1-octanesulfonic acid and 0.5mM triethylamine. Qualitative and quantitative analysis of metabolites was performed by comparing retention times and peak areas with internal standard 3, 4-DHBA.

3.2.4 Optimization of salicylic acid concentration

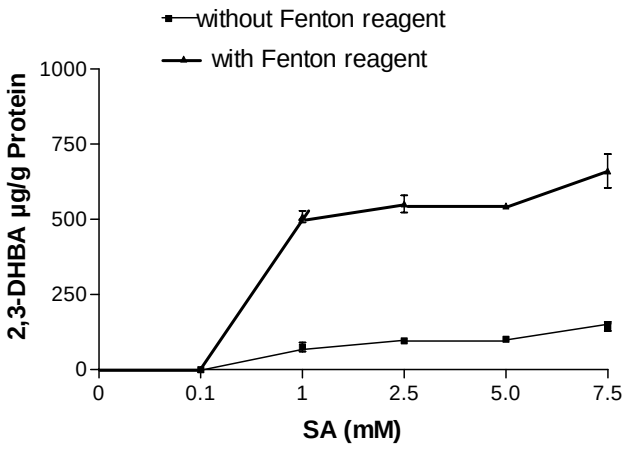

Figure 3. Different concentration of SA induced hydroxyl radical production estimated by the spin-trapping product 2,3-DHBA in SK-N-SH cells with or without Fenton reagent. Fenton reagent, but not SA itself, markedly increased 2, 3-DHBA formation above control levels. Two-way analysis of variance showed an additional affect of Fenton reagent ($P<0.001$).

Different concentrations of SA were added to confluent cells, with or without Fenton reagent. The concentrations used were 1μM, 1mM, 2.5mM, 5mM and 7.5mM. Treated cells were harvested after incubated for 24hours. 2, 3-DHBA was undetected in cell homogenate in the presence of SA concentrations up to 1mM but was clearly measurable at 1mM and remained relatively stable at higher concentrations (Figure 3). The addition of Fenton reagent markedly increased the amount of 2, 3-DHBA produced. Cell morphology was unchanged in the presence of SA concentrations up to 2.5mM but visible cell death occurred at the two highest concentrations.

3.2.5 Optimization of incubation time

Cultures were incubated with 1mM SA and Fenton reagent for different time periods from 1h to 48 hours. 24 hours incubation was the optimal incubation time when a clear reproducible signal of 2, 3-DHBA was present (Figure 4).

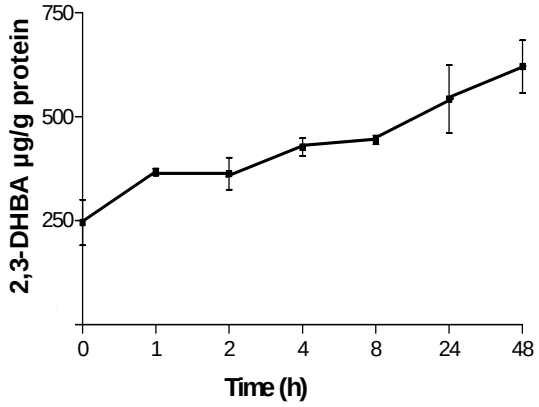

Figure 4. 1mM SA plus Fenton reagent induced hydroxyl radical production estimated by the spin-trapping product 2,3-DHBA in SK-N-SH cells at different time. Fenton reagent induced hydroxyl radical production in a time dependent manner.

3.2.6 Optimization of incubation time and concentration of DAM

Different concentrations of DAM were added to confluent cultures and incubated for 24 hrs. 0.1mg/ml DAM was chosen as the optimal pigment concentration, which results in a clear 2, 3-DHBA signals. This was then further investigated at different incubation periods and 24 hrs was chosen as a practical incubation time for the remaining experiments (Figure 5 and Figure 6).

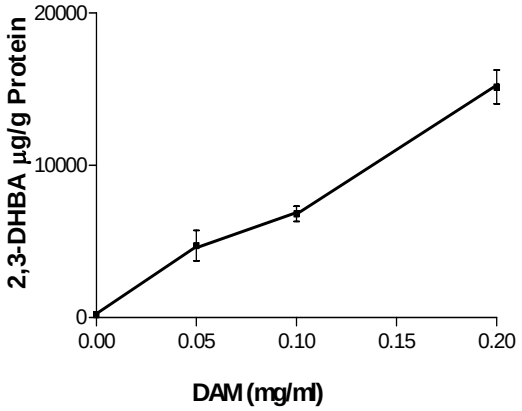

Figure 5. 1mM SA plus different concentration of DAM induced hydroxyl radical production estimated by the spin-trapping product 2, 3-DHBA in SK-N-SH cells. DAM induced hydroxyl radical production in a concentration dependent manner.

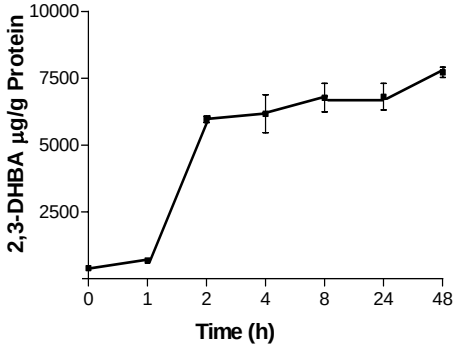

Figure 6. 1mM SA plus 1mg/ml DAM induced hydroxyl radical production estimated by the spin-trapping product 2, 3-DHBA in SK-N-SH cells. DAM induced hydroxyl radical production in a time dependent manner.

3.2.7 Optimization the concentration of NM

Confluent cultures were treated with different concentrations of NM from 0.05mg/ml to 0.2mg/ml. 0.1mg/ml NM was chosen as a standard concentration for use in the following experiments as it was associated with a clear signal, but did not use too much of the rare human pigment (Figure 7).

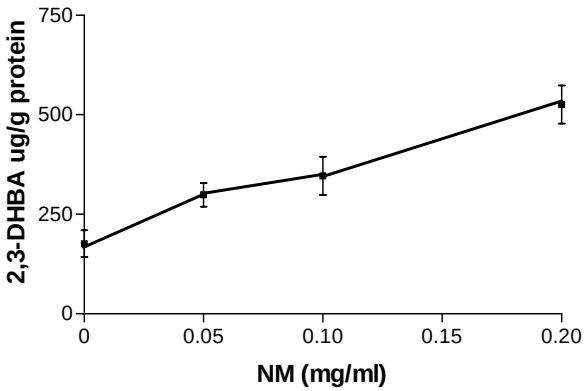

Figure 7. OnemM SA plus different concentration of NM induced hydroxyl radical production estimated by the spin-trapping product 2,3-DHBA in SK-N-SH cells. NM induced hydroxyl radical production in a concentration dependent manner.

4. Results

4.1 Phagocytosis of melanins

Incorporation of both DAM and NM was observed in both cell types under the light microscope, thus this phenomenon was further investigated using electron microscopy. Following 24 hr incubation, both the native NM and the synthetic DAM pigment could be seen as electron dense granules both within the cell bodies of the SK-N-SH (Figure 8 B and C) and U373 cells and lying external to the cells. Each melanin type appeared present at equiconcentrations within each cell type but the U373 cells incorporated a greater amount of both melanin types than SK-N-SH cells. Examination of cells in which the incorporation of melanin was incomplete suggested that the melanin was incorporated into the cell via an invagination of the cell membrane (Figure 8A).

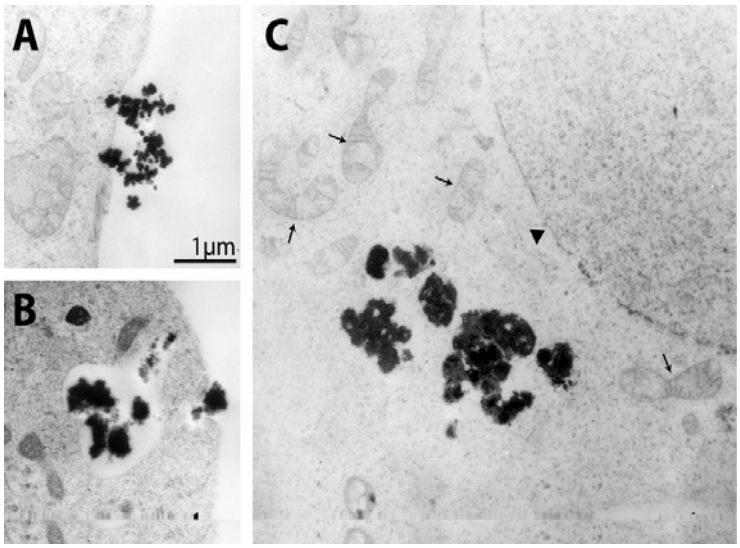

Figure 8: Electron microscopy of melanin uptake. A: Invagination of the cell membrane as DAM is phagocytosed into SK-N-SH cells. B: Phagocytosed DAM in the cytoplasm of SK-N-SK cells. C: Phagocytosed NM in the cytoplasm of SK-N-SK cells. The nuclear membrane (arrowhead) and mitochondrial membranes (arrows) can be clearly seen but no membrane is evident surrounding incorporated NM. This work was done by Double at el in Prince of Wales medical research institute, Australia.

Following the complete incorporation of the melanins both NM and DAM were present as loosely defined dark colored granules of heterogeneous size in the cytoplasm. While other cellular membranes, such as he nuclear membrane and mitochondrial membranes

could be clearly discerned individual melanin granules did not appear to be membrane-defined (Figure 10C), although occasionally a partial membrane could be seen.

4.2 LDH activity

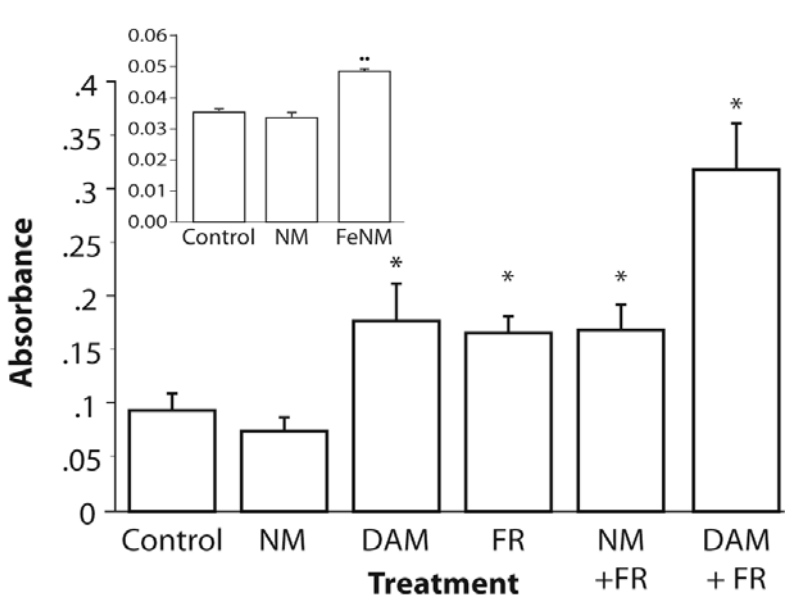

Figure 9: LDH activity in SK-N-SH cells was significantly increased by DAM (p=0.02), and FR (p=0.05), but not NM, compared with untreated cells. Co incubation of FR with NM increased LDH activity above control values (p= 0.4) but not above that seen in FR-treated cultures (p= 0.92). In contrast, DAM and FR added together significantly increased LDH activity above levels in both control and FR-treated cultures (p<0.0001 and p=0.0002 respectively). Inset: 0.1 mg/ml FeNM significantly increased LDH activity above those in control and NM-treated cultures (**both $p < 0.001$) *$p < 0.05$ compared with control, NM: 0.1 mg/ml neuromelanin, 0.1 mg/ml DAM: synthetic dopamine melanin, FR: Fenton reagent (400µM $FeSO_4$ + 200µM H_2O_2), FeNM: iron-saturated NM.

In SK-N-SH cells, LDH activity was significantly enhanced by Fenton reagent to 1.75 times that of untreated control cells (p=0.05). Similarly, 0.1mg/ml DAM significantly increased lactate activity to 1.9 times that of control cells (p=0.02). The affects of DAM and Fenton reagent added together were additive; lactate activity increased to 3.4 times that of basal levels when these treatments were combined (p<0.0001). In contrast to the effect of DAM, however, lactate activity in the presence of 0.1mg/ml NM did not differ to that in control cultures (p= 0.58). In the presence of both Fenton reagent and 0.1 mg/ml NM lactate activity increased to 1.78 times control activity (p= 0.04), however

this increase was equivalent to that stimulated by Fenton reagent alone (Figure 9). In a separate series of experiments the effect of 0.1 mg/ml iron-saturated NM (FeNM) on stimulation of LDH activity was compared with 0.1 mg/ml EDTA-treated NM and untreated cultures. LDH activity was significantly increased in FeNM-treated SK-N-SH cells compared with control and with EDTA-treated cultures (Figure 9, $p < 0.001$ compared with both control- and EDTA-NM-treated cultures).

4.3 Lipid peroxidation

Following 24 hrs incubation with Fenton reagent, lipid peroxidation in SK-N-SH cells was 19 times that of untreated cells (Figure 10, p=0.02). Similarly, following treatment with 0.1 mg/ml DAM lipid peroxidation was 23 times that of control levels (p= 0.005) and when cells were treated with varying concentrations of DAM a dose-dependent increase in lipid peroxidation was measurable (Figure 10). In contrast, lipid peroxidation following treatment with 0.1 mg/ml NM did not differ to that in untreated cells (p=0.54). Co-incubation of Fenton reagent with either DAM or NM resulted in additive effects, compared to the levels elicited by Fenton reagent and the melanins alone. Further, the iron chelator desferroxamine did not induce lipid peroxidation (p = 96, not shown) but when added together with Fenton reagent attenuated lipid peroxidation to control levels (p= 0.37) and when added with DAM plus Fenton reagent (p=0.07) and NM plus Fenton reagent (p=0.06) attenuated lipid peroxidation to levels equivalent to those induced by the melanin pigments alone (Figure 10).

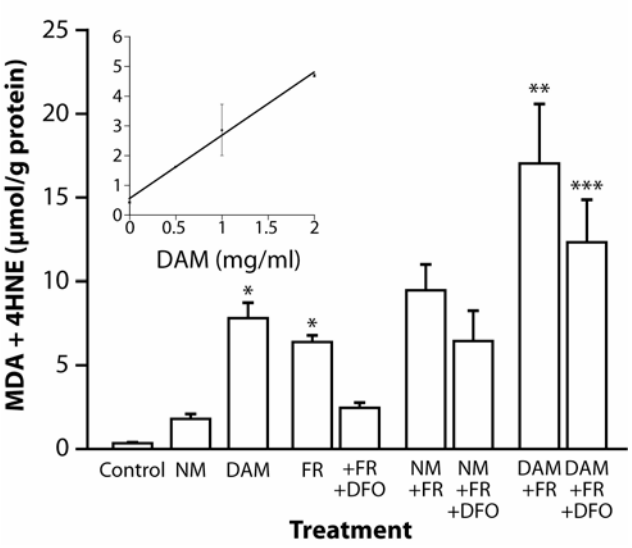

Figure 10: Lipid peroxidation in SK- N-SH cultures following 0.1 mg/ml NM was not different to control levels (p=0.54). In contrast, 0.1 mg/ml DAM induced significantly greater lipid peroxidation compared with control cultures (p= 0.005), as did FR (p=0.02). Lipid peroxidation induced by addition of NM to FR was not different to that produced by FR alone (p=0.20) but DAM and FR stimulated significantly greater lipid peroxidation than FR. The iron chelator DFO (100 µM) did not induce lipid peroxidation (p = 96, not shown) but when added together with FR attenuated lipid peroxidation to control levels (p= 0.37) and when added with DAM plus FR (p=0.07) and NM plus FR (p=0.06) attenuated lipid peroxidation to levels equivalent to those induced by the melanin pigments alone. Inset: The increase in lipid peroxidation induced by DAM was dose-dependent. *p < 0.05 compared with control, **p=0.0002 compared with FR, *** p= 0.02 compared with FR, NM: 0.1 mg/ml neuromelanin, 0.1 mg/ml DAM: synthetic dopamine melanin, FR: Fenton reagent (400µM $FeSO_4$ + 200µM H_2O_2).

In contrast, lipid peroxidation in U373 cells exposed to NM or DAM did not differ to that measured in untreated cells (Figure 11).

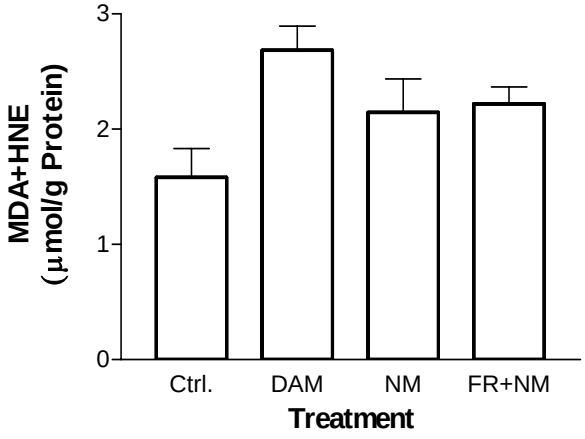

Figure 11: Lipid peroxidation in U373 cultures following 0.1 mg/ml DAM or 0.1 mg/ml NM or FR plus 0.1 mg/ml NM was not different to control levels (p>0.05).

4.4 Hydroxyl radical production

One mM salicylic acid was chosen as the optimal concentration for the spin-trapping experiments as this concentration was associated with normal cell morphology and cell survival equivalent to that in untreated cells (data not shown). Incubation of 1 mM salicylic acid in SK-N-SH cells with Fenton reagent induced a time-dependent increase in the hydroxyl radical-stimulated product 2, 3-DHBA which plateaued at 24 hrs thus this incubation time was chosen for all subsequent experiments. One mM salicylic acid alone did not stimulate 2, 3-DHBA but the addition of Fenton reagent increased 2, 3-DHBA production to three times that of control levels (Figure 12, p = 0.19). The addition of 0.1 mg/ml NM did not increase 2, 3-DHBA levels above control (p= 0.80), in contrast, the addition of the same concentration of DAM increased 2, 3-DHBA 20

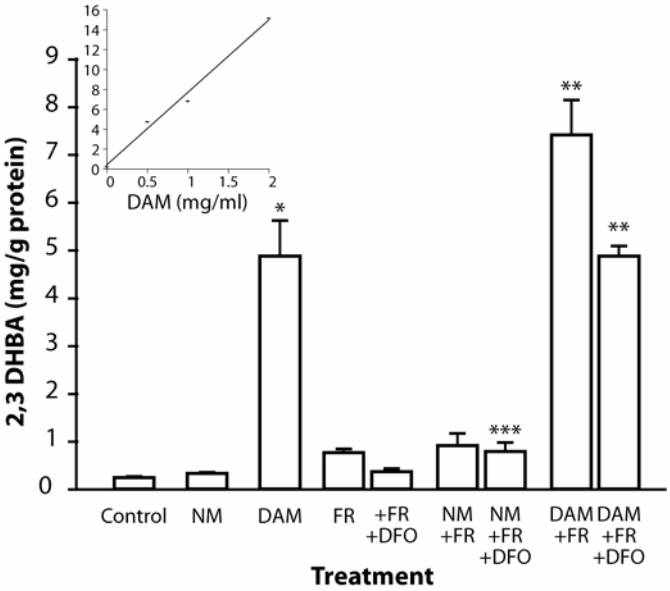

Figure 12: Hydroxyl radical production estimated by the the spin-trapping product 2, 3-DHBA in SK-N-SH cultures. DAM, but not NM (p= 0.80), markedly increased 2, 3-DHBA formation above control levels. The addition of FR to NM did not increase 2, 3-DHBA levels above those stimulated by FR alone (p = 0.76) but the addition of FR to DAM significantly increased 2, 3-DHBA to ten times that of Fenton alone. The iron chelator DFO reduced FR-induced 2, 3-DHBA to control levels (p =0.39) and, when added together with FR and the melanins, educed 2, 3-DHBA to levels associated with the melanins alone (p = 0.29 and p = 0.99 for NM and DAM respectively). Levels of 2, 3-DHBA following treatment with DAM + FR + DFO, however, remained significantly higher than those stimulated by FR alone. *p < 0.0001, compared with control. **p < 0.0001 compared with FR. Inset: The effect of DAM on 2, 3-DHBA production was concentration dependent. NM: 0.1 mg/ml neuromelanin, 0.1 mg/ml DAM: synthetic dopamine melanin, FR: Fenton reagent (400µM $FeSO_4$ + 200µM H_2O_2).

times that of control levels (Figure 12, $p < 0.0001$). Further, the effect of DAM was concentration dependent (Figure 12, inset). The addition of Fenton reagent to NM did not increase 2, 3-DHBA levels above those stimulated by Fenton alone ($p = 0.76$), but the addition of Fenton reagent to DAM significantly increased 2, 3-DHBA to ten times that of Fenton alone ($p < 0.0001$). The iron chelator DFO effectively reduced Fenton-induced 2, 3-DHBA to control levels ($p = 0.39$) and, when added together with Fenton reagent and the melanins, reduced 2, 3-DHBA to levels associated with the melanins alone ($p = 0.29$ and $p = 0.99$ for NM and DAM respectively).

Consistent with results obtained in the lipid peroxidation experiments, hydroxyl radical production in U373 cells incubated with either NM or DAM did not differ to that in untreated cells (Figure 13).

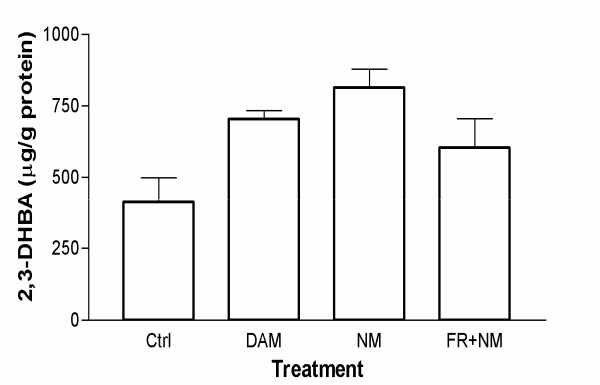

Figure 13: Hydroxyl radical production estimated by the the spin-trapping product 2, 3-DHBA in SK-N-SH cultures. DAM or NM or FR plus NM did not increase 2, 3-DHBA formation above control levels ($p>0.05$). NM: 0.1 mg/ml neuromelanin, 0.1 mg/ml DAM: synthetic dopamine melanin, FR: Fenton reagent (400μM $FeSO_4$ + 200μM H_2O_2).

4.5 Flow cytometry on SK-N-SH cells

The synthetic melanin DAM (0.1mg/ml) stimulated a time-dependent increase in total cell death in SK-N-SH cell (Figure 14A). Further, the proportion of SK-N-SH cells expressing apoptotic staining characteristics following incubation with 0.1 mg/ml DAM for 24 hrs was three times higher than that of untreated cells ($p <0.001$, Figure 14C-F). In contrast to the positive apoptosis control camptothecin-induced apoptosis, however,

DAM-associated apoptosis was not attentuated by the caspase-inhibitor ZVAD ($p = 0.09$, Figure 14C).

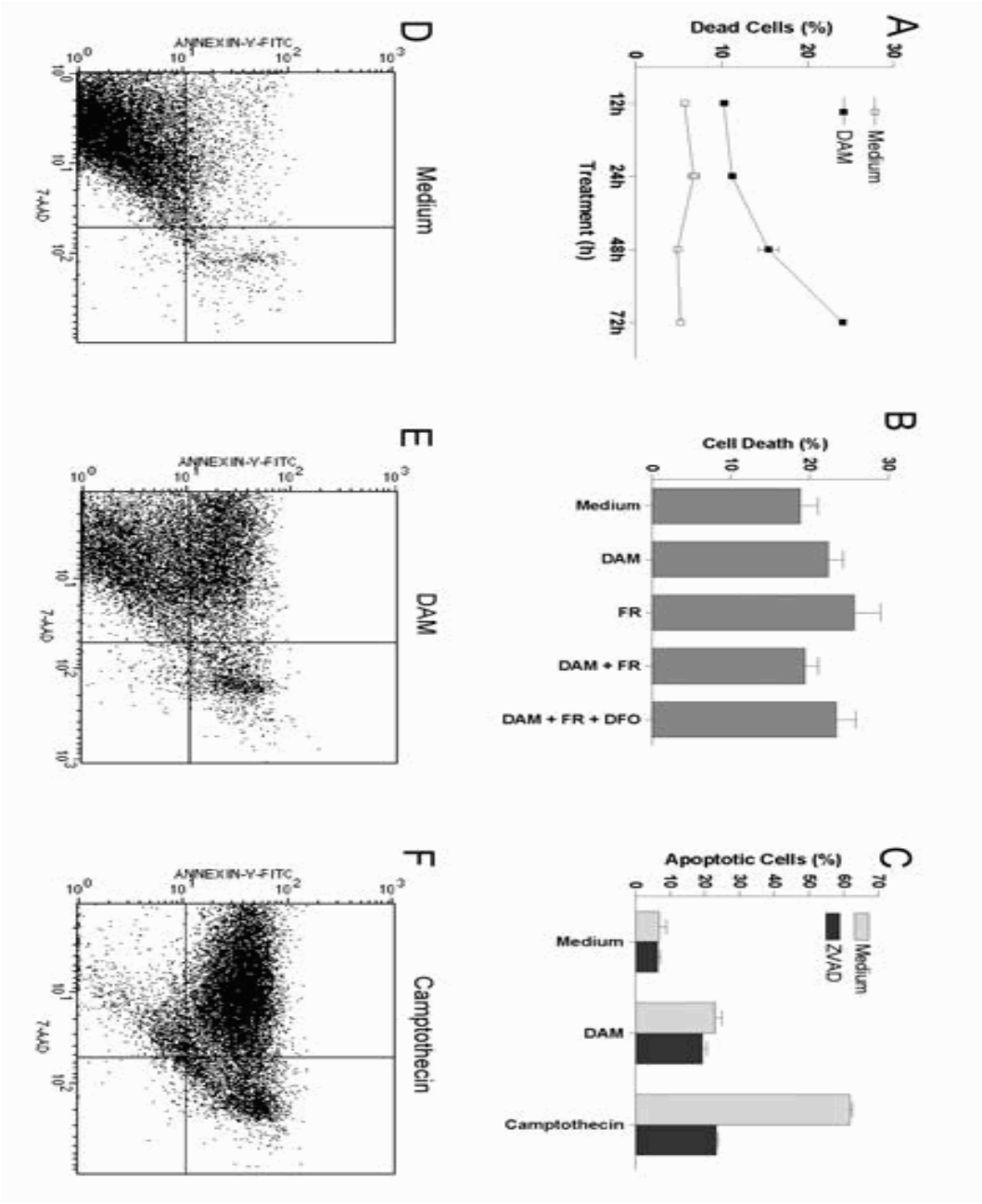

Figure14. DAM induces apoptotic cell death in SK-N-SH cells. A: SK-N-SH cells were incubated with DAM (0.1 mg/ml, black squares) or medium alone (white squares) for the indicated time. Cells were stained with 7-AAD and analyzed by flow cytometry. 7-AAD-positive cells were scored as dead cells and shown in percent. Triplicate analysis, data represent means ± S.E.M. B: SK-N-SH cells were incubated with Medium alone, DAM, Fenton reagent, DAM+FR, or DAM+FR+DFO for 24h. Data are mean values from 3-8 independent experiments. Cells were stained with 7-AAD and analyzed by flow cytometry. 7-

AAD-positive cells were scored as dead cells and shown in percent. C: SK-N-SH cells were incubated with medium alone, DAM (0.1 mg/ml) or Camptothecin (2 μM) in presence or absence of zVAD-fmk (ZVAD, 100 μM). Cells were stained with Annexin-V-FITC/7-AAD and analyzed by flow cytometry. Annexin-V-FITC-positive/7-AAD-negative cells were scored as aopototic cells and shown in percent. Triplicate analysis, data represent means ± S.E.M. D, E, F: Representative pictures of FACS-analysis derived from C. D: untreated cells, E: DAM (0.1 mg/ml)-treated cells, F: Camptothecin (2 μM)-treated cells.

Unfortunately, native NM, but not synthetic DAM, shows autofluorescence under confocal microscope (Figure 15); therefore the results from NM-treated cells are artificial. We conclude that flow cytometry method, which is based on capturing the fluorescence of samples, is not a proper way to detect NM-treated cell's survival.

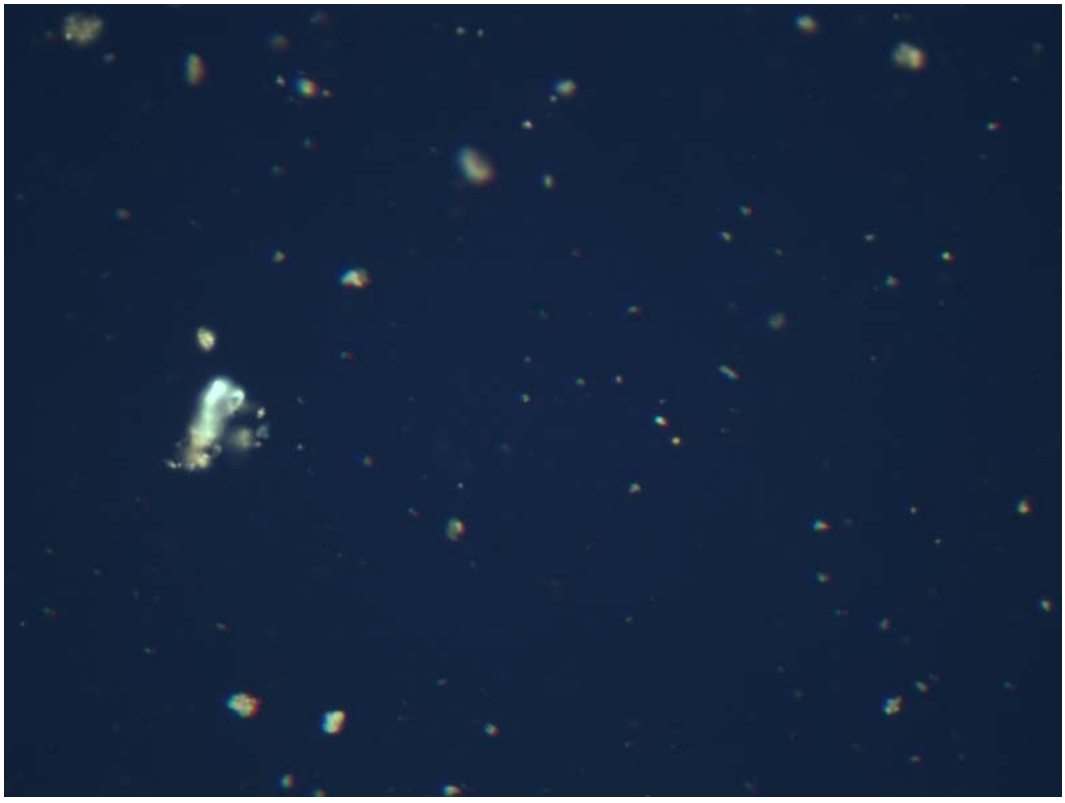

Figure 15. Isolated NM itself under confocal mocroscope, it shows autofluorescence. This work was done by Dr. Double in Prince of Wales Medical Research Institute.

5. DISCUSSION

5.1 A new method and a novel model

Salicylic acid was used as a spin trap for •OH as it passes freely through the cell membrane and forms quantifiable amounts 2, 3-DHBA when it reacts with •OH. Following interaction with •OH the resultant 2, 3-DHBA can be quantified using isocratic reverse-phase HPLC with electrochemical detection. The method provides a convenient and reproducible way for quantification of •OH in cell culture systems suitable for the investigation of oxidative mechanisms in vitro.

It is very important to choose standard experimental conditions. The concentration of SA, DAM or NM and incubation time were important factors to be taken into consideration for the assay. Low concentrations of SA might not form quantifiable amounts of 2, 3-DHBA, while concentrated SA might be toxic to the culture. To optimize conditions, different concentrations of SA were added to cells from 1μM to 10mM. 1mM SA was identified as the lowest concentration forming detectable quantities of 2, 3-DHBA in the absence of changes in cell morphology and was therefore chosen as the standard concentration of SA in the following experiments.

The incubation time was another important factor for the experiments. Because 2, 3-DHBA production in cell homogenates increased in a time-dependent manner, the optimal time was one long enough for the complete reaction among SA, DAM or NM and/or Fenton reagent. A long incubation period may result in the cells suffering a lack of nutrition so that any change of metabolism, altered PH of the medium, bacteria or fungus infection may affect the accuracy of 2, 3-DHBA detection. Cells were harvested at different times after being treated and the homogenates were analyzed. 24 hours was

chosen as the optimal time as it is sufficient for the reaction but offers little chance of infection.

Finally the concentration of DAM and NM is crucial. What makes studying NM challenging is the limited amount of NM contained in human brain. Therefore, NM is very rare and to find out the proper working concentration of it was one of the most important parts of this experiment. First, different concentrations of DAM was added to cells rather than using NM directly. The concentration range covered 0.025mM to 0.5M. The results showed the formation of 2, 3-DHBA increased in a concentration-dependent manner up to 0.1mg/ml. Therefore, 0.1mg/ml NM and DAM were chosen as the working concentration used in most of the experiment.

Interestingly in our cell culture system, and in contrast to the human and animal studies previously utilizing this method (Giovanni et al. 1995; Thome et al. 1997) 2, 5-DHBA was a very small peak was sometimes below the limits of detection. 2, 5-DHBA is an enzymatic production induced by cytochrome P-450 and it doesn't represent the oxidative situation. Only 2, 3-DHBA is formed from the reaction between •OH and salicylicate, which reflects the oxidation situation. It is an advantage of the use of this technique in cell culture that the influence of induced metabolism of SA by cytochrome P-450 appears to be rather less than in a whole human or animal.

This technique provides a new, quick and safe method for the quantification of •OH in cell culture systems suitable for the investigation of oxidative mechanisms in vitro. In addition, it may provide a convenient in vitro system for screening potential neuroprotective agents for Parkinson's disease in vitro.

The SK-N-SH cells were dopaminergic cells; they may serve as a model for studying neuronal damage in general and neurodegeneration in the SN of PD patients, in particular.

The NM-containing neurons of the human adult substantia nigra cannot be grown in culture for technical reasons, The use of animal models for this work is inappropriate as

the midbrains of commonly used laboratory animals do not contain NM pigments in the central nervous system (Marsden 1961). Thus the use of a human neuron-derived cell line is a useful approach to address the effects of NM on dopaminergic neuron function. Daniel Offen et al used PC12 cell line, which is rat pheochromocytoma PC12, rather than human derived (Offen et al. 1997a; Offen et al. 1997b; Offen et al. 1999). Two different types of cell cultures were used: a human-derived neuroblastoma cell line, which exhibits dopaminergic characteristics (SK-N-SH) and a human-derived gliablastoma cell line (U373), which exhibits glial characteristics. These two cell lines model the major cell types within this region.

Previous functional studies have utilised synthetic dopamine melanin, DAM, to investigate the influence of NM in cell culture systems (Offen et al. 1997a; Offen et al. 1997b; Offen et al. 1999), but this is the first work to use internalised NM isolated from the healthy human brain as a model of intraneuronal pigment in vitro. NM and DAM were incorporated into both the glial and neuronal cell line by a phagocytosis-like mechanism similar to that previously reported (Offen et al. 1999). Consistent with the physiological functions of glial cells, a larger number of U373 cells incorporated melanin than the SK-N-SH cells, although equal concentrations of NM and DAM were incorporated within each individual cell. In contrast to the incorporation of DAM into mouse cerebellar granule cells (Offen et al. 1999), neither DAM nor NM granules were contained within membrane-delimited vesicles within our SK-N-SH or U373 cells. This is consistent with the lack of a persistent membrane-bound organelle associated with NM granules reported in the human midbrain (D'Agostino and Luse 1964), (Moses et al. 1966) (Duffy and Tennyson 1965) (Schwyn et al. 1970). Double et al have shown that human NM differs significantly in its structure compared with the synthetic pigment, suggesting that the synthetic pigment commonly used in published work pertaining to NM to date is an inappropriate model of NM (Double 2000b).

5.2 The function of NM and DAM

Our results agree with previously published work using cell line (Offen et al. 1997b) and primary mesencephalic culture models, in that we found functional changes indicative of cellular stress (increased LDH activity and increased lipid peroxidation) induced by a range of concentrations of DAM in SK-N-SH cells. In contrast, U373 cells appeared resistant to the effects of melanin at concentrations associated with marked degenerative changes in SK-N-SH cells. This finding is consistent with the relative robustness of glial cells in the brain, which can survive and even proliferate under conditions where neuronal cells degenerate (Hirsch et al. 2003).

Flow cytometry confirmed that DAM-induced toxicity resulted in cell death in SK-N-SH cells and that this death occured via apoptotic mechanisms, consistent with a previous report (Offen et al. 1997b). Radical trapping experiments suggest that cell injury and death associated with DAM may occur, at least partially, via stimulation of $^{\bullet}OH$ production, consistent with previously in vitro reports that DAM toxicity can be attenuated by antioxidants (Offen et al. 1997a).

Iron is been suggested to be a critical cofactor in the production of $^{\bullet}OH$ by DAM (Pilas et al. 1988), and in our system is present in the culture medium. Toxicity induced by co-incubation of Fenton reagent and DAM was additive and iron chelation in cultures treated with both of these agents reduced the $^{\bullet}OH$ production associated with Fenton reagent, but not that associated with DAM, suggesting that the DAM polymer itself might produce $^{\bullet}OH$. In contrast, NM at the same concentration failed to induce any functional evidence of damage to SK-N-SH cells, nor was it associated with increased $^{\bullet}OH$ formation or apoptosis. The relative toxicity of DAM, but not NM in cell lines, as well as the finding that human NM injected into the substantia nigra and caudate putamen of rats is not associated with evidence of neurotoxicity (Aime et al. 1996).

Unlike NM, DAM and Fenton reagent appear to be toxic to SK-N-SH cells. DAM stimulates significantly higher •OH production and lipid peroxidation than that stimulated by NM. In the presence of Fenton reagent and DAM, •OH production and lipid peroxidation are increased in an additive manner. The iron chelator desferrioxamine reduced •OH production and lipid peroxidation to levels stimulated by DAM alone, suggesting that DAM-induced lipid peroxidation and •OH production occur via iron-dependent pathways, consistent with the findings of Offen et al, who reported (described his results here) in a PC12 cell line (Offen et al. 1999).

5.3 The interaction between NM and iron

High and low-affinity iron-binding sites in NN have recently been reported in vitro (Double et al. 2003) and suggested that in the healthy brain NM might function as a endogenous iron chelator, reducing the availability of redox-active iron and thus serving a protective function within the vulnerable dopaminergic neurons (Double et al. 2003). Consistent with this suggestion is the finding that the nigral ventral tier cells most effected in PD contain less NM than the less vulnerable, heavily pigmented cells in the dorsal tier of the SN, suggesting that NM may confer an advantage on the cells in which is found (Gibb 1992). It has been previously shown in vitro that NM can significantly reduce basal levels of lipid peroxidation in rat brain homogenate (Double et al. 1999). At the concentration used in the current experiments, however, both NM and DAM were unable to attentuate the toxic effects of the added oxidative stimulus whereby a total of 224 µg iron/ mg NM was added to the culture wells. The atomic absorbtion experiments suggest that NM is saturated with iron at 100 µg/mg NM, thus the added iron probably exceeded the chelating capacity of NM. The iron chelator desferoximine, however, effectively reduced Fenton-induced indices of cellular damage, suggesting a pivatol role of iron in the toxicity observed. In Parkinson's disease, where nigral iron

levels are increased, the saturation of high affinity iron binding sites on NM may also overwhelm the protective capacity of this molecule, leading instead to an increase in redox iron activity, and subsequent cellular damage (Ben-Shachar et al. 1991a), (Double et al. 2003). This hypothesis is supported by our current finding of increased LDH activity stimulated by iron-saturated FeNM and our previous work in which iron-saturated NM induced significantly greater membrane damage in vitro compared with NM in which iron levels were low (Double et al. 1999). Interestingly, biophysical data suggest that the iron-binding abilities of NM in the parkinsonian brain are reduced compared to that in the healthy brain (Lopiano et al. 2000) (Bolzoni et al. 2002). This would further increase the likelihood of iron saturation of NM in the parkinsonian brain, thus putting these cells at greater risk for iron-mediated damage. Indeed post-mortem data indicate that redox-active iron associated with NM granules is significantly increased within the parkinsonian substantia nigra (Jellinger et al. 1992) (Good et al. 1992) (Zecca and Swartz 1993) consistent with the hypothesis that the NM/iron interaction contributes to a heightened oxidative load in the vulnerable pigmented neurons. Iron-mediated damage may be stimulated by the release of iron from its binding site on NM. The release of iron from its binding sites in NM into the cellular milieu would thus increase the concentration of free redox-active iron within the cell, resulting in the production of free radical species.

5.4 The differences between NM and DAM

Our current data demonstrate that DAM represents a poor functional model of NM in that it displays a marked toxicity unrepresentative of the effects of the native melanin. These results support previous studies of these two molecules, which suggest that the synthetic pigment represents a poor structural model for NM (Enochs et al. 1993)

(Double et al. 2000). Unlike NM, which is likely to undergo slow oxidation over a period of years rendering it redox inactive, DAM is produced over a time periods measured in hours or days, and thus may be incompletely oxidised. A range of concentrations of DAM increase LDH and lipid peroxidation and •OH production, which means DAM induces cellular stress, thus it is toxic to cells. In contrast, NM at the same concentration failed to induce any functional evidence of damage to SK-N-SH cells, nor was it associated with increased ˙OH formation.

Double et al and Aime et al suggested that the synthetic product differ in some respects as compared to human neuromelanin (Double et al. 2000) (Aime et al. 1996). For example, there is no amino acid and DOPA can be detected in DAM, but in NM, no matter if it was treated with proteinase or not, it contains certain amount of amino acid and DOPA. On the other hand, DAM comprises large amount of DA, while NM has less (Double et al. 2000). An earlier study has shown that dopamine is extremely toxic to PC12 cells; this toxicity, however, is progressively reduced by autoxidation of the dopamine and removal of the subsequent insoluble product (Offen et al. 1997b)). It is believed that dopamine also undergoes autoxidation in vivo, a process, which ultimately results in the formation of NM, via pathways yet to be fully described. During autoxidation of dopamine a range of radicals (hydrogen peroxide, hydroxyl radicals and superoxide), to which the toxicity of dopamine is ascribed, are produced (Swartz et al. 1992) (Aime et al. 1994). Further, enzymatic metabolism of produces, quinone species suggested to be involved in mechanistic pathways leading to cell damage, such as mitochondrial dysfunction and protein aggregation (Sulzer and Zecca 2000), as well as also mediating the synthesis of ˙OH and oxyradical species. These multiple pathways arising from dopamine catabolism may thus contribute directly to the high oxidative load in these neurons.

5.5 NM from Parkinson patients and control subjects

It also cannot be ruled out that the structure of NM in PD may be different from that in normal brain (Lopiano et al. 2000), which may foster increased release of iron to the cytoplasmic pool and the catalysis of radical production (Double et al. 1999). Several precise methods with a subcellular resolution such as laser microprobe mass analysis and energy dispersive X-ray microanalysis have demonstrated an accumulation of iron in neuromelanin granules in the SNpc of PD patients (Good et al. 1992; Jellinger et al. 1992; Kienzl et al. 1999). This accumulation of nigral iron seems to occur within the NM granules, where it was found that iron concentration is higher than in neuromelanin granules of normal subjects (Jellinger et al. 1992) (Good et al. 1992). In normal conditions, NM binds high amounts of iron ions under the form of small Fe^{3+} oxyhydroxy particles and (Gerlach et al. 1995; Aime et al. 1997), to a low extent, under the form of mononuclear Fe^{3+} ions. If the neuromelanin chelating frame is destroyed, iron ion would become available as Fenton-active low molecular weight chelates. Iron can then catalyze the oxidation of catecholamines to electrophilic semiquinoid or quinoid species, which are the attacking agents to amine, cysteine, or arginine residues of proteins (Aime et al. 2000).

The NM used in this experiment was extracted from control subjects rather than from the brains of individuals with PD. Up to 90% of pigmented neurons are lost in PD, thus it is unfeasible to isolate sufficient NM from parkinsonian brains for use in the functional experiments described in the current work.

To sum up, our results consistent with our hypothesis that ssynthetic DAM, but not native NM, is toxic to cell line. Neuronal cells were relatively more vulnerable to cell damage induced by DAM and an oxidative stimulus compared to glial cells. But

contradiction with our hypothesis, toxicity stimulated by oxidative stress was attenuated neither by native NM nor by synthetic DAM.

6. Summary

Neuromelanin (NM) is a complex polymer pigment found in catecholaminergic neurons of the human substantia nigra and locus ceruleus. The structure of this molecule is poorly characterised, and the physiological function of it in the brain is unknown. In vitro data, based upon synthetic dopamine melanins (DAM), suggest that these pigments may exhibit radical scavenging properties, but in the presence of iron, DAM acts as a proxidant. These data suggested that NM may be associated with the especial vulnerability of pigmented dopaminergic cells in Parkinson´s disease (PD), a disorder in which nigral iron levels are increased and the relatively specific loss of the pigmented neurons of the substantia nigra. Given the rarity of NM, and the difficulty of isolating this material from the human brain, all functional studies of NM published to date have utilised a synthetic dopamine melanin in place of the native pigment. In the current work we investigated the effects of NM from the healthy human brain and synthetic DAM on cell health and oxidative status in human-derived cell lines.

Methods SK-N-SH, a human neuroblastoma cell line, and U 373, a human glioblastoma cell line was chosen to represent human neuronal and glial cell types. NM was isolated from the SN of adult human subjects from Australia and Germany with no history of neurological or neurodegenerative diseases. Synthetic DAM was prepared by autooxidation of dopamine. DAM and NM samples were added to the cultures with fresh media to final concentrations of 0.05 or 0.1 mg/ml. In some experiments cells were incubated with Fenton reagent (400µM $FeSO_4$ plus 200µM H_2O_2) in the presence or absence of melanin or the iron chelator desferoxamine mesylate (100µM). The cells were incubated at 37 °C at 5% CO2 for varying periods of time as described. Lactate dehydrogenase (LDH) activity and Lipid peroxidation were measured. Hydroxyl radical production in the cultures was estimated used a modification of the salicylic acid spin-trapping method. All experiments were performed three times in triplicate and analysed

using regression analysis and one- or two-way Analysis of Variance followed by Bonferroni's t test corrected for multiple comparisons as appropriate.

Results Following 24 hr incubation, both the native NM and the synthetic DAM pigment could be seen as electron dense granules both within the cell bodies of the SK-N-SH and U373 cells. The melanin was incorporated into the cell via an invagination of the cell membrane. DAM but not NM significantly increased the LDH activity and lipid peroxidation as well as the hydroxyl radical production. Co-incubation of Fenton reagent with either DAM or NM resulted in additive effects, compared to the levels elicited by Fenton reagent and the melanins alone. When added the iron chelator desferoximine together with Fenton reagent attentuated lipid peroxidation and hydroxyl radical production to control levels. In contrast, lipid peroxidation and hydroxyl radical production in U373 cells exposed to NM or DAM did not differ to that measured in untreated cells.

Discussion Human neuron-derived cell line is a useful approach to address the effects of NM on dopaminergic neuron function. This is the first work to use internalised NM isolated from the healthy human brain as a model of intraneuronal pigment in vitro. Cell line functional studies showing cellular changes induced by DAM but not NM demonstrated that DAM is relatively toxic to cells but not NM. DAM represents a poor functional model of NM in that it displays a marked toxicity unrepresentative of the effects of the native melanin. Both NM and DAM were unable to attentuate the toxic effects of the added oxidative stimulus, this probably due to the exceeding the chelating capacity of NM.

Future studies should point to the characterization and role of NM under in vivo conditions. The development of strategies to protect the neuromelanin in dopaminergic neurons may have important therapeutic implications not only for PD.

7. References

Afifi A. K. and Bergman R. A. (1998) Functional Anatomy: Text and Atlas. pp 275-303. McGraw-Hill, New York.

Agid Y., Ruberg M., Javoy-Agid F., Hirsch E., Raisman-Vozari R., Vyas S., Faucheux B., Michel P., Kastner A., Blanchard V. and et al. (1993) Are dopaminergic neurons selectively vulnerable to Parkinson's disease? Adv Neurol 60, 148-164.

Aime S., Bergamasco B., Biglino D., Digilio G., Fasano M., Giamello E. and Lopiano L. (1997) EPR investigations of the iron domain in neuromelanin. Biochem Biophys Acta 1361, 49-58.

Aime S., Bergamasco B., Casu M., Digilio G., Fasano M., Giraudo S. and Lopiano L. (2000) Isolation and 13C-NMR characterization of an insoluble proteinaceous fraction from substantia nigra of patients with Parkinson's disease. Mov Disord 15, 977-981.

Aime S., Fasano M., Bergamasco B., Lopiano L. and Quattrocolo G. (1996) Advance in Neurology, pp 263-270. Lippincott-Raven Publisher, Philadelphia.

Aime S., Fasano M., Bergamasco B., Lopiano L. and Quattrocolo G. (1996) Nuclear magnetic resonance spectroscopy characterization and iron content determination of human mesencephalic neuromelanin. Adv Neurol 69, 263-270.

Aime S., Fasano M., Bergamasco B., Lopiano L. and Valente G. (1994) Evidence for a glycidic-lipidic matrix in human neuromelanin, potentially responsible for the enhanced

iron sequestering ability of substantia nigra. J Neurochem 62, 369-371.

Aimi Y. and P.L. M. (1996) Lack of toxicity of human neuromelanin to rat brain dopaminergic neurons. Parkinsonism Relat. Disord 2, 69-74.

Ambani L., Van Woert M. and S M. (1975) Brain peroxidase and catalase in Parkinson disease. Arch Neurol 32, 114-118.

Barden H. (1969) The histochemical relationship of neuromelanin and lipofuscin. J Neuropathol Exp Neurol 28, 419-441.

Barden H. (1975) Histochemical relationship and nature of neuromelanin. Aging 1, 79-117.

Beal M. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Ann Neurol 31, 119-130.

Beal M. (2000) Energetics in the pathogenesis of neurodegenerative diseases. Trends Neurosci 23, 298-304.

Benjamin C. L., Lai M., MSc, K.C. J. and Tsui M. (2001) Epidemiology of Parkinson's disease. BC Medical Journal 43, 133-137.

Ben-Shachar D., Eshel G., Finberg J. and Youdim M. (1991) The iron chelator desferrioxamine (desferal) retards 6-hydroxydopamine-induced degeneration of nigrostriatal neurons. J Neurochem 56, 1441-1444.

Ben-Shachar D., Riederer P. and Youdim M. B. H. (1991) Iron-melanin interaction and lipid peroxidation: Implications for Parkinson's disease. J Neurochem 57, 1609-1614.

Ben-Shachar D. and Youdim M. B. H. (1991) Intranigra iron injection induces behavioral and biochemical "parkinsonism" in rats. J Neurochem 57, 2133-2135.

Berg D., Gerlach M., Youdim M. B., Double K. L., Zecca L., Riederer P. and Becker G. (2001) Brain iron pathways and their relevance to Parkinson's disease. J Neurochem 79, 225-236.

Boka G., Anglade P., Wallach D., Javoy-Agid F., Agid Y. and Hirsch E. (1994) Immunocytochemical analysis of tumor necrosis factor and its receptors in Parkinson's disease. J Comp Neurol 287, 373-392.

Bolzoni F., Giraudo S., Lopiano L., Bergamasco B., Fasano M. and Crippa P. R. (2002) Magnetic investigations of human mesencephalic neuromelanin. Biochem Biophys Acta 1586, 210-218.

Brayda-Bruno M. and Levi A. C. (1979) Ultrastructure of neuromelanin granules in dog substantia nigra at different ages. Boll Soc Ital Biol Sper 55, 1902-1908.

Bridelli M. G., De Mattei M. and Levi A. C. (1982) Preliminary spectroscopic studies on the neuromelanic pigment of human substantia nigra nucleus. Ital J Biochem 31, 315-321.

Cotzias G. C., Papavasiliou P. S., Vanwoert M. H. and Sakamoto A. (1964) Melanogenesis and Extrapyramidal Diseases. Fed Proc 23, 713-718.

Cowen C. (1986) The melanoneurons of the human cerebellum (nucleus pigmentosus cerebellaris) and homologues in the monkey. J Neuropathol Exp Neurol 45, 205-221.

D'Agostino A. J. and Luse S. A. (1964) Electron microscopic observations on the human substantia nigra. Neurology 12, 529-536.

D'Amato R. J., Lipman Z. P. and Snyder S. H. (1986) Selectivity of the parkinsonian neurotoxin MPTP: toxic metabolite MPP+ binds to neuromelanin. Science 231, 987-989.

Damier P., Hirsch E., Zhang P., Agid Y. and Javoy-Agid F. (1993) Glutathione peroxidase, glial cells and Parkinson's disease. Neuroscience 52, 1-6.

Dauer W. and Przedborski S. (2003) Parkinson's disease: mechanisms and models. Neuron 39, 889-909.

Dawson T. M. and Dawson V. L. (2003) Molecular pathways of neurodegeneration in Parkinson's disease. Science 302, 819-822.

de Pedro-Cuesta J., Gudmundsson G., Abraira V., Gudmundsson G., Love A., Tulinius H., Veiga J., Almazan J. and Petersen I. (1996) Whooping cough and Parkinson's disease. The Europarkinson Preparatory Activity Research Group. Int J Epidemiol 25, 1301-1311.

de Rijk M. C., Tzourio C., Breteler M. M., Dartigues J. F., Amaducci L., Lopez-Pousa S., Manubens-Bertran J. M., Alperovitch A. and Rocca W. A. (1997) Prevalence of parkinsonism and Parkinson's disease in Europe: the EUROPARKINSON Collaborative Study. European Community Concerted Action on the Epidemiology of Parkinson's

disease. J Neurol Neurosurg Psychiatry 62, 10-15.

DeMattei M., Levi A. C. and Fariello R. G. (1986) Neuromelanic pigment in substantia nigra neurons of rats and dogs. Neurosci Lett 72, 37-42.

Dexter D., Carayon A., Vidailher M., Agid F. and Agid Y. (1990) Decreased ferritin levels in brain in Parkinson's disease. J. Neurochem 55, 16-20.

Dexter D.T., Wells F. R., Lees A. J. Agid F., Agid Y., Jenner P., Marsden C. D. (1989) Increased nigral iron content and alteration in other metal ions occurring in brain in Parkinson's disease. J Neurochem 52, 1830-1836.

Dexter D. T., Carter C. J., Wells F. R., Javoy-Agid F., Agid Y., Lees A., Jenner P. and Marsden C. D. (1989) Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J. Neurochem 52, 381-389.

Dexter D. T., Jenner P., Schapira A. H. and Marsden C. D. (1992) Alterations in levels of iron, ferritin, and other trace metals in neurodegenerative diseases affecting the basal ganglia. The Royal Kings and Queens Parkinson's Disease Research Group. Ann Neurol 32 Suppl, S94-100.

Di Monte D., Chan P. and Sandy M. (1992) Glutathione in Parkinson's disease: a link between oxidative stress and mitochondrial damage? Ann Neurol 32 Suppl, S1112-S1115.

D'Mello S. R., Galli C., Ciotti T. and Calissano P. (1993) Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth

factor I and cAMP. Proc Natl Acad Sci U S A 90, 10989-10993.

Double K. (2000a) Structural characteristics of human substantia nigra neuromelanin. J Neurochem. 2000 75, 2583-2589.

Double K. and Crocker A. (1993) Quantitative electromyographic change following modification of central dopaminergic transmission. Brain Research 604, 342-344.

Double K., Maywald M., Schmittel M., Riederer P. and Gerlach M. (1997) In vitro studies of ferritin iron release and toxicity. J Neurochem 70, 2492-2499.

Double K., Riederer P. and Gerlach M. (1999) The significance of neuromelanin in Parkinson's disease. Drug News Dev 12, 330-340.

Double K. L., Ben-Shachar D., Youdim M. B., Zecca L., Riederer P. and Gerlach M. (2002) Influence of neuromelanin on oxidative pathways within the human substantia nigra. Neurotoxicol Teratol 24, 621-628.

Double K. L., Gerlach M., Schunemann V., Trautwein A. X., Zecca L., Gallorini M., Youdim M. B., Riederer P. and Ben-Shachar D. (2003) Iron-binding characteristics of neuromelanin of the human substantia nigra. Biochem Pharmacol 66, 489-494.

Double K. L., Gerlach M., Youdim M. B. and Riederer P. (2000b) Impaired iron homeostasis in Parkinson's disease. J Neural Transm Suppl, 37-58.

Duffy P. E. and Tennyson V. M. (1965) Phase and electron microscopic observations of Lewy bodies and melanin granules in the substantia nigra and locus ceruleus in

Parkinson's disease. J Neuropathol Exp Neurol 24, 398-414.

Ebadi M. and Srinivasan S. (1996) Oxidative stress and antioxidant therapy in Parkinson's disease. Progress in Neurobiology 48, 1-19.

Enochs W. S., Nilges M. J. and Swartz H. M. (1993) Purified human neuromelanin, synthetic dopamine melanin as a potential model pigment, and the normal human substantia nigra: characterization by electron paramagnetic resonance spectroscopy. J Neurochem 61, 68-79.

Faucheux B., Martin M., Beaumont C., Hauw J., Agid Y. and Hirsch E. (1998) Proteins involved in the regulation of intracellular iron (H- and L- ferritin, iron regulatory protein) in the mesencephalon of patients with Parkinson's disease. Abst. Soc. Neurosci 574, 16.

Fenichel G. M. and Bazelon M. (1968) Studies on neuromelanin. II. Melanin in the brainstems of infants and children. Neurology 18, 817-820.

Foley P. and Riederer P. (2000) Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson's disease. J Neurol 247, 82-94.

Friedman A. and Galazka-Friedman J. (2001) The current state of free radicals in Parkinson's disease. Nigral iron as a trigger of oxidative stress. Adv Neurol 86, 137-142.

Galazka-Friedman J., Bauminger E. R., Friedman A., Barcikowska M., Hechel D. and Nowik I. (1996) Iron in parkinsonian and control substantia nigra--a Mossbauer spectroscopy study. Mov Disord 11, 8-16.

Galazka-Friedman J., Bauminger E. R., Koziorowski D. and Friedman A. (2004) Mossbauer spectroscopy and ELISA studies reveal differences between Parkinson's disease and control substantia nigra. Biochem Biophys Acta 1688, 130-136.

Gassen M. and Youdim M. (1997) The potential role of iron chelators in the treatment of Parkinson's disease and related neurological disorders. Pharm. Toxicol 80, 159-166.

Gerlach M., Ben-Shachar D., Riederer P. and Youdim M. (1994) Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem 63, 793-807.

Gerlach M., Double K. L., Riederer P., Hirsch E. C., Jellinger K., Jenner K., Trautwein A. X. and Youdim M. B. H. (1997) Iron in the Parkinsonian substantia nigra. Mov disorder 12, 258-260.

Gerlach M., Trautwein A. X., Zecca L., Youdim M. B. and Riederer P. (1995) Mossbauer spectroscopic studies of purified human neuromelanin isolated from the substantia nigra. J Neurochem 65, 923-926.

Gibb W. R. (1992) Melanin, tyrosine hydroxylase, calbindin and substance P in the human midbrain and substantia nigra in relation to nigrostriatal projections and differential neuronal susceptibility in Parkinson's disease. Brain Res 581, 283-291.

Gibb W. R. and Lees A. J. (1987) The progression of idiopathic Parkinson's disease is not explained by age-related changes. Clinical and pathological comparisons with post-encephalitic parkinsonian syndrome. Acta Neuropathol (Berl) 73, 195-201.

Giovanni A., Liang L., Hastings G. T. and Zigmond J. M. (1995) Estimating hydroxyl

radical content in rat brain using systemic and intraventricular salicylate: Impact of methamphetamine. J. Neurochem 64(4):1819-25

Goetz M., Dirr A., Freyberger A., Burger R. and Riederer P. (1993) The thiobarbituric acid assay reflects susceptibility to oxygen induced lipid peroxidation in vitro rather than levels of lipid hydroperoxides in vivo: a methodological approach. Neurochemistry International 22, 255-262.

Goldman S. and Tanner C. M. (1998) Parkinson's disease and movement disorders, 3rd Edition, pp 133-158. Beltimore: williams and wilkins.

Good P. F., Hsu A., Werner P., Perl D. and Olanow C. (1998) Protein nitration in Parkinson's disease. J Neuropathol Exp Neurol 57, 338-342.

Good P. F., Olanow C. W. and Perl D. P. (1992) Neuromelanin-containing neurons of the substantia nigra accumulate iron and aluminum in Parkinson's disease: a LAMMA study. Brain Res 593, 343-346.

Gorell J. M., Johnson C. C., Rybicki B. A., Peterson E. L., Kortsha G. X., Brown G. G. and Richardson R. J. (1997) Occupational exposures to metals as risk factors for Parkinson's disease. Neurology 48, 650-658.

Gu M., Owen A. D., Toffa S. E., Cooper J. M., Dexter D. T., Jenner P., Marsden C. D. and Schapira A. H. (1998) Mitochondrial function, GSH and iron in neurodegeneration and Lewy body diseases. J Neurol Sci 158, 24-29.

Gutteridge J., Halliwell B. and Treffry A. (1983) Effect of ferritin containing fractions

with different iron loading on lipid peroxidation. J. Biochem 209, 557-560.

Gutteridge J., Quinlan G., Clark I. and Halliwell B. (1985) Aluminum salts accelerate peroxidation of membrane lipids stimulated by iron salts. Biochem Biophys Acta 835, 441-447.

Halliday G. and McRitchie D. (1996) Midbrain neuropathology in idiopathic Parkinson's disease and diffuse Lewy body disease. Journal of Clinical Neuroscience 3, 52-60.

Halliwell B. (1992) Reactive oxygen species and the central nervous system. J Neurochem 59, 1609-1623.

Halliwell B. and Gutteridge J. (1986) Iron and free radical reactions: two aspects of antioxidant protection. Trends biol Sci 11, 1372-1375.

Harrison P. M. and Arosio P. (1996) The ferritins: Molecular properties, iron storage function and cellular regulation. Biochem Biophys Acta 1275, 161-203.

Hastings T. (1995) Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. J Neurochem 64, 919-924.

Hattori N., Shimura H., Kubo S., Kitada T., Wang M., Asakawa S., Minashima S., Shimizu N., Suzuki T., Tanaka K. and Mizuno Y. (2000) Autosomal recessive juvenile parkinsonism: a key to understanding nigral degeneration in sporadic Parkinson's disease. Neuropathology 20 Suppl, S85-90.

Hellenbrand W., Bauer G., Boeing H., Seidler A., Robra BP. (1996) Diet and Parkinson's disease II: a possible role for the past intake of specific nutrients. Neurology 47, 644-650.

Hirsch E. C. (2000) Glial cells and Parkinson's disease. J Neurol 247, S58-S62.

Hirsch E. C., Graybiel A. M. and Agid Y. A. (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. Nature 334, 345-348.

Hirsch E. C. (1992) Why are nigral catecholaminergic neurons more vulnerable than other cells in Parkinson's disease? Ann Neurol 32 Suppl, S88-93.

Hirsch E. C., Breidert T., Rousselet E., Hunot S., Hartmann A. and Michel P. P. (2003) The role of glial reaction and inflammation in Parkinson's disease. Ann N Y Acad Sci 991, 214-228.

Hlliwell B., Kaur H. and Ingelman-Sundverg M. (1991) Hydroxylation of salicylate as an assay for hydroxyl radicals: a cautionary note. Free Radic. Biol. med 10, 439-441.

Hornykiewicz O. and Kish S. J. (1987) Biochemical pathophysiology of Parkinson's disease. Adv Neurol 45, 19-34.

Ian R. A. and Mackenzie M. D. (2001) The pathology of Parkinson's disease. BC Medical Journal 43, 142-147.

Jellinger K., Kienzl E., Rumpelmair G., Riederer P., Stachelberger H., Ben-Shachar D.

and Youdim M. B. (1992) Iron-melanin complex in substantia nigra of parkinsonian brains: an x-ray microanalysis. J Neurochem 59, 1168-1171.

Jenner P. and Olanow C. (1998) Understanding cell death in Parkinson's disease. Ann Neurol 44, S72-S84.

Kastner A., Hirsch E. C., Lejeune O., Javoy-Agid F., Rascol O. and Agid Y. (1992) Is the vulnerability of neurons in the substantia nigra of patients with Parkinson's disease related to their neuromelanin content? J Neurochem 59, 1080-1089.

Kienzl E., Jellinger K., Stachelberger H. and Linert W. (1999) Iron as catalyst for oxidative stress in the pathogenesis of Parkinson's disease? Life Sci 65, 1973-1976.

Kish S., Morito C. and Hornykiewicz O. (1985) Glutathione peroxidase activity in Parkinson's disease brain. Neurosci Lett 58, 343-346.

Krol E. S. and Liebler D. C. (1998) Photoprotective actions of natural and synthetic melanins. Chem. Res. Toxic 11, 1434-1440.

Lan J. and Jiang D. (1997) Excessive iron accumulation in the brain: a possible potential risk of neurodegeneration in Parkinson disease. Neural Transm 104, 649-660.

Langston J. W., Ballard P. A., Tetrud J. W. and Iriwn I. (1983) Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219, 979-980.

Le Couteur D. G., Muller M., Yang M. C., Mellick G. D. and McLean A. J. (2002) Age-environment and gene-environment interactions in the pathogenesis of Parkinson's

disease. Rev Environ Health 17, 51-64.

Le W. D., Rowe D. B., Jankovic J., Xie W. and Appel S. H. (1999) Effects of cerebrospinal fluid from patients with Parkinson disease on dopaminergic cells. Arch Neurol 56, 194-200.

Lennox G., Lowe J., Landon M., Byrne E. J., Mayer R. J. and Godwin-Austen R. B. (1989) Diffuse Lewy body disease: correlative neuropathology using anti-ubiquitin immunocytochemistry. J Neurol Neurosurg Psychiatry 52, 1236-1247.

Litvan I. (1998) Parkinsonian features: when are they Parkinson disease? Jama 280, 1654-1655.

Lopiano L., Chiesa M., Digilio G., Giraudo S., Bergamasco B., Torre E. and Fasano M. (2000) Q-band EPR investigations of neuromelanin in control and Parkinson's disease patients. Biochem Biophys Acta 1500, 306-312.

Mann D. M. and Yates P. O. (1983) Possible role of neuromelanin in the pathogenesis of Parkinson's disease. Mech Ageing Dev 21, 193-203.

Mann V., Cooper J., Daniel S., Srai K., Jenner P., Marsden C. and Schapira A. (1994) Complex I, iron, and ferritin in Parkinson's disease. Ann Neurol 36, 876-881.

Maret G., Testa B., Jenner P., el Tayar N. and Carrupt P. A. (1990) The MPTP story: MAO activates tetrahydropyridine derivatives to toxins causing parkinsonism. Drug Metab Rev 22, 291-332.

Marsden C. D. (1989) Brain melanin, pp 395-417. Academic Press, New York.

Marsden C. D. (1961) Pigmentation in the nucleus substantia nigra of mammals. J. Anatomy 95, 256-261.

Meara J., Bhowmick B. K. and Hobson P. (1999) Accuracy of diagnosis in patients with presumed Parkinson's disease. Age Ageing 28, 99-102.

Meglio L. and Oteiza I. (1999) Aluminium enhances melanin-induced lipid peroxidation. Neurochem Res 24, 1001-1008.

Melcon M. O., Anderson D. W., Vergara R. H., and Rocca W. A.. (1997) Prevalence of Parkinson’s disease in Junin, Buenos Aires Province, Argentina. Mov disorder 12, 197-205.

Mitchell S. L., Kiely D. K., Kiel D. P. and Lipsitz L. A. (1996) The epidemiology, clinical characteristics, and natural history of older nursing home residents with a diagnosis of Parkinson's disease. J Am Geriatr Soc 44, 394-399.

Mochizuki H., H. M. and Y M. (1997) Apoptosis in neurodegenerative disorders. J. Neural Transm Suppl. 50, 125-140.

Mochizuki H., Nishi K. and Mizuno Y. (1993) Iron-melanin complex is toxic to dopaminergic neurons in nigrostriatal co-culture. Neurodegeneration 2, 1-7.

Moos T. (2000) Absence of ferritin protein in substantia nigra pars compacta neuron. A reappraisal to the role of iron in Parkinson disease pathogenesis. Mov Disord 15, 319A.

Mayeux R., Marder K., Cote L. J., Denaro J., Hemenegildo N., Mejia H., Tang M. X., Lantigua R., Wilder D., Gurland B, et al (1995) The frequency of idiopathic Parkinson's disease by age, ethnic group, and sex in northern Manhattan, 1988–1993. Am J Epidemiol 142, 820-827.

Morens D. M., Davis J. W., Grandinetti A., Ross G. W., Popper J. S. and White L. R. (1996) Epidemiologic observations on Parkinson's disease: Incidence and mortality in a prospective study of middle-aged men. Neurology 46, 1044-1050.

Mori H. L., Kondo T., Yokochi M., Matsumine H., Nakagawa-Hattori Y., Miyake T., Suda K. and Mizuno Y. (1998) Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 51, 890-892.

Moses H. L., Ganote C. E., Beaver D. L. and Schuffman S. S. (1966) Light and electron microscopic studies of pigment in human and rhesus monkey substantia nigra and locus ceruleus. Anat Rec 155, 167-183.

Nau-Staudt K., Nau W. M., Haefliger I. O. and Flammer J. (2001) Lipid peroxidation in porcine irises: dependence on pigmentation. Curr Eye Res 22, 229-234.

Ngim C. and Devathasan G. (1989) Epidemiologic study on the association between body burden mercury level and idiopathic Parkinson's disease. Neuroepidemiology 8, 128-141.

Offen D., Gorodin S., Melamed E., Hanania J. and Malik Z. (1999) Dopamine-melanin is actively phagocytized by PC12 cells and cerebellar granular cells: possible implications for the etiology of Parkinson's disease. Neurosci Lett 260, 101-104.

Offen D., Ziv I., Barzilai A., Gorodin S., Glater E. and Hochman A. E. (1997a) Dopamine-melanin induces apoptosis in PC 12 cells: Possible implications for the etiology of Parkinson's disease. Neurochem Int. 31, 207-216.

Offen D., Ziv I., Panet H., Wasserman L., Stein R., Melamed E. and Barzilai A. (1997b) Dopamine-induced apoptosis is inhibited in PC12 cells expressing Bcl-2. Cell Mol Neurobiol 17, 289-304.

Olanow C. (1992) An introduction to the free radical hypothesis in Parkinson's disease. Ann Neurol 32, S2-S9.

Ozawa T., Hayakawa M., Katsumata K., Ikebe S. and Mizuno Y. (1997) Alterations in the distribution of glutathione in the substantia nigra in Parkinson's disease. J Neural Transm 104, 661-677.

Pahwa R. and Koller W. C. (1995) Defining Parkinson’s Disease and Parkinsonism, in Etiology of Parkinson’s Disease (Ellenberg J. H., Koller W. C. and Langston J. W., eds), pp 1-54. Marcel Dekker, New York.

Pall H. S., Williams A. C., Blake D. R., Lunec J., Gutteridge J. M., M. H. and Taylor A. (1987) Raised cerebrospinal-fluid copper concentration in Parkinson's disease. Lancet 2, 238-241.

Pedrell M. D., Navarro M., Faura C. C. and Horga J. F. (1995) Verapamil-induced Parkinsonism. Am J Med 99, 436.

Perry T., Goden D. and Hansen S. (1982) Parkinson's disease: a disorder due to nigral

glutathione deficiency. Neurosci Lett 33, 305-310.

Pilas B., T. S., Kalayanarman B. and Swartz H. M. (1988) The effect of melanin on iron-associated decomposition of hydrogen peroxide. Free radical Biol. Med. 4, 285-293.

Playfer J. R. (1997) Parkinson's disease. Postgraduate Medicine Journal 73, 257-264.

Polymeropoulos M. H., Higgins J. J., Golbe L. I., Johnson W. G., Ide S. E., Di Iorio G., Sanges G., Stenroos E. S., Pho L. T., Schaffer A. A., Lazzarini A. M., Nussbaum R. L. and Duvoisin R. C. (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science 274, 1197-1199

.

Porebska-Budny M., Sakina N. L., Stepien K. B., Dontsov A. E. and Wilczok T. (1992) Antioxidative activity of synthetic melanins. Cardiolipin liposome model. Biochem Biophys Acta 1116, 11-16.

Qureshi G., Baig S., Bednar I., Sodersten P., Forsberg G. and Siden A. (1995) Increased cerebrospinal fluid concentration of nitrite in Parkinson's disease. Neuroreport 6, 1642-1644.

Reichmann H. and Janetzky B. (2000) Mitochondrial dysfunction- a pathogenetic factor in Parkinson's disease. J Neurol 247, 63-68.

Reinhardt J. (1992) Side-effects: mercury contribution to body burden from dental amalgam. Adv Dent Res 6, 110-113.

Riederer P., Konradi C., Hebenstreit G. and Youdim M. B. (1989) Neurochemical perspectives to the function of monoamine oxidase. Acta Neurol Scand 126, 41-45.

Riederer P., Sofic E., Raysch W. D., Schmidt B., Reynolds G. P., Jellinger K. and Youdim M. B. (1989) Transition metals, ferritin, glutathione, and ascorbic in parkinsonian brain. J Neurochem 52, 515-520.

Ross B. M., Moszcynska A., Ehrlich J. and Kish S. J. (1998) Low activity of key phospholipid catabolic and anabolic enzymes in human substantia nigra: possible implications for Parkinson's disease. Neuroscience 83, 791-798.

Rozanowska M., Sarna T., Land E. J. and Truscott T. G. (1999) Free radical scavenging properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. Free Radic Biol Med 26, 518-525.

Saggu H., Cooksey J., Dexter D., Wells F., Lees A., Jenner P. and Marsden C. (1989) A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra. J Neurochem 53, 692-697.

Sanchez-Ramos J. R., Overvik E. and Ames B. N. (1994) A marker of oxyradical mediated DNA damage(8-hydroxy- 2-deoxyguanosine) is increased in nigro-striatum of Parkinson's disease brain. Neurodegeneration 3, 197-204.

Sarna T., Menon I. A. and Sealy R. C. (1985) Photosensitization of melanins: a comparative study. Photochem Photobiol 42, 529-532.

Sarna T., Pilas B., Land E. J. and Truscott T. G. (1986) Interaction of radicals from water

radiolysis with melanin. Biochem Biophys Acta 883, 162-167.

Savory J., Rao J. K., Huang Y., Letada P. R. and Herman M. M. (1999) Age-related hippocampal changes in Bcl-2:Bax ratio, oxidative stress, redox-active iron and apoptosis associated with aluminum-induced neurodegeneration: increased susceptibility with aging. Neurotoxicology 20, 805-817.

Scalia M., Geremia E., Corsaro C., Santoro C., Baratta D. and Sichel G. (1990) Lipid peroxidation in pigmented and unpigmented liver tissues: protective role of melanin. Pigment Cell Res 3, 115-119.

Schapira A. (1990) Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 54, 823-827.

Schapira A. (1996) Neurotoxicity and the mechanisms of cell death in Parkinson's disease, Huntington's disease, hereditary paraplegia and Friedrich's ataxia. Biochem Biophys Acta 1410, 159-170.

Schapira A., Mann Y., Cooper J., Dexter D., Daniel S., Jenner P., Clark J. and Marsden C. (1990) Anatomic and disease specificity of NADH CoQ1 reductase (complex I) deficiency in Parkinson's disease. J Neurochem 55, 2142-2145.

Schoenberg B. (1987) Descriptive epidemiology of Parkinson's disease: disease distribution and hypothesis formulation. Adv Neuorol 45, 277-283.

Schwyn R. C., King J. S. and Fox C. A. (1970) Pigments in the red nucleus and substantia nigra in man and in representative old and new world monkeys. Bol Estud

Med Biol 26, 139-160.

Shima T., Sarna T., Swartz H. M., Stroppolo A., Gerbasi R. and Zecca L. (1997) Binding of iron to neuromelanin of human substantia nigra and synthetic melanin: an electron paramagnetic resonance spectroscopy study. Free Radic Biol Med 23, 110-119.

Shukla A., Ahukla G. S. and Srimal R. C. (1996) Cadmium-induced alteration in blood-brain barrier permeability and its possible correlation with decreased microvessel antioxidant potential in rat. Human and Experimental toxicology 15, 400-405.

Singleton A. B., Farrer M., Johnson J., Singleton A., Hague S., Kachergus J., Hulihan M., Peuralinna T., Dutra A., Nussbaum R., Lincoln S., Crawley A., Hanson M., Maraganore D., Adler C., Cookson M. R., Muenter M., Baptista M., Miller D., Blancato J., Hardy J. and Gwinn-Hardy K. (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science 302, 841.

Smythies J. (1996) On the functional of neuromelanin. Proc R Soc Lond B Biol Sci 263, 487-489.

Sofic E., Lange K. W., Jellinger K. and Riederer P. (1992) Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. Neurosci Lett 142, 128-130.

Sofic E., Paulus W., Jellinger K., Riederer P. and Youdim M. B. (1991) Selective increase of iron in substantia nigra zona compacta of parkinsonian brains. J Neurochem 56, 978-982.

Spencer J., Jenner P., Daniel S., Lees A., Marsden C. and Halliwell B. (1998) Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: Possible mechanism of formation involving reactive oxygen species. J. Neurochem 71, 2112-2122.

Stacy M. and Jankovic J. (1992) Differential diagnosis of Parkinson's disease and the parkinsonism plus syndromes. Neurol Clin 10, 341-359.

Sulzer D. and Zecca L. (2000) Intraneuronal dopamine-quinone synthesis: a review. Neurotox Res. 1, 181-195.

Swan G. A. (1963) Chemical structure of melanins. Ann N Y Acad Sci 100, 1005-1019.

Swartz H. M., Sarna T. and Zecca L. (1992) Modulation by neuromelanin of the availability and reactivity of metal ions. Ann Neurol 32 Suppl, S69-75.

Tanner C. M., Hubble J. P. and Chan P. I. U. (1997) Movement disorders: Neurologic Principles and Practice, pp 137-152. McGraw-Hill Company, Inc., New York.

Tanner C. M., Ottman R. and Goldman S. M. (1999) Parkinson disease in twins: An etiologic study. JAMA 281, 341-346.

Thome J., Zhang j., Davids E., Foley P., Weijers H.-G., Wiesbeck G. A., Boening J., Riederer P. and Gerlach M. (1997) Evidence for increased oxidative stress in alcohol-dependent patients provided by quantification of in vivo salicylate hydroxylation products. Alcohol Clin Exp Res 21, 82-85

Toshihide S., Tadeusz S., Harold M. S., Antonella S., Rosalba G. and Luigi Z. (1997) Binding of Iron to Neuromelanin of Human Substantia Nigra and Synthetic Melanin: An Electron Paramagnetic Resonance Spectroscopy Study. Free radical Biol. Med. 23, 110-119.

Van Woert M. H. and Ambani L. M. (1974) Biochemistry of neuromelanin. Adv Neurol 5, 215-223.

von Baumgarten F., Baumgarten H. G. and Schlossberger H. G. (1980) The disposition of intraventricularly injected 14C-5, 6-DHT-melanin in, and possible routes of elimination from the rat CNS. An autoradiographic study. Cell Tissue Res 212, 279-294.

Wang Y. (1991) The incidence and prevalence of Parkinson's disease in the People's Republic of China. Chinese J Epidemiol 12, 363-365.

Wooten G. F., Currie L. J., Bovbjerg V. E., Lee J. K. and Patrie J. (2004) Are men at greater risk for Parkinson's disease than women? J Neurol Neurosurg Psychiatry 75, 637-639.

Xie C. X., Mattson M. P., Lovell M. A. and Yokel R. A. (1996) Intraneuronal aluminium potentiates iron-induced oxidative stress in cultured rat hippocampal neurons. Brain Res 743, 271-277.

Yoshida E., Mokuno K., Aoki S., Takahashi A., Riku S., Murayama T., Tanagi T. and Kato K. (1994) Cerebrospinal fluid levels of superoxide dismutases in neurological diseases detected by sensitive enzyme immunoassays. J Neurol Sci 124, 25-31.

Youdim M. B.and Riederer P. (1994a) Understanding Parkinson's disease. Sci Am 276, 52-59.

Youdim M. B., Ben-Shachar D. and Riederer P. (1993) The possible role of iron in the etiopathology of Parkinson's disease. Mov Disord 8, 1-12.

Youdim M. B., Ben-Shachar D. and Riederer P. (1994b) The enigma of neuromelanin in Parkinson's disease substantia nigra. J Neural Transm Suppl 43, 113-122.

Zack M. M. and Langston J. W. (1995) Is Parkinson's Disease a single entity with a single cause? A cautionary note, in Etiology of Parkinson's Disease (Ellenberg J. H., Koller W. C. and Langston J. W., eds), pp 55-63. Marcel Dekker, New York.

Zareba M., Bober A., Korytowski W., Zecca L. and Sarna T. (1995) The effect of a synthetic neuromelanin on yield of free hydroxyl radicals generated in model systems. Biochem Biophys Acta 319, 142-148.

Zecca L., Fariello R., Riederer P., Sulzer D., Gatti A. and Tampellini D. (2002a) The absolute concentration of nigral neuromelanin, assayed by a new sensitive method, increases throughout the life and is dramatically decreased in Parkinson's disease. FEBS Lett 510, 216-220.

Zecca L., Gallorini M., Schunemann V., Trautwein A. X., Gerlach M., Riederer P., Vezzoni P. and Tampellini D. (2001a) Iron, neuromelanin and ferritin content in the substantia nigra of normal subjects at different ages: consequences for iron storage and neurodegenerative processes. J Neurochem 76, 1766-1773.

Zecca L., Pietra R., Goj C., Mecacci C., Radice D. and Sabbioni E. (1994) Iron and other metals in neuromelanin, substantia nigra, and putamen of human brain. J Neurochem 62, 1097-1101.

Zecca L., Shima T., Stroppolo A., Goj C., Battiston G. A., Gerbasi R., Sarna T. and Swartz H. M. (1996) Interaction of neuromelanin and iron in substantia nigra and other areas of human brain. Neuroscience 73, 407-415.

Zecca L. and Swartz H. M. (1993) Total and paramagnetic metals in human substantia nigra and its neuromelanin. J Neural Transm 5, 203-213.

Zecca L., Tampellini D., Gatti A., Crippa R., Eisner M., Sulzer D., Ito S., Fariello R. G. and Gallorini M. (2002b) The neuromelanin of human substantia nigra and its interaction with metals. J Neural Transm 109, 663-672.

Zecca L., Tampellini D., Gerlach M., Riederer P., Fariello R. G. and Sulzer D. (2001b) Substantia nigra neuromelanin: structure, synthesis, and molecular behavior. J Clin Pathol: Mol Pathol 54, 414-418.

Acknowledgements

My life in Germany is nearly three years and it has been brought me much happiness and precious memory, because I have met so many nice people and had much pleasure with them and got a lot of help from them.

Prof. Dr. Helmut Beckmann, Prof. Dr. Peter Riederer and Prof. Dr. Manfred Gerlach have given me the valuable chance to study here and offered generous help for me. Without them, I would not have had the possibility to study here. Prof. Dr. Peter Riederer and Prof. Dr. Manfred Gerlach have not only taught me the thinking way of scientific research and led me to the right direction of my doctor work but also instilled the German culture me. I feel speechless gratitude for him and only hope this thesis could express my heartfelt appreciation

Dr. Kay Double has given me valuable advices for the scientific work and a great deal of help not only for science but also for my life. Her wisdom and efforts has helped me to clear away many difficulties in my work. Without her, I couldn't finish my doctor work. I feel so lucky to have an extraordinary supervisor and truly, deeply appreciate for her.

My thanks are also extending to the following people:

To Dr. Carsten Scheller and PD. Eleni Koutsilieri for the flow cytometry work.

To Dr. Edna Gruenblatt and Dr. Angelica Schmitt for the help when I have problem.

To Francine Griffiths, Phillip Beart, Linda Mercer, Glenda Halliday, Emma Kettle in Prince of Wales Medical Research Institute and Howard Florey Institute, University of Melbourne, Australia for the work of Electron photography and Lactate dehydrogenase (LDH) activity.

Mr. Rainer Burger for HPLC measurement.

The last but not least: to my husband and my daughter, thanks for their support and understanding me.

During the period of my stay in Germany, I have received a great deal of help from many people. I am grateful to all of them, even if I have not mentioned their names here.

CURRICULUM VITAE

Li Jie

Personal Data

Date of Birth: 12 June, 1971
Place of birth: Tianjin, China
Marital Status: Married, with one child
E-mail: tjlijie612@yahoo.com
Telephone: +86-22-27831240(H), +49-13512410436 (Mobile)
Mail Address: P.O.Box 109, Tianjin Medical University, Qixiangtai Rd. 22, Heping District, Tianjin, P.R.China, 300070

Education

10, 2001-Now Clinical neurochemistry, Clinic and Policlinic for Psychiatry and Psychotherapy, University of Würzburg, Würzburg, Germany
Doctoral student, major in neurochemistry, research into the etiology and new medication of Parkinson's disease and other neurodegeneration diseases by biochemistrical and molecular biological methods

8, 1998-7, 2001 Department of Biochemistry and Molecular Biology, Tianjin Medical University, Tianjin, China
Received Master's degree of Medicine, research into the pathogenesis of Alzheimer's disease

8, 1990-7, 1995 Department of Clinical Medicine, Tianjin Medical University, Tianjin, China
Received Bachelor's Degree of Medicine

Scientific Experience

7, 1995-8, 1998 and 7, 2001-10, 2001 Department of Psychiatry, Tianjin Medical University
Clinician and lecturer

- Conduct clinical activities, such as treating patients with psychotherapies and medication
- Research into the cause of psychiatric and neurological diseases, and conduct clinical trial of new medicine
- Teach Psychiatry to classes of Clinical Medical Department

Training

1, 1996-12, 1996 Trained in Neurological and Internal Medical Department of the General Hospital of Tianjin Medical University as a clinician.