Investigating a Novel Antioxidant Approach to the Treatment of Tardive Dyskinesia

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Abstract

Extrapyramidal side effects are the most frequently experienced side effects associated with neuroleptic drug use, while tardive dyskinesia (TD) remains one of the most debilitating of these side-effects. TD is a serious motor disorder, especially of the orofacial region, caused by prolonged treatment with neuroleptic drugs.

Various hypotheses exist to explain this severely disfiguring syndrome but the precise pathology remains unclear. In recent years, a growing body of evidence has been mounting in support of the free radical hypothesis. The striatum, the brain region associated with regulation and control of movement, is highly susceptible to oxidative stress, primarily because of the oxidative metabolism of dopamine in striatal neurons. Indeed, increased oxidative stress in the striatum has been implicated in various neurological disorders of movement including TD.

There is currently no pharmacological treatment that has been found to be universally effective in the treatment of TD in clinical practice, although antioxidants have been purported to have possible therapeutic value in the treatment of the disorder. N-acetylcysteine (NAC) is an antioxidant and effective free radical scavenger, and is also able to increase the intracellular cysteine concentrations in order to maintain glutathione (GSH) synthesis.

With regard to conditions in which oxidative stress and free radicals are implicated, two therapeutic strategies, utilizing antioxidants may be considered:
Preventative: In this strategy patients are healthy and oxidative stress and free radicals are not abnormally increased. Healthy patients are supplemented with antioxidants in order to prevent the induction of oxidative stress that may occur later through some or other process. This can be related to the daily taking of vitamins by healthy individuals.
Therapeutic: In this strategy anti-oxidants are utilized as treatment options. In these cases, oxidative stress has already been induced and antioxidant therapy is employed in order to minimize the effect of oxidative stress and possibly even reverse these effects.

In the current study both of these strategies were examined. The purpose of the current study was, firstly, to establish a valid animal model of TD, followed by the evaluation of the behavioural and neurochemical effects of chronic NAC administration at various doses in a non-pathological state, i.e. in healthy rats, and, lastly, to assess the behavioural and neurochemical effects of various doses of NAC in rats also receiving chronic haloperidol treatment (TD model).

The treatment of the rats with haloperidol was exploited as a *in vivo* animal model of striatal oxidative stress since numerous studies have not only suggested that TD is a condition of overt oxidative stress in striatal regions of the brain, but also that haloperidol has been found to increase oxidative stress both *in vivo* and *in vitro*.

In the validation phase of the study, rats were treated with either haloperidol (1.5 mg/kg/day) of vehicle. In the non-pathological state, rats received either vehicle or NAC at doses of 10 mg/day, 100 mg/day or 300 mg/day orally for 21 days. In the pathological study utilizing the previously established animal model of TD, rats received either haloperidol and NAC vehicles (control), haloperidol (1.5 mg/kg/day) and NAC vehicle (TD model), or haloperidol (1.5 mg/kg/day) in combination with either 10 mg/day, 100 mg/day or 300 mg/day doses of NAC for 21 days. Behaviour was assessed by counting the number of vacuous chewing movements (VCMs) on days 0, 7, 14, 17, 19 and 21 during a 2 minute rating session. On day 21, after the final behavioural evaluation, animals were sacrificed by decapitation, and striatal tissue were dissected out and fixed in liquid N_2 for later utilization in the neurochemical assays, namely the measurement of the degree of lipid peroxidation, levels of superoxide and the determination of oxidized versus reduced glutathione (GSSG:GSH).

In the TD model, haloperidol treatment induced significant increases in VCMs, superoxide radicals and lipid peroxidation, supporting the hypothesis that TD and chronic neuroleptic administration is associated with increased free radical production and subsequent cell damage. However, haloperidol had no effect on the GSSG:GSH ratio.

The current study demonstrated that in a non-pathological state 10 mg/day and 100mg/day NAC doses had no effect on either behaviour or neurochemical markers of oxidative stress. In contrast, at a dose of 300 mg/day, NAC induced a significant increase in the number of VCMs compared to control. This increase in VCMs, while initially increasing, showed a decrease with time, suggesting that these abnormal movements may only be temporary. At a dose of 300 mg/day, NAC also caused a significant increase in superoxide radicals in the striatum of the animals, indicating that NAC may act as a pro-oxidant at these doses by increasing free radicals such as superoxide. However, this increase in superoxide radicals was associated with a decrease in lipid peroxidation, and it is proposed that the increase in superoxide radicals may induce certain adaptive processes which may protect against excessive oxidative stress and damage and may also explain the reduction in VCMs seen in the last week of treatment.

In the animal model of TD, only 100 mg/day NAC dose was able to significantly decrease VCMs when compared to the haloperidol group. However, all of the doses were able to significant reduce superoxide radicals which were induced by haloperidol administration. Only the 100 mg/day and 300 mg/day NAC doses were able to effect a decrease in lipid peroxidation levels

comparable to basal values, while this decrease was also associated with an increase in the GSSG:GSH ratio that could possibly indicate that reduced glutathione was consumed through its scavenging effects on superoxide (and possibly other free radicals as well) and converted to oxidized glutathione and in this way mediating the decrease in lipid peroxidation.

The results of the current study is thus in support of the free radical hypothesis of TD and indicates that NAC may have potential in protecting against haloperidol-induced motor abnormalities. High doses of NAC were able to protect against lipid peroxidation, although the negative effect that high doses NAC exert on motor behaviour warrants further investigation to explore whether these behavioural deficits are only temporary or more permanent in character. The data also support the hypothesis that antioxidants may become pro-oxidative under certain circumstances, which may be dependant on the redox environment of the biological system in which it is administered or put another way, whether the organism is in a state of oxidative stress or not.

Abstrak

Ekstrapiramidale newe-effekte is die newe-effekte wat mees algemeen met die langdurige gebruik van tipiese antipsigotiese geneesmiddels voorkom, terwyl tardiewe diskinesie (TD) steeds die mees uitmergelende van hierdie newe-effekte is. TD is 'n ernstige motoriese versteuring, veral van die mond en gesig, wat deur langtermyngebruik van antipsigotika veroorsaak word.

Daar is tans verskeie hipoteses wat poog om hierdie ernstige siektetoestand te verklaar, maar die presiese patologie is steeds onbekend. Die afgelope paar jaar is daar egter 'n groeiende hoeveelheid data ter ondersteuning van die vryradikaalhipotese van TD. Die striatum is die deel van die brein wat die regulering van beweging beheer en is hoofsaaklik vanweë die metabolisme van dopamien in stiatale neurone hoogs vatbaar vir die effekte van oksidatiewe stres. 'n Hoë mate van van oksidatiewe stres hou inderdaad verband met verskeie neurologiese afwykings in beweging, waaronder TD.

Daar is tans geen farmakologiese middel met bewese effektiwiteit vir die behandeling van TD in kliniese praktyk nie, alhoewel antioksidante na bewering belowend lyk. N-asetielsisteïen (NAS) is 'n effektiewe antioksidant en opruimer van vry radikale wat ook die vermoë besit om intrasellulêre sisteïenvlakke te verhoog en sodoende glutatioonsintese onderhou.

Ten opsigte van die toestande waarin oksidatiewe stres en vry radikale 'n rol kan speel, kan twee strategieë wat anti-oksidante gebruik, oorweeg word, naamlik:

Voorkomend: in hierdie strategie is die pasiënt gesond en die vlakke van oksidatiewe stres en vry radikale is nie abnormaal hoog nie. Gesonde pasiënte kry aanvullings van antioksidante ten einde aanvang van oksidatiewe stres te voorkom wat later deur een of ander proses veroorsaak kan word. Dit is dieselfde as die daaglikse gebruik van vitamiene deur gesonde persone.

Terapeuties: in hierdie strategie word antioksidante vir behandeling gebruik. In hierdie gevalle het oksidatiewe stres reeds begin en behandeling met antioksidante word gegee om die effek van oksidatiewe stres te minimaliseer en moontlik om te keer.

In die huidige studie is albei hierdie strategieë ondersoek. Die doel van die huidige studie was om eerstens 'n geldige diermodel vir TD te vestig gevolg deur beoordeling van die gedrag en neurochemiese effekte van kroniese toediening van NAS teen verskillende dosisse aan

gesonde rotte, en laastens om die gedrag en neurochemiese effekte van verskillende dosisse NAS te beoordeel in rotte wat ook kroniese behandeling met haloperidol kry (die TD-model). Behandeling van rotte met haloperidol is as 'n *in vivo*-diermodel van strialate oksidatiewe stres gebruik omdat talle studies nie net getoon het dat TD 'n toestand van uitgesproke oksidatiewe stres in striatale gebiede van die brein is nie, maar ook dat haloperidol oksidatiewe stres *in vivo* en *in vitro* verhoog.

Vir die valideringsfase van die studie is rotte óf met haloperidol (1.5 mg/kg/dag) óf met draer behandel. In die gesonde toestand het rotte vir 21 dae draer of NAS teen dosisse van 10 mg/dag, 100 mg/dag of 300 mg/dag oraal ontvang. In die patologiese studie wat die gevalideerde TD-model gebruik het, het rotte vir 21 dae haloperidoldraer en NAS-draer (kontrole), of haloperidol (1.5 mg/kg/dag) en NAS-draer (TD-model) of haloperidol in kombinasie met NAS 10 mg/dag, 100 mg/dag of 300 mg/dag oraal ontvang. Gedrag is beoordeel deur doellose koubewegings (DKB's) tydens 'n sessie van 2 minute op dae 0, 7, 14, 17, 19 en 21 te meet. Na die finale beoordeling van gedrag op dag 21 is die diere onthoof, die striatums uitgedissekteer en in vloeibare N_2 gefikseer vir latere gebruik vir neurochemiese analise, naamlik meting van die mate van lipiedperoksidasie, superoksiedvlakke en bepaling van geoksideerde teenoor gereduseerde glutatioon (GSSG:GSH).

In die TD-model het haloperidol betekenisvolle toenames in DKB's, superoksiedradikale en lipiedperoksidasie veroorsaak wat die hipotese ondersteun dat TD en kroniese toediening van neuroleptiese geneesmiddels met 'n toename in produksie van vry radikale en gevolglike selskade gepaardgaan. Haloperidol het egter geen effek op die GSSG:GSH-verhouding gehad nie.

Die huidige studie het verder getoon dat dosisse van 10 mg/dag en 100 mg/dag NAS in 'n gesonde staat geen effek op gedrag of die neurochemiese merkers van oksidatiewe stres het nie. In teenstelling hiermee het 300 mg/dag NAS tot 'n betekenisvolle toename in die voorkoms van DKB's gelei. DKB's het aanvanklik toegeneem en daarna weer afgeneem wat toon dat hierdie abnormale bewegings slegs tydelik kan wees. Teen 300 mg/dag het NAS ook 'n betekenisvolle verhoging in die vlakke van superoksied veroorsaak wat aandui dat NAS by hierdie dosisse as 'n pro-oksidant kan optree deur vry radikale soos superoksied te laat toeneem. Hierdie toename in superoksiedradikale het egter met 'n vermindering in lipiedperoksidasie gepaardgegaan en dit word voorgestel dat die toename in superoksiedradikale, sekere aanpassingsprosesse mag induseer en so teen oormatige oksidatiewe stres en skade kan beskerm en dit kan ook die afname in DKB's in die laaste week van behandeling verklaar.

In die diermodel van TD het geen dosis van NAS betekenisvolle effekte op haloperidol-geinduseerde DKB's getoon vergelyke met kontrole, alhowel die dosis van 100 mg/dag NAS 'n betekenisvolle verskil in DKB's vergeleke met die haloperidolgroep veroorsaak. Al die dosisse het die vlakke van superoksied, geïnduseer deur haloperidol, egter betekenisvol verlaag. Slegs die dosisse van 100 mg/dag en 300 mg/dag NAS kon lipiedperoksidasie verlaag terwyl hierdie verlaging met 'n verhoging in die GSSG:GSH-verhouding gepaardgegaan het wat moontlik kan aandui dat gereduseerde glutatioon in die opruiming van superoksied (en moontlik ook ander vry radikale) verbruik is en na geoksideerde glutatioon omgeskakel is wat op dié manier teen lipiedperoksidasie beskerm het.

Die resultate van die huidige studie ondersteun die vryradikaalhipotese van TD en toon dat NAS moontlik die potensiaal besit om teen haloperidol-geïnduseerde motoriese abnormaliteite te beskerm. Hoë dosisse NAS kon teen lipiedperoksidasie beskerm, hoewel die negatiewe effekte van hoë dosisse NAS op motoriese gedrag verdere studies vereis om te bepaal of hierdie gedragsafwykings slegs tydelik of meer permanent is. Die data ondersteun ook die hipotese dat antioksidante onder sekere toestande pro-oksidatief kan optree wat afhanklik kan wees van die redoksomgewing van die biologiese sisteem waarin die antioksidant toegedien word, of anders gestel, of die organisme in 'n toestand van oksidatiewe stres is of nie.

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Publications and Congress Proceedings

The results of the current study were presented at a congress as follows:

JOUBERT, C., HARVEY, B.H. & BERK, M. 2006. The antioxidant, N-acetyl cysteine (NAC), increases vacuous chewing movements in rats: Evidence for striatal oxidative stress. (Paper presented as podium presentation at the 4th ICPPS Congress held in Vanderbijlpark from 20 – 23 September 2006).

Exerpts of the study have been published as follows:

HARVEY, B.H., JOUBERT, C., DU PREEZ, J.L. & BERK, M. 2007. Effect of chronic N-acetylcysteine administration on oxidative stress in rat striatum. *Neurochemical Research,* (Article in Press).

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Chapter 1 Introduction

1.1 PROBLEM STATEMENT

Neuroleptic treatment is associated with a number of side-effects, including the induction of several movement disorders such as drug-induced parkinsonism, akathisia, dystonia and tardive dyskinesia (TD) (Hirsch & Weinberger, 2003), and indicative of the toxic effects of these agents on the striatum and its function in regulating movement. Of these disorders, TD is arguably the most serious disturbance and follows after long-term neuroleptic treatment and is characterized by a late onset (Munetz & Cornes, 1982). Some of the most troubling aspects of this disorder are that it is often resistant to treatment and in some cases may be irreversible (Munetz & Cornes, 1982).

In 1956, the first case of chlorpromazine-induced TD was reported and soon after other cases were added. Since then, several hypotheses have been put forward to explain this devastating disorder, for example the dopamine supersensitivity hypothesis, the GABA hypothesis (Fibiger & Lloyd, 1984), excitotoxicity hypothesis (McGeer & McGeer, 1976) and the free radical/oxidative stress hypothesis (Lohr *et al.*, 1990). In recent years much evidence has accumulated in support of the latter hypothesis.

A great deal of emphasis has been placed on the role of free radicals and oxidative stress in various disease states, including cancer, hypertension, diabetes and numerous others. Free radicals and other reactive oxygen species (ROS) have also been implicated in a number of neurodegenerative diseases, particularly Alzheimer's disease (AD) (Markesbery, 1997; Kontush, 2001), Parkinson's disease (PD)(Fahn & Cohen, 1992; Valko *et al.*, 2007) as well as TD (see Lohr *et al.*, 2003 for a review). Numerous studies have implicated free radicals with ensuing lipid peroxidation as causing damage to sensitive cellular targets that may be related to disease pathogenesis. In this regard, various preclinical studies (Bolkenius *et al.*, 1996; Roghani & Behzadi, 2001) have also been undertaken in order to evaluate the potential benefit that antioxidant therapy may hold for diseases in which free radicals are implicated (see Mazza *et al.*, 2007 for a review). Regarding the use of antioxidant therapy in PD, a comprehensive clinical open study suggested that treatment with high doses of both Vitamin A and C delayed the use of levodopa or DA agonists by 2.5 years (Fahn, 1992). Similarly, a study conducted by the Alzheimer's Disease Cooperative Study (Sano *et al.*, 1997), suggest that vitamin E may slow

disease progression in patients with moderately severe AD. Studies employing antidoxidant therapy in TD have also been conducted (Adler *et al.*, 1998; Brown *et al.*, 1998; Mahadik *et al.*, 2001), however, these studies report conflicting evidence, with some studies reporting a clinical improvement of TD, while others do not.

This hypothesis has also been tested in the vacuous chewing movement (VCM) animal model of TD (Bhattacharya *et al.*, 2002; Naidu & Kulkarni, 2002; Abilio *et al.*, 2003; Naidu *et al.*, 2003b) by reversing certain markers related to oxidative stress as well as the behavioural aspects of neuroleptic treatment in rats. The VCM model is based on the observation that chronic administration of a neuroleptic drug (especially first generation neuroleptics) induces a pronounced increase in VCMs in rats compared to vehicle treated animals. The behavioural model on which the current study is based utilizes the determination of the degree of orofacial dyskinesia in rats as a measure of TD. This model has been previously validated under our current laboratory setup and conditions (Bester, 1999).

N-acetylcysteine (NAC) is an antioxidant probably best known for its use in toxicology as a treatment for paracetamol poisoning (Flanagan & Meredith, 1991). NAC effectively scavenges several free radical and reactive oxygen species (ROS) including superoxide, but can also be easily deacetylated to cysteine, an important precursor of cellular glutathione (GSH) synthesis (Gillissen & Nowak, 1998). The GSH system is especially important for cellular defence against ROS (Dringen *et al.*, 2000). In TD, as with most other neurodegenerative diseases, prevention rather treatment of the potential permanent damage associated with the disorder can be of great value. With regard to prevention, an antioxidant like NAC can be administered prior to the induction of oxidative stress and in this manner prevent the pathogenesis of the illness. In addition, if oxidative stress is already induced and the illness is manifest, NAC may be administered to prevent further oxidative damage and worsening of the symptoms. Therefore, the central dogma is that an antioxidant like NAC may either be used as a preventative agent against the development of conditions that precipitate increased oxidative stress, or be used as a treatment option once oxidative stress has been induced.

1.2 PROJECT AIMS

The current study can be divided into three separate but related parts:

- The first part of the study sees the setting up and validation of a rat model of TD. In this model, rats are treated chronically with the typical neuroleptic haloperidol to induce an increase in VCMs in rats. In addition to the latter observations, the involvement of striatal oxidative stress will be evaluated by measuring the formation of superoxide radicals, the degree of lipid peroxidation, and the oxidised versus reduced status of GSH.

- Secondly, the effect of increasing doses of chronic NAC administration on the above-mentioned bio-behavioural parameters will be determined. These effects will be evaluated in healthy rats, i.e. rats which are not treated with haloperidol, and in this manner reproducing conditions of normal oxidative status.

- The final objective of the current study will be to utilise the established rodent model of TD in order to evaluate the effect of increasing doses of NAC on haloperidol-induced striatal toxicity, as assessed by behavioural (VCMs) and neurochemical markers of oxidative stress. Thus study hopes to establish the ability of NAC to reverse the abovementioned bio-behavioural effects associated with chronic haloperidol administration.

1.3 PROJECT LAYOUT

1.3.1 General Procedures

Each group will consist of 24 rats, with the entire group of rats being used for behavioural evaluation. Twelve rats from each group will randomly be assigned for the measurement of superoxide formation and lipid peroxidation, and the remaining 12 rats will be used for the assessment of the glutathione redox status.

During and after the respective treatment for each of the three phases of the study, the following behavioural and neurochemical assays will be employed:

- At various time points during the treatment period (i.e. days 0, 7, 14, 17, 19, 21), behaviour of the animals will be assessed in terms of the number of VCMs in a 2 minute rating session. Following the final rating session on day 21, the animals will be sacrificed by decapitation, and the striata of the brains dissected and kept at -72°C for neurochemical assays to follow.

- The formation of superoxide will be measured by assessing the striata of 12 rats from each group, using the Nitro Blue Tetrazolium (NBT) assay.

- Utilising the same brain homogenates that were used in the abovementioned NBT assay, lipid peroxidation will be measured using the Thiobarbituric Acid (TBA) Assay.

- The remaining 12 striata of each group will be individually utilised to evaluate the glutathione redox state using LC-MS.

1.3.2 Treatment Regimes

1.3.2.1 Establishment of the Animal Model of TD

In order to establish the rodent model of TD under the current laboratory and experimental conditions, rats will be treated for 3 weeks with haloperidol at a dose of 1.5 mg/kg/day via intra peritoneal (i.p.) injections, with control animals receiving vehicle for the same time period.

1.3.2.2 Evaluation of the Effects of NAC alone

For the second part of the study, rats will receive NAC at a dose of 10 mg/day, 100 mg/day or 300 mg/day by direct gastric administration for 3 weeks. The control animals will receive vehicle via the same administration technique for the duration of the treatment period.

1.3.2.3 The Reversal of Haloperidol-Induced Toxicity with NAC

Finally, animals will be treated by co-administering haloperidol (1.5 mg/kg/day ip) and NAC at doses of 10 mg/day, 100 mg/day or 300 mg/day by direct gastric administration for 3 weeks. Control animals will receive vehicle as above for the duration of the treatment period.

Chapter 2 Literature Review

2.1 TARDIVE DYSKINESIA

2.1.1 Background Information

In 1952, chlorpromazine was introduced into clinical psychiatry as a treatment for psychosis and schizophrenia (Dukes, 1988). Since conventional treatment at the time included electronconvulsion therapy, insulin shock therapy, metrazol, hydrotherapy, fever therapy and frontal lobotomy (Palmer, 2006), chlorpromazine represented a major breakthrough, and soon several other drugs were added to clinical practice. These drugs have been used for a variety of indications, including the management of schizophrenia, mania, certain organic psychoses (Dukes, 1988), and certain mood disorders (Buckley, 2001). However, while they had a profound benefit on psychiatry it later became apparent that these new neuroleptic drugs were not without risk and side effects, and by 1956 the first case of chlorpromazine-induced movement disorder was diagnosed (Jankovic, 2006). Thereafter, as use of these agents increased worldwide, the incidence of a severe motor disorder we know today as tardive dyskinesia (TD), began to escalate in patients treated with neuroleptics until it reached almost epidemical proportions (Lohr *et al.*, 2003). In many cases the side effect burden of these drugs was more severe than the disorder they were prescribed for. As well as improved efficacy, improving the side effect profile of neuroleptic agents was one of the driving forces for the development of the new generation agents that represented a rational and targeted pharmacological approach to treating psychosis.

The introduction of second generation neuroleptic drugs, otherwise referred to as the atypical neuroleptics, saw a significant reduction in the incidence of these neuroleptic associated motor-side effects (Barak *et al.*, 1995; Street *et al.*, 1996; Wittenberg *et al.*, 1996; Turrone *et al.*, 2005). Nevertheless, despite the introduction of these drugs extrapyramidal symptoms (EPS) are still the most frequently experienced side effects associated with long-term neuroleptic use (Kane, 2001), while TD remains an ever-present reality. Indeed, atypical neuroleptics are not totally free of the risk of TD (Dolder & Jeste, 2003) and several cases have been reported in the literature (Gafoor & Brophy, 2003; Karama & Lal, 2004; Ertugrul & Demir, 2005). Also, despite their improved side effect risk, many patients do not have access to second generation drugs,

especially in the developing world, who continue to use the older class of neuroleptics. This is of mayor concern and suggests that drug-induced motor disturbances may remain a global concern for the near future (Tammenmaa *et al.*, 2004). Acute EPS include parkinsonism, akathisia and dystonia while TD is a long-term, chronic (Kane, 2001) and frequently irreversible (Jeste *et al.*, 1979) motor disorder. TD represents the most debilitating of the EPS disorders, and contributes significantly to the stigma and social isolation associated with schizophrenia (Kane, 2001).

2.1.2 Clinical Description

Tardive dyskinesia (TD) is a highly disfiguring syndrome that consists of complex hyperkinetic movements (Hirsch & Weinberger, 2003). Stereotypically it can be defined as involuntary, coordinate, patterned, repetitive, rhythmic, ritualistic, purposeless (seemingly purposeful) movement, posture or utterance (Jankovic, 2006). The orofacial area (tongue, mouth and face) is most commonly involved in TD (Hirsch & Weinberger, 2003), but some patients present with more complex stereotype motor abnormalities of the trunk and limbs (Yovtcheva *et al.*, 2000). These can include hand and toe waving, touching and picking, rubbing of the face, scalp or other body parts, head nodding, body rocking, shallow and rapid breathing, pelvic thrusting, crossing and uncrossing of legs, shifting of body weight from one leg to the other, pacing or marching in one place, alternating sitting and standing, vocalization and noise-making (Hirsch & Weinberger, 2003; Jankovic, 2006).

The most serious aspect of this extremely embarrassing and demoralizing disorder is that it may persist years after drug withdrawal and can be irreversible in a number of patients (Jeste *et al.*, 1979). Moreover, drug withdrawal actually worsens TD (Schultz *et al.*, 1995; Goldberg, 2002), with drug re-introduction reversing the syndrome. Similarly, anticholinergic agents typically used to treat parkinsonism have no effect and may even worsen the symptoms of TD (Miller *et al.*, 2005). These attributes, together with distinct evidence that TD can also be induced by new generation antipsychotics, and that it may be an irreversible side effect, has prompted great interest in understanding the pathophysiology of TD.

2.1.3 Pathophysiology

Several hypotheses have been put forward in an attempt to explain the development of TD. The current study will be focussing on the free radical hypothesis of this disorder, and for this reason will be discussed in more detail. Alternative hypotheses will only be mentioned in brief.

2.1.3.1 The Free Radical Hypothesis

Eukaryote cells produce free radical molecules as a normal part of oxidative metabolism (Ahmad, 1995). However, in recent years a disturbance in free radical production and free radical scavengers have become increasingly important with regards to their possible involvement in various diseases (Valko *et al.*, 2007). Likewise, an increase in free radical formation has been implicated in the development of TD (Lohr *et al.*, 2003).

The therapeutic actions of neuroleptics are primarily antagonism at dopamine (DA) serotonin (5HT) and norepinephrine (NE) receptors (Lee *et al.*, 1997). All clinically effective neuroleptics are characterized by a high affinity for dopamine D_2 receptors (Kapur & Remington, 2001) and this action in limbic brain areas, especially the nucleus accumbens (Rushlow *et al.*, 1997; Hussain *et al.*, 2002), is considered critical for the clinical efficacy of these drugs (Lidsky *et al.*, 1997) (see Figure 2-1 for a detailed description). This chronic blockade results in various adaptive neuroplastic events, including a reactive up-regulation of DA receptors and/or an increased pre-synaptic DA release in order compensate for these changes (Muller & Seeman, 1977). The latter action results in a secondary increase in DA synthesis and an increase in DA metabolism. The latter is particularly important as the metabolic pathway of DA is associated with the co-production of oxidative free radicals. Thus, the degradation of DA by monoamine oxidase (MAO) leads to the formation of hydrogen peroxide as a by-product as well as the generation of toxic quinones, while the auto-oxidation of DA can lead to the production of superoxide and hydroxyl radicals (Loeffler *et al.*, 1998). Increased free radical formation leads to oxidative stress which together with products of lipid peroxidation has been implicated in various s neurological disorders including TD.

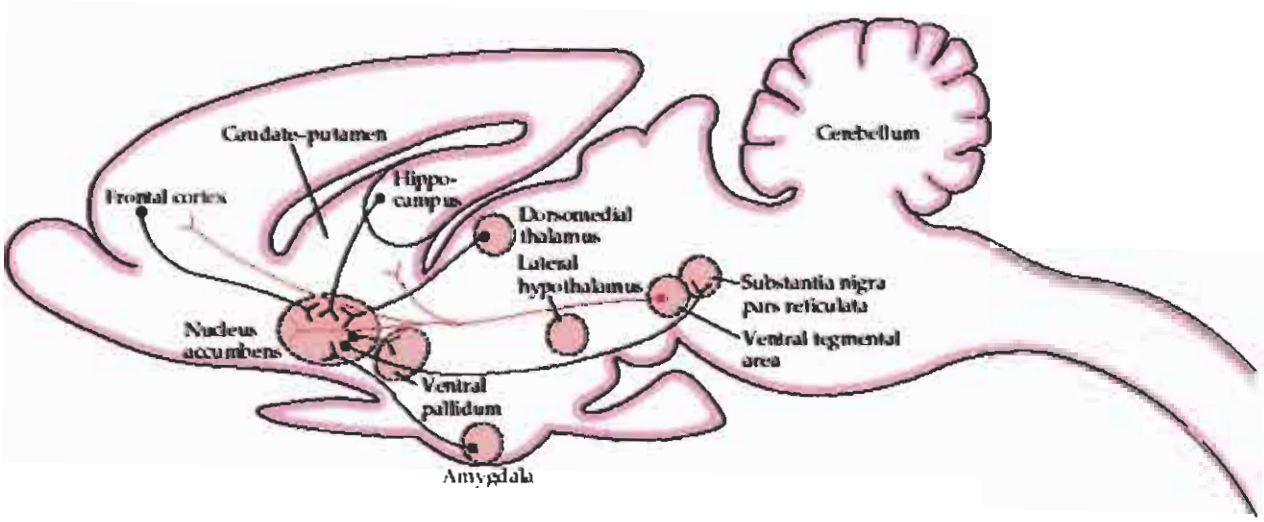

Figure 2-1: The Mesolimbic Dopamine System of the rat (Feldman & Quenzer, 1984)

In vitro experiments have shown the involvement of reactive oxygen species (ROS) in the neurotoxic effect of haloperidol (Behl *et al.*, 1996; Sagara, 1998). Further, increases in oxidative

stress parameters and decreases in antioxidant protective enzymes have been found in cases of TD (Lohr *et al.*, 1990; Zhang *et al.*, 2003). In an earlier study in rats (Parikh *et al.*, 2003), chronic treatment with haloperidol for 45 and 90 days produced severe oxidative stress in the brain, as determined by an increase in lipid peroxidation, and accompanied by marked changes in antioxidant defence enzymes as well as associated oxidative tissue injury.

Another mechanism whereby neuroleptics could cause oxidative damage is through the inhibition of the mitochondrial respiratory chain (Dean, 2006). For example, the production of a haloperidol metabolite, haloperidol-pyridinium (Avent *et al.*, 1996) inhibits complex I of the mitochondrial respiratory chain which in turn leads to oxidative stress in a number of brain areas (McLennan *et al.*, 1996). The metabolite also shows structural similarities to the known dopamine neurotoxin, MPP+, and may cause neuronal injury through selective toxicity of dopaminergic neurons (Wright *et al.*, 1998). However, no similar toxic metabolites for other neuroleptics have been identified to date, and the relevance of this theory remains to be proven.

In addition, since pre-clinical studies have shown that antioxidants (e.g. tocopherol, better known as vitamin E) attenuate haloperidol-induced vacuous chewing movements, these results further support the free radical/oxidative stress hypothesis of TD (Abilio *et al.*, 2003). Similarly, other authors (Naidu *et al.*, 2003b) have established that the co-administration of quercetin, a bioflavonoid with strong antioxidant properties, together with haloperidol reverses the behavioural and biochemical changes associated with haloperidol administration in rats.

Clinical studies in TD have also shown potential for the role of tocopherol in the treatment of TD (Adler *et al.*, 1998), although its efficacy has been questioned by others (Dorevitch *et al.*, 1997; Sachdev, 2000). Nevertheless, in a review article on this subject (Rotrosen et al., 1996), it was concluded that clinical studies, in general, are in support of the notion that tocopherol may indeed improve TD, especially after chronic administration, and also when used early in treatment.

2.1.3.2 Other Hypotheses

2.1.3.2.1 The Dopamine Supersensitivity Hypothesis

Briefly, the dopamine supersensitivity hypothesis proposes that the chronic blockade of postsynaptic DA receptors in the limbic areas of the brain needed for the effective treatment of psychosis will also include non-specific blockade in brain areas related to motor control, resulting in an up-regulation of DA receptors (Margolese *et al.*, 2005). This implies that neurons may produce more DA receptors (increased density) and/or receptors with altered activation states that are more sensitive to DA (Drucker *et al.*, 1994). In nigro-striatal motor regions of the

brain, this supersensitivity of DA receptors may lead to a hyperdopaminergic state which may clinically manifest as dyskinesia (Tan *et al.*, 2005).

Despite its theoretical value and its popularity for many years, the hypothesis has a number of limitations, and is not able to explain the disease process fully (Fibiger & Lloyd, 1984). Even though the mechanism of DA receptor supersensitivity is well accepted and does occur secondary to neuroreceptor blockade, it is not able to account for the fact that some patients receiving DA blockers never develop TD or that there is usually a delay of months and even years to the onset of the disease (Munetz & Cornes, 1982), this in spite of the fact that receptor supersensitivity develops within days or weeks of drug initiation (Lohr *et al.*, 2003). Moreover, it cannot explain the fact that advanced age (Yassa & Jeste, 1992), female gender (Yassa & Jeste, 1992) and other factors increase the risk for TD, or that TD can persist for years after drug withdrawal(Munetz & Cornes, 1982). Clearly, something else is happening that may well have its origins in DA receptor blockade, but ultimately becomes independent of the chronic activation state of the DA receptor.

2.1.3.2.2 The GABA Hypothesis

It has been proposed that TD may be a result of neuroleptic induced destruction of GABA containing neurons in the striatum. In addition, hypofunction of nigral GABA-containing neurons may play a pathophysiologic role in the disease (Lahti *et al.*, 1989). Indeed, TD has been reported to be associated with a regional reduction in GABA function in the basal ganglia, such as a significant decrease in the activity of the GABA synthesizing enzyme, glutamate decarboxylase (Johansson *et al.*, 1990).

2.1.3.2.3 The Excitotoxicity Hypothesis

Some authors (McGeer & McGeer, 1976) have suggested that striatal excitotoxicity may be implicated in the development of TD. The excitatory neurotransmitters, glutamate and aspartate, are well recognized to evoke cell death under conditions of excessive stimulation (Sanganahalli *et al.*, 2006). It has been shown that the substantia nigra may undergo damage under conditions of excessive glutamatergic cortical-striatal activation (Andreassen & Jorgensen, 2000; Andreassen *et al.*, 2003), which could precipitate EPS. Also, it has been demonstrated that neuroleptic-induced modulation of DA can exert profound effects on the function and activity of glutamatergic systems. For example, D_2 receptor agonists are able to block the release of glutamate, while D_2 receptor antagonists increase the activity of the glutamate system (Yamamoto & Davy, 1992; Konradi & Heckers, 2003).

In summary, one or more of the abovementioned mechanisms may be involved in the development of TD. There is evidence that suggests that excitotoxicity and oxidative stress may act together, since these are closely related processes (Andreassen & Jorgensen, 2000). However, the development of TD remains unclear, and further work on the role of free radicals and their natural control mechanisms needs to be addressed.

2.1.4 Histopathology, Morphological & Genetic Correlates

The potential permanent character of the disorder indicates that long-lasting cerebral changes are involved in the pathogenesis of TD (Andreassen *et al.*, 1999). In 1978 (Nielsen & Lyon, 1978) it was suggested that persistent anatomical changes can follow long-term neuroleptic treatment. These authors noted a significant cell loss (10%), in the ventrolateral striatum after long-term treatment of rats with flupenthixol.

Exposure to neuroleptics may be associated with hypertrophy of certain brain regions. In patients treated with conventional neuroleptics it appears that a higher drug dose is associated with higher caudate (Chakos *et al.*, 1994), putamen (Elkashef *et al.*, 1994) and thalamus volumes (Harrison, 1999). Specifically, the left globus pallidus seems to show a more pronounced increase in volume (Gur *et al.*, 1998). However, higher doses of atypical neuroleptics are associated more with higher thalamic volumes (Gur *et al.*, 1998) and some studies have reported a decrease in the size of the basal ganglia with atypical neuroleptics (Corson *et al.*, 1999). Much of these data need replication and confirmation, especially since earlier studies (Mion *et al.*, 1991) found that the volume of the caudate nuclei of patients with TD were significantly smaller when compared to patients without the disorder. The occurrence of these brain abnormalities in patients with TD, as well as in rats displaying abnormal oro-facial movements (Nielsen & Lyon, 1978; Chakos *et al.*, 1998) lends support to the theory that the use of neuroleptics might accelerate a natural degenerative process (Andreassen & Jorgensen, 2000).

There is also strong evidence for a genetic contribution to the development of TD (Tan *et al.*, 2003). Genetic linkage studies have identified the role of the Cytochrome P450 2D6 gene (Kapitany et al., 1998; Ellingrod et al., 2002) in TD. In patients carrying the dopamine D3gly allele and the cytochrome P 450 17α-hydroxylase A2-A2 genotype there also seems to be a greater risk of developing TD (Segman *et al.*, 2002). This may explain one of the major detractors for the DA supersensitivity hypothesis of TD, namely why some patients on neuroleptics develop TD while others that receive a similar treatment regime do not.

2.1.5 Risk Factors

A recent clinical study (Miller *et al.*, 2005) found that TD was significantly associated with increased age, duration of neuroleptic treatment, treatment with typical neuroleptics, anticholinergic treatment, the presence of EPS and akathisia and substance abuse (Miller *et al.*, 2005). Negative symptoms, smoking (Nilsson *et al.*, 1997), female gender (Morgenstern *et al.*, 1987; Yassa & Jeste, 1992; Kasper *et al.*, 2006) and cognitive impairment (Quinn *et al.*, 2001) have also been identified as possible risk factors, although the literature seems somewhat conflicting on the precise role of gender. Ageing has been found to be one of the most consistent risk factors for the development of TD. Various mechanisms have been suggested to associate increased age with the risk of developing TD, but the most interesting is age-related loss or changes in oxidative status and endogenous antioxidant mechanisms (Rikans & Hornbrook, 1997; Sohal, 2002; Kasper *et al.*, 2006). This may contribute to increased vulnerability of the aging brain to oxidative stress associated with TD, and lends further support to the free radical hypothesis.

2.1.6 Treatment

There are currently no pharmacological treatments that have been proved to be universally effective in the treatment of TD in clinical practice (Hirsch & Weinberger, 2003). Prevention rather than cure seems to remain essential, while the use of neuroleptics should be reserved for patients for whom there are no other treatment options (Murray, 2005). When chronic treatment is warranted, the lowest effective dose should be used and the need for these drugs should be reassessed over four to six months (The Canadian Movement Disorder Group, 2004). Moreover, patients should be placed on an atypical agent as soon as possible, preferably at the start of treatment (Wickman & Cold, 2006). The improved motor side effect of these agents has been linked to various pharmacological actions, including their increased 5-$HT_{2A/2C}$ antagonism (Kapur & Remington, 2001), alpha-adrenergic actions, 5-HT_{1A} agonism, anti-muscarinic actions as well as possible effects on glutamate (see Harvey *et al.*, 1999 for a review), or alternatively because of low affinity binding to dopaminergic receptors thus disinhibiting DA actions in the motor and frontal cortical areas (Kapur & Remington, 2001; Westerink, 2002).

Should TD emerge, withdrawal of the drug seems to be the most logical option. However, this is discouraged when treating chronic schizophrenia (Wickman & Cold, 2006) since, as highlighted earlier, this may be associated with worsening of symptoms of TD and may also precipitate psychoses. Rather, switching to an atypical neuroleptic may be the most efficacious strategy (Hirsch & Weinberger, 2003) , although these agents themselves are not without risk (Gafoor & Brophy, 2003; Karama & Lal, 2004; Ertugrul & Demir, 2005). More recent studies have also indicated that TD induced by older generation agents can be treated with atypical agents such

as olanzapine (Kinon *et al.*, 2004), clozapine (Jurjus *et al.*, 1997) and risperidone (Koponen, 1996).

2.1.7 Concluding remarks

Despite the proven efficacy of neuroleptic drugs currently available, schizophrenia remains a devastating disorder, while neuroleptics in general are plagued by EPS such as restless leg syndrome, parkinsonism and dystonia, all of which contribute to the eventual development of TD. While TD and EPS remain the leading causes of non-compliance amongst patients receiving neuroleptics, this and the fact that the disorder is often irreversible and resistant to treatment holds major consequences for the long-term treatment outcome of schizophrenia and undoubtedly warrants further investigation into this disorder.

2.2 FREE RADICALS

"A free radical is a molecule or molecular fragment with an unpaired valence electron, i.e. it does not contribute to the bonding within the molecule and is, in that sense free" (Tarr & Samson, 1993). The unpaired electron in free radicals often undergoes reactions in which the electron is lost (oxidation) or where another electron is gained (reduction) thereby producing a more stable, electron paired species. This tendency to pair unpaired electrons is the reason why free radicals have such high reactivity. Free radicals are therefore often the reactants or products in oxidation-reduction reactions (Tarr & Samson, 1993).

2.2.1 The Role of Free Radicals in the Body

It was previously believed that the production of free radicals during metabolism was just an unfortunate result of aerobic life (Hensley *et al.*, 2000). However free radicals possess numerous important functions in cells making them essential to life. For example, they are important for extracting usable energy for metabolism, and are able to break down large molecules into their more basic parts, a process which is important in lysosomes for the metabolism of macromolecules and also the destruction of pathogens (Lohr *et al.*, 2003). Free radicals and ROS also play an important part in cell signalling, since they are able to act as second messengers which propagate pro-inflammatory and growth-stimulatory signals (Hensley *et al.*, 2000).

2.2.2 The Biological Formation of ROS

The production of oxygen radicals in the body follows the reduction of oxygen, which is necessary for the generation of energy (Lohr *et al.*, 2003). Different species of ROS differ in their site of formation, physiological function, reactivity and biological half life. In the mitochondria, arachidonic acid metabolism and enzymes such as xanthine oxidase, nitric oxide synthase (NOS), monoamine oxidases and the cytochrome (CY) P450 series all have the ability to produce ROS in the brain (Schulz *et al.*, 2000). In the electron transport chain of the mitochondria for example, the energy produced by oxidation is used to build high-energy phosphates, such as adenosine triphosphate (ATP) (Lohr *et al.*, 2003), and results in the production of free radicals (Lohr *et al.*, 2003). ROS includes oxygen free radicals and other related substances such as superoxides, hydrogen peroxide, singlet oxygen, nitric oxide (NO), and peroxynitrite (Chong *et al.*, 2005).

2.2.2.1 Superoxide ($O_2^{\cdot -}$)

The one-electron transfer to molecular oxygen (O_2) yields $O_2^{\cdot -}$. It is a relatively weak oxidant that can also act as a reducing agent for transition metals and metal complexes (Ahmad, 1995). The most important reaction of $O_2^{\cdot -}$ involves the spontaneous or enzymatic dismutation to hydrogen peroxide. It can also be protonated to form hydroperoxyl radicals ($HOO^{\cdot}$) (Ahmad, 1995).

2.2.2.2 Hydrogen peroxide (H_2O_2)

H_2O_2 is formed by the disproportionation of $O_2^{\cdot -}$, either spontaneously or catalyzed by SOD. Even though H_2O_2 is not a radical itself and shows only moderate chemical reactivity it is still of critical importance, since H_2O_2 is able to cross biological membranes freely and is required for the formation of more potent oxidants, such as the hydroxyl radical ($OH^{\cdot}$), which is formed upon reaction with metal chelates, and oxoferryl complexes which is formed when H_2O_2 reacts with hemoproteins (Ahmad, 1995).

2.2.2.3 Hydroxyl radical ($OH^{\cdot}$)

The $OH^{\cdot}$ radical is considered the most reactive of all the radicals and is mainly formed by the reaction of hydrogen peroxide with reduced forms of transition metal ions:

$H_2O_2 + Me+ \rightarrow OH^{\cdot} + OH- + Me2+$

Hydroxyl radicals can also be formed from peroxylnitrite or by the reaction of superoxide with hypochlorite (Beal *et al.*, 2005).

Superoxide and hydrogen peroxide are both able to initiate oxidative stress. Neither of these molecules are strong oxidants but they can be converted into more hazardous oxidants by certain reactions in tissues. Superoxide can be produced from molecular oxygen via a variety of enzymes, including those of the respiratory chain, xanthine oxidase, cyclo-oxygenase and NADPH-oxidase. Superoxide can, either spontaneously or via enzymatic reaction, dismutate to hydrogen peroxide and in the presence of transition metals hydrogen peroxide can be converted to extremely strong oxidative hydroxyl radicals. Myeloperoxidase is able to produce hypochlorus acid from hydrogen peroxide, while superoxide can react with nitric oxide to form peroxynitrite. These radicals can cause damage to sensitive cellular targets (Ratnam *et al.*, 2006), and the resultant lipid peroxidation of membranes caused by the reaction of free radicals with these sensitive targets can severely alter membrane function, structure and fluidity (Tarr & Samson, 1993; Brown *et al.*, 1998).

2.3 OXIDATIVE STRESS

In the body, ROS are constantly formed via numerous metabolic processes. Antioxidant defence systems protect against ROS, but when these systems are overwhelmed, oxidative stress is initiated (Dorado-Martinez *et al.*, 2001), which has been defined as "a condition in which cellular antioxidant defences are no longer sufficient to keep the levels of ROS below a toxic threshold" (Schulz *et al.*, 2000).

The brain is at a severe disadvantage with regard to the generation and detoxification of ROS (Dorado-Martinez *et al.*, 2001). The brain utilizes 20% of the body's oxygen supply but comprises only 2% of the body weight. Some areas of the brain also have a high iron content which is able to catalyze the generation of ROS (Dringen *et al.*, 2000), such as $OH^{\cdot}$ which can be generated via the Fenton reaction (Markesbery, 1997). Another reason for the brain's vulnerability is that it is rich in lipids with unsaturated fatty acids; these are the targets for lipid peroxidation. Furthermore the brain also contains only low to moderate antioxidant activity when compared to other peripheral organs (Dringen *et al.*, 2000).

2.3.1.1 Oxidative stress and disease

Oxidative stress has been associated with several diseases and conditions which include ischemia and reperfusion injury (Granger *et al.*, 1981; Ahmad, 1995), cancer (Valko *et al.*, 2007) rheumatoid arthritis (Valko *et al.*, 2007) as well as a number of CNS diseases such as schizophrenia and even chemically-induced neurological disorders (Gilgun-Sherki *et al.*, 2001). Oxidative stress also plays an important role in aging and age-related neurodegenerative diseases (Dorado-Martinez *et al.*, 2001), such as Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease and Parkinson's disease (Gilgun-Sherki *et al.*, 2001).

2.3.1.2 Neurodegenerative diseases

Neurodegenerative diseases follow a route of slow onset, gradual advancement leading finally to an unavoidable conclusion (Schulz *et al.*, 2000). Apoptosis appears to be of major importance in the neuropathology of neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis, in which the nervous system's functionality is decreased due to the death of neurons. While the cause of apoptotic cell death in neurodegeneration is unclear, growing evidence suggests that oxidative stress is involved in the propagation of cellular injury that underlies the neuropathology in these various conditions (Yap *et al.*, 2007). During normal aging, the brain suffers both morphological and functional changes, and a growing body of evidence seems to suggest that the long-term effects of oxidative stress, mitochondrial dysfunction and inflammatory insults are major contributing factors (Mariani *et al.*, 2005). With regard to the process of neurodegeneration it has become apparent that oxidative stress and inflammation are interrelated and possibly even inseparable processes (Hensley *et al.*, 2000).

2.3.1.2.1 Alzheimer's disease (AD)

AD is the leading cause of dementia in the elderly and is characterized by memory dysfunction, loss of lexical access, spatial and temporal disorientation and impairment of judgment (Mariani *et al.*, 2005). The selective loss of neurons and synapses in the hippocampus and cerebral cortex associated with the disease correlates with the clinical symptoms (Zhu *et al.*, 2004). The precise pathology of AD remains unclear, but much attention has been given to the massive loss of the neurotransmitter acetylcholine and the possible role of oxidative stress in its development (Mariani *et al.*, 2005). Another factor associated with AD is the increased production of amyloid-β (Aβ), and the accumulation of Aβ in the form of senile plaques is characteristic of the disease. Aβ is produced as an antioxidant in the brain as a response to oxidative stress. However Aβ, like many antioxidants, has the ability to become pro-oxidative at high concentrations (see also Kontush, 2001 for a review; Butterfield, 2002). Increased Aβ production is followed by chelation of transition metal ions by Aβ, accumulation of Aβ-metal lipoprotein aggregates, production of reactive oxygen species and neurotoxicity, implying a possible role for increased Aβ, with associated increase in oxidative stress, in the pathology of AD (Kontush, 2001). Increased markers of lipid peroxidation, as a consequence of oxidative stress, have been found in the brains of patients suffering from AD (Lovell *et al.*, 1995). Nitric Oxide (NO) and NO derived species has also been reported to contribute significantly to lipid peroxidation in AD affected patients (Williamson *et al.*, 2002). Age is a strong risk factor and age dependant increase in oxidized protein, lipids and DNA have been found in patients with AD (Mariani *et al.*, 2005). Abnormalities of the mitochondria also seems to play a pivotal role in the disease state of Alzheimer's although the precise connection is still unknown and warrants further investigation (Reddy & Beal, 2005).

2.3.1.2.2 Parkinson's disease (PD)

PD is a result of neurodegeneration in the substantia nigra and striatum, leading to a critical depletion of dopamine (Mariani *et al.*, 2005), accompanied by the development of Lewy bodies in neurons (Wang *et al.*, 2006). While the precise pathology of PD remains unclear, evidence in support of free radical involvement is substantial. The oxidation of dopamine to yield toxic semiquinones (Mariani *et al.*, 2005) as well as the enzymatic metabolism of dopamine by MAO-B that generates an excess formation of free radical such as H_2O_2, $OH^{\cdot}$ and $O_2^{\cdot -}$ has been implicated in the aetiology of PD (Fahn & Cohen, 1992; Mariani *et al.*, 2005). A decrease in the antioxidants tocopherol and ascorbate (vitamin E and C); have also been shown to be present in the plasma of patients with vascular PD. Several neurotoxins that destroy the dopamine neurons of the substantia nigra have also been reported to generate their toxic effects via oxidative stress (Fahn & Cohen, 1992) lending further support to this hypothesis. The immune system and inflammatory responses also seem to play a pivotal role in the aetiology of PD. In a recent study (Wang *et al.*, 2006), it was reported that activation of certain immune factors by microglia, which are the primary immune cells of the brain, are able to induce the release of free radicals ($O_2^{\cdot -}$ and NO) and it was suggested that these free radicals may be major contributors to miroglia-mediated dopaminergic neurotoxicity in neuron–glia cultures. This study also supports the abovementioned hypothesis, namely that oxidative stress and inflammation may be interrelated processes.

2.3.1.3 Normal Aging

Aging has been defined as "the accumulation of changes responsible for the sequential alterations that accompany advancing age and the associated progressive increases in the chance of disease and death" (Harman, 1991). These changes may be attributed to disease, environment, immune dysfunction, and to an inborn aging process. Among the many theories put forward, the free radical theory of aging shows the greatest promise. This theory, proposed by Harman in 1956, postulates that aging is caused by the gradual load of free radical reactions over time (Ashok & Ali, 1999). There is not only strong evidence in support of the oxidative stress hypothesis of aging but also a great lack of evidence for the contrary. The amount of oxidative damage increases as an organism ages and is postulated to be a major causal factor of senescence (Sohal & Weindruch, 1996). The severity and extent of brain damage increases with age, which may be due to the impaired ability of the brain to utilize antioxidants in cases of oxidative injury or as a consequence of altered basal levels of these antioxidants (Moor *et al.*, 2006). Decreased levels of antioxidant such as ascorbate have also been found in the brain of aged rats (Moor *et al.*, 2006). Other authors (Rebrin *et al.*, 2003) have shown that the redox potential of glutathione in the mitochondria of mice becomes more pro-oxidative with increasing age, with the GSSG:GSH ratio shifting in favour of GSSG.

2.3.2 Defence Mechanisms against ROS

The generation of ROS is an essential and unavoidable part of aerobic life. In order to cope with this, aerobic organisms posses elaborate defence systems that protect the body against toxic oxygen forms (Ahmad, 1995).

2.3.2.1 Primary antioxidant defences

'Antioxidant activity involves the donation of a single electron to a free radical species. In doing so, the radical character is transferred to the antioxidant, yielding the antioxidant-derived radical which are much less reactive than the original molecules (Ahmad, 1995).

Antioxidants can be classified into two major groups, namely enzymatic and non-enzymatic antioxidants. Some of these antioxidants are produced in the body and include enzymes, low molecular weight molecules and enzyme co-factors while others such as the non-enzymatic antioxidants are mostly obtained from dietary sources (Ratnam *et al.*, 2006).

2.3.2.1.1 Enzymatic Antioxidants

Enzymatic antioxidants include superoxide dismutase, catalase and glutathione peroxidase. Superoxide dismutase and catalase both have a high affinity and rate of reaction with ROS and are also among the most potent natural antioxidants known (Ratnam et al., 2006). Glutathione is able to scavenge both peroxynitrite and hydroxyl radicals and is also able to convert hydrogen peroxide to water (Best, 2006).

Superoxide Dismutase (SOD):

The major function of superoxide dismutase is to catalyze the dismutation of superoxide ($O2^{\cdot -}$) to oxygen and hydrogen peroxide. In humans there a three types of SODs. The first is found in the cytosol, namely CuZn-SOD, the second is mitochondrial Mn-SOD and finally extracellular SOD (Ahmad, 1995).

The formation of ROS and free radicals in the mitochondria leads to an upregulation in the expression of Mn-SOD, and represents a probable self-defence mechanism to protect the mitochondria against oxidative damage. This increase in Mn-SOD must be accompanied by a concurrent increase in catalase and/or glutathione peroxidase which reacts with H_2O_2 in order to prevent the excessive build-up of H_2O_2 (Ahmad, 1995).

Catalase (CAT):

One of the products formed during dismutation of superoxide is hydrogen peroxide (H_2O_2). Catalase (CAT) is responsible for the dismutation of H_2O_2 and therefore acts in succession to SOD. During this process two molecules of H_2O_2 are dismutated simultaneously, where one acts as an oxidant and the other as a reductant in two consecutive steps (Ahmad, 1995) as follows:

$CAT\text{-}Fe(III) + H_2O_2 \rightarrow Complex\ I$

$Complex\ I + H_2O_2 \rightarrow CAT\text{-}Fe(III) + {}_2H_2O + O_2$

CAT displays non-saturation kinetics, meaning that the activity of the enzyme is able to increase linearly with the amount of H_2O_2 present in the biological system. CAT is primarily responsible for the decomposition of H_2O_2 (Ahmad, 1995), and can also act on toxic compounds such as phenols, formaldehyde and alcohols by peroxidative reaction (Ratnam et al., 2006). Large quantities of CAT are found throughout the body, with the highest activity being found in the liver, followed by erythrocytes, then the lungs (Ratnam et al., 2006). CAT is present primarily in the peroxisomes, where many of the H_2O_2 producing enzymes can be found (Ahmad, 1995).

Glutathione peroxidase:

Superoxide dismutase initiates the antioxidant process by transforming superoxide radicals to hydrogen peroxide. Hydrogen peroxide is then metabolized by catalase, which is then further metabolized by different forms of glutathione peroxidase, intra- and extracellularly (Berger, 2005).

2.3.2.1.2 Non-Enzymatic Endogenous Antioxidants

ROS that are not effectively scavenged by the antioxidant enzymes can be scavenged by a variety of lipid- and water-soluble antioxidants (Ahmad, 1995). The antioxidants, tocopherol, ascorbate and carotene form a network of interlinked processes protecting the cell against oxidative damage. These antioxidants either act catalytically through their interactions with other antioxidants or through direct enzymatic systems involved in their regeneration. They are thus able to act either synergistically or additively to reduce oxidative stress (Ahmad, 1995) and thereby counteract the negative effects of ROS. Another important naturally occurring antioxidant is glutathione, which is described in more detail below.

Glutathione:

Glutathione is the most abundant antioxidant occurring in the intracellular space and is considered the principal body defence against free radicals (Gillissen & Nowak, 1998). It is of fundamental importance in the cellular detoxification of drugs, environmental chemicals and oxidants (McLellan & Wolf, 1999). Glutathione is synthesized by the enzyme γ-glutamylcysteine synthase which makes use of glutamate and cysteine as substrates to form a dipeptide. A second enzyme, glutathione synthase, then combines glycine with the dipeptide to form glutathione. ATP serves is a co-substrate for both enzymes (Dringen et al., 2000) and cysteine is considered the rate-limiting step in the synthesis of glutathione (Gillissen & Nowak, 1998). Since glutathione is unable to cross the blood-brain-barrier (Fukami et al., 2004), de novo synthesis is important in the CNS.

Glutathione can exist in two forms, the reduced form (usually abbreviated as GSH) and an oxidized form (GSSG). GSH and GSSG are interconvertable by the action of two enzymes, namely glutathione peroxidase and glutathione reductase. Reduced GSH modulates the redox state (ratio of oxidizing to reducing equivalents) of the cell, a role which is critical for cell survival. GSSG is formed in antioxidant reactions that involve GSH, and can accumulate with increased oxidative processing in the cell (Bains & Shaw, 1997).

Glutathione is able to demonstrate its antioxidant properties through two mechanisms. Firstly by reacting directly with radicals in a non-enzymatic way, and then enzymatically in the reduction of peroxides by means of the enzyme, glutathione peroxidase (GPx) (Ahmad, 1995; Best, 2006). Oxidation of GSH leads to the formation of a glutathione radical, which is then neutralized by combining the radical with another glutathione radical to produce glutathione disulfide (GSSG) (Best, 2006). The enzyme, glutathione reductase (GR), then acts to regenerate GSH within cells from the oxidized form, GSSG. By transferring a reduction equivalent from NADPH to GSSG, GSH is regenerated by the enzyme (GR) (Dringen et al., 2000). See Figure 2-2 for a detailed description.

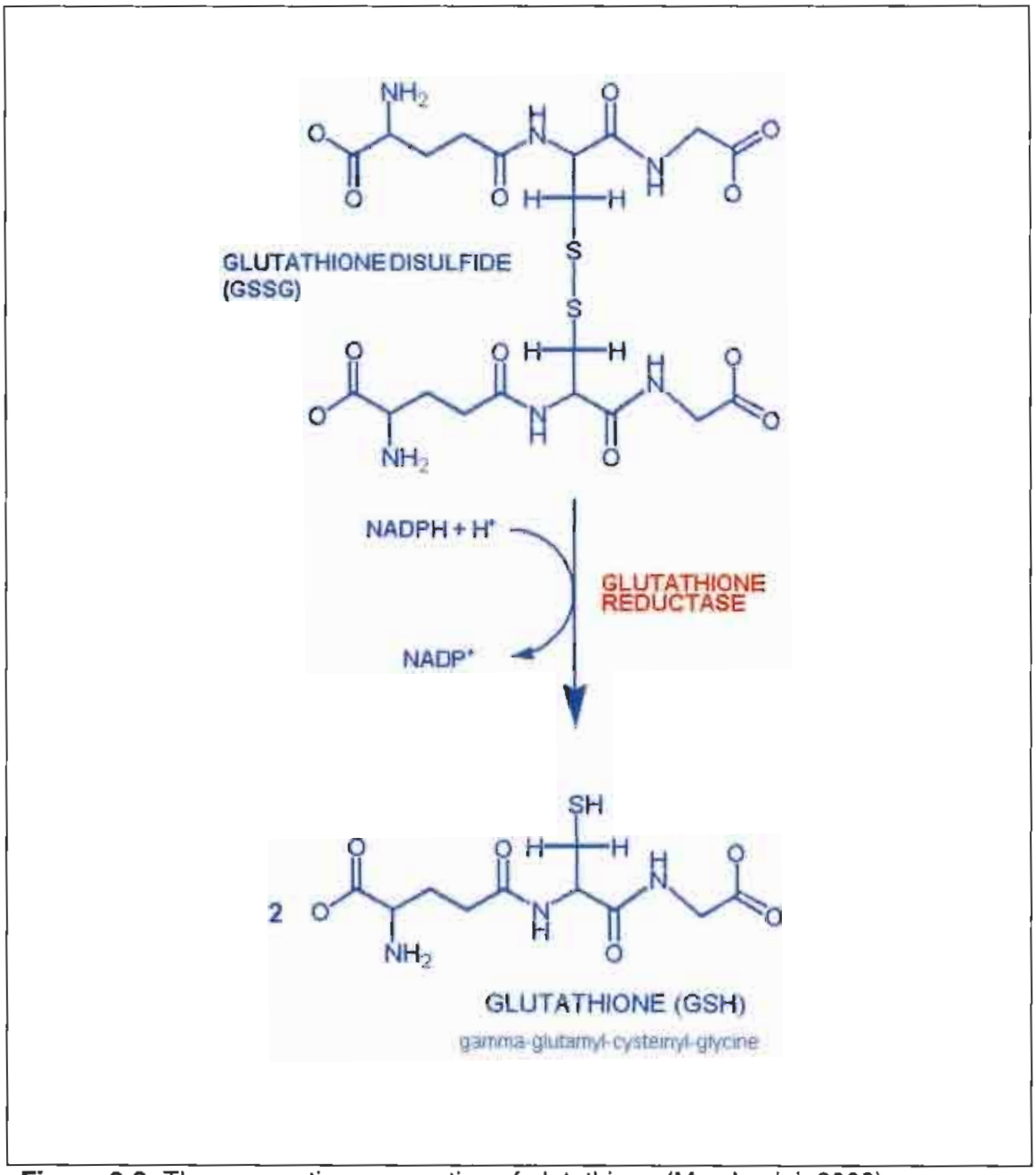

Figure 2-2: The enzymatic regeneration of glutathione (Marchesini, 2003).

2.3.2.1.3 Non-Enzymatic Dietary Antioxidants

Without dietary antioxidants the human antioxidant defence system would not be complete. Antioxidants such as ascorbate, vitamin E, Coenzyme Q10 (CoQ10), carotenoids and polyphenols are obtained from dietary sources and have important roles in maintaining human health (Ratnam *et al.*, 2006). These antioxidants and their proposed mechanisms of action are outlined in Table 2-1 below:

Table 2-1: Summary of numerous antioxidants and their mechanisms of action (Ratnam *et al.*, 2006).

Antioxidant	**Mechanism of action**
SOD	Dismutation of superoxide to H_2O_2
CAT	Decomposes H_2O_2 to molecular oxygen and water
NAC	Scavenging of H_2O_2 and peroxide
	Deacetylation of precursor for GSH synthesis
GSH	Intracellular reducing agent
EGCG	Metal chelation
	Scavenging of superoxide, H_2O_2, OH and singlet oxygen
	Tocopherol regeneration
Lycopene	Trapping of singlet oxygen
Ellagic acid	Scavenging of H_2O_2
	Stimulation of glutathione-S-transferase
CoQ10	Inhibition of lipid peroxidation
	Reduces mitochondrial oxidative stress
I3C	Inhibition of DNA-carcinogen adduct formation
	Suppression of free radical production
Genistein	H_2O_2 scavenging
Quercetin	H_2O_2 scavenging, one of the potent antioxidant among polyphenols
Ascorbate	Scavenging of superoxide anion by forming semidehydroascorbate radical which is subsequently reduced by GSH
Tocopherol	Direct scavenging of superoxide
	Upregulation of antioxidant enzymes
	Inhibition of lipid peroxidation

SOD = superoxide dismutase, CAT = catalase, NAC = N-acetyl cysteine, GSH = glutathione, EGCG = epigallocatechin-3-O-gallate, CoQ10 = coenzyme Q10, I3C = indole-3-carbinol.

Importance of Primary Antioxidants:

Antioxidants, whether enzymatic or non-enzymatic, are able to counteract free radicals and prevent the damage caused by such radicals (Ratnam *et al.*, 2006). They are able to inhibit or delay oxidation of a substrate (Berger, 2005), and most antioxidants have been shown to be pharmacologically active. They reduce the damaging effects of free radicals by quenching them before they can react with biologic targets, preventing chain reactions or preventing the activation of oxygen to highly reactive oxygen products (Ratnam *et al.*, 2006). When ROS are formed in excess or in inappropriate environments or when anti-oxidant defences are insufficient, these radicals can damage DNA, lipids, proteins and carbohydrates (Gillissen & Nowak, 1998).

2.3.2.2 Secondary antioxidant defences

Secondary defences are a varied host of enzymes that are involved in a repair system in response to oxidative challenge and/or injury. They eliminate molecules or cell components that have been damaged by oxidants or free radicals, which were not inactivated by the primary defence systems. These enzymes repair membrane phospholipids, proteins and DNA. This involves the digestion of critically damaged RNA and proteins. Phospholipids in turn are repaired by the removal of oxidized portions followed by lysophospholipid reacylation, and where DNA is concerned, proper base replacement occurs (Ahmad, 1995).

2.4 PRO-OXIDANTS

It seems plausible that dietary or pharmaceutical augmentation of endogenous antioxidant defence mechanisms may be useful in the prevention of ROS mediated carcinogenicity and various other diseases (Lee & Lee, 2006). However, many studies have shown that antioxidant supplementation may very well be a double-edged sword. Several antioxidants, which include vitamins and phenolic phytochemicals, have been shown to exhibit pro-oxidant, rather than antioxidant properties. Most free radical scavengers are able to act as either antioxidants or pro-oxidants depending on their structure and the biological conditions (Lee & Lee, 2006), as previously discussed for amyloid-β (refer to § 2.3.1.2.1).

2.4.1 Antioxidants that can act as pro-oxidants

2.4.1.1 Daidzein

Flavonoids are natural antioxidants. Daidzein is a flavonoid that belongs to the isoflavone subclass and is found in fruits, nuts and soybeans and is a well known antioxidant. However, in a recent study (Choi, 2006), it was reported that with chronic administration, daizein may act as

a pro-oxidant by decreasing GSH levels in rats. The author concluded that the excessive use of daidzein is not likely to produce beneficial effects and that a more critical consideration is warranted in high consumption.

2.4.1.2 Nordihydroguaiaretic acid

Nordihydroguaiaretic acid (NDGA) is a polyphenol present in the herbal product Chaparral, a product that is most commonly used for its antioxidant effects. In clone-9 rat hepatocyte cultures, NDGA has been shown to cause an increase in lipid peroxidation and DNA double strand breaks, the latter a marker for cell viability. These pro-oxidant effects where exhibited at a concentration range of 20-100uM, while concentrations of less than 10uM have previously been shown to be antioxidative in rat alveolar macrophages. The authors conclude that it would appear that the potential of NDGA to act as an antioxidant or pro-oxidant may be related to the concentration used (Sahu *et al.*, 2006).

2.4.1.3 Ascorbate

The antioxidant ascorbate has been shown in various studies to react as a pro-oxidant (Podmore *et al.*, 1998; Lee *et al.*, 2001). In the presence of metals, ascorbate may react to form •OH radicals and is used as a •OH generating system with Fe2+ and H_2O. The pro-oxidant abilities of ascorbate are concentration dependant. At low concentrations ascorbate acts to reduce transition metals and promote free radical formation, but at high concentrations ascorbate scavenges •OH and $O_2^{\cdot -}$. Ascorbate has also been reported to induce apoptosis in tumour cell lines. This ability has been attributed to ascorbate's capacity to act as a pro-oxidant (Rietjens *et al.*, 2002).

The well known antioxidant beta-carotene which is responsible for the production of retinol has also been found to exhibit pro-oxidant activity (Paolini *et al.*, 1999; Murata & Kawanishi, 2000).

2.5 CONCLUDING REMARKS

In our modern day environment, people are exposed to a variety of toxins, which are frequently potent oxidants. This, together with the fact that our diets are highly unbalanced and deprived of essential antioxidants, makes it clear that supplementation with antioxidants may be of great value in protecting against disease in which oxidative stress is implicated (Ratnam *et al.*, 2006). Nevertheless, it is now evident that over zealous use of antioxidants may have negative effects which suggest that the use of antioxidants for "protection" against possible illness linked to oxidative stress be viewed with caution. In the following section, one such anti-oxidant will be discussed in more detail, namely N-acetyl cysteine (NAC). NAC forms an integral part of this

study making it essential that the physico-chemical characteristics, and its physiological and pharmacological attributes be considered.

2.6 N-ACETYLCYSTEINE

2.6.1 Introduction

As has been mentioned in §2.3.2.1.2, glutathione plays a major role in protecting the body against ROS and subsequent oxidative stress. ROS and oxidative stress are implicated in a number of serious diseases, and substances that increase the levels of antioxidants, such as glutathione, may have clinical use in providing some protection against ROS mediated diseases. When considering central nervous system disorders of this nature, unfortunately supplementation with glutathione (GSH) is not viable since glutathione crosses the blood-brain barrier only poorly (Fukami *et al.*, 2004). Cysteine is generally regarded as the rate-limiting compound in the synthesis of glutathione (Powell *et al.*, 2001) (see § 2.3.2.1.2) However cysteine itself is not ideal for therapeutic use, as neural toxicity has been described with high doses (Fukami et al., 2004). For this reason, supplementation with a cysteine pro-drug that is easily absorbed and converted to GSH seems to be a logical alternative.

2.6.2 N-Acetylcysteine: Chemistry and Pharmacokinetics

N-acetylcysteine (NAC) is a thiol (sulfhydryl-containing) compound, with a molecular weight of 163.2 and the chemical formula $C_5H_9NO_3S$ (Kelly, 2005). The structure of NAC comprises the amino acid L-cysteine combined with an acetyl group that is attached to the amino group.

The addition of the acetyl group aids absorption and distribution of the compound upon oral administration (Best, 2006), while it also protects the molecule against oxidation. In gastric fluid most thiol-containing compounds are up to 75-100% oxidized, while the oxidation of NAC is restricted to only 16% (Kelly, 2005). Amino acids such as cysteine, which contain a sulphur group, are able to act as antioxidants, and NAC itself also displays antioxidative properties (Best, 2006). The sulfhydryl group (SH) is also responsible for most of the metabolic activity displayed by NAC (Kelly, 2005). The oral bioavailabilty of NAC is estimated at between 4 and 10% due to the majority of NAC being metabolized to other compounds. However, the conversion of NAC to these latter compounds account for most of the antioxidant activity of authentic NAC (Kelly, 2005). Thus, while NAC is readily absorbed after oral administration, extensive first pass metabolism by cells of the small intestine and liver results in the incorporation of NAC into protein peptide chains and the formation of a variety of metabolites of NAC. Only a small percentage of the intact molecule arrives in plasma and subsequently also in

tissues (Kelly, 2005). Peak concentration levels are reached 2-3 hours later and its half-life is approximately 6 hours (Best, 2006). NAC has been shown to increase the levels of cysteine (rate limiting compound in glutathione synthesis) and to preserve protein thiol levels (important antioxidants) in the brains of rats subjected to oxidative challenges (Karageorgos *et al.*, 2006) suggesting that NAC or its metabolites readily enters the brain and thus offering antioxidant protection against damaging free radicals. Numerous other studies also supports brain penetration of NAC (Christen *et al.*, 2001; Sekhon *et al.*, 2003; Cocco *et al.*, 2005) as well as the upregulation of both antioxidant enzymes and GSH in the brain (Farbiszewski *et al.*, 2000). These studies clearly support the use of NAC as a protective and beneficial agent when the brain is subjected to increased levels of free radicals and subsequent oxidative stress.

2.6.3 Indications for the pharmacological use of NAC

2.6.4 Primary indications for the use of NAC

2.6.4.1 Paracetamol Poisoning

NAC has been in clinical use since the 60's (Wong *et al.*, 2003) in toxicology for the treatment of paracetamol poisoning (Flanagan & Meredith, 1991). Paracetamol is metabolized in the liver, and the hepatorenal toxicity of paracetamol is mediated by a reactive metabolite normally detoxified by reduced glutathione (Flanagan & Meredith, 1991). This metabolite, n-acetyl benzoquinoneimine, reacts to deplete the hepatic glutathione pool (Ercal & Gurer-Orhan, 2002). If glutathione is depleted, covalent binding to macromolecules and/or oxidation of thiol enzymes can lead to cell death and liver damage (Flanagan & Meredith, 1991). NAC is able to act as a cysteine donor and replenish the depleted GSH levels. The recommended dosage for NAC in paracetamol poisoning is 140 mg/kg followed by 17 subsequent doses of 70 mg/kg every 4 hours (Ercal & Gurer-Orhan, 2002).

2.6.4.2 NAC as a Mucolytic Agent

NAC is also an effective mucolytic agent and is used regularly in the treatment of chronic bronchitis and several other pulmonary diseases, mainly as it is able to decrease the severity of cough and relieve diaphragm fatigue. NAC is able to break mucus down into smaller less viscous units. This is achieved by the sulfhydryl groups of NAC that are able to react and split disulfide bonds in bronchial mucus. Therefore NAC is often referred to as a 'slime loosener' (Ercal & Gurer-Orhan, 2002).

2.6.5 Other indications for the use of NAC

NAC has been found to be beneficial in a number of illnesses, including biliary obstruction (Pastor et al., 1997), diabetic associated myocardial dysfunction (Xia et al., 2006) and cocaine addiction (Rees, 2005). However, since the present study focuses on TD, the possible use of NAC in central nervous system disorders, particularly those linked to increased oxidative stress, are highlighted.

2.6.5.1 Parkinson's disease

Oxidative stress, as mentioned previously, has been implicated in the aetiology of PD. A recent study revealed that NAC protects mice against MPTP (1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine)-induced nigrostriatal dopaminergic toxicity in an animal model of PD by reducing the loss of striatal dopaminergic fibres (Park *et al.*, 2004). Since activation of c-Jun NH_2-terminal kinase (JNK)seems to play a role in the pathology of PD, pre-treatment with NAC was found to effectively prevent JNK translocation from cytoplasm to nucleus thereby effectively preventing MPTP-induced JNK activation and consequently apoptosis of nigral dopaminergic neurons (Park *et al.*, 2004).

2.6.5.2 Tardive dyskinesia

A recent study in rats (Sadan *et al.*, 2005) which was undertaken and published during the time of the current study, evaluated the effect of NAC-amide (1000 mg/kg/day) on the rat model of TD. The authors reported that NAC-amide administration significantly attenuated haloperidol induced VCMs, lipid peroxidation and protein oxidation.

2.6.5.3 Alzheimer's disease

The pathology of Alzheimer's disease is associated with the increased production of amyloid-β (Aβ) and the accumulation thereof in the form of senile plaques. Aβ has the ability to act act as a prooxidant (refer to §2.3.1.2.1) and accumulation Aβ and associated oxidative stress seem to play a causal role in Alzheimer's disease (refer to §2.3.1.2.1). In a resent study (Tucker *et al.*, 2006) the adiminstration of NAC to mice showed a significant anti-amyloid action. Furthermore, ApoE-deficient mice are a model for AD and the mice undergo increased oxidative neurodamage and cognitive decline when kept on a folate-free diet. The administration of NAC to these animals was able to decrease oxidative damage and cognitive decline as well as restoring glutathione synthase and GSH levels (Tchantchou *et al.*, 2005). In a controlled trail the adiminstration of NAC to AD patients also showed great promise (Adair et al., 2001).

2.6.6 Safety and Side Effects

As with most drugs and antioxidants the side effects of NAC administration are mostly dose related. The most frequently experienced side-effects include nausea, vomiting and other gastrointestinal disturbances. The intravenous administration of NAC used in paracetamol poisoning may in some cases cause allergic reactions such as rash and angioedema (Ercal & Gurer-Orhan, 2002) or even anaphylactic shock in extreme cases (Best, 2006). In chronic liver disease, the dosage of NAC may need to be carefully adjusted (Ercal & Gurer-Orhan, 2002).

2.6.7 NAC as an antioxidant

The antioxidant activity of NAC involves two mechanisms. Firstly NAC can act as a free radical scavenger and effectively scavenges hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), hydroxyl radicals (Wang *et al.*, 2006) as well as peroxyl radicals (Margaill *et al.*, 2005). Secondly NAC has the ability to increase intracellular GSH by acting as a precursor for the synthesis of GSH (Wang *et al.*, 2006) thus increasing the rate of endogenous glutathione synthesis (Margaill *et al.*, 2005).

2.6.8 NAC as a pro-oxidant

Even though NAC is primarily used for its antioxidant ability, NAC has the potential, as do most other antioxidants, to act as a pro-oxidant under specific conditions. A recent study (Wang *et al.*, 2006) showed that in acute ethanol-induced liver damage, pre-treatment with NAC is able to act as an antioxidant to protect against ethanol induced damage. However, these authors also reported that post-treatment with NAC aggravated the ethanol-induced hepatic lipid peroxidation and actually worsened acute ethanol-induced liver damage. They hypothesized that the local redox environment might influence the physiochemical properties of NAC. When administered before ethanol, the body is in a state of intracellular redox balance and NAC is able to behave as an antioxidant to attenuate ethanol-induced oxidative stress. However, when administered after ethanol, NAC may interact with ROS produced by ethanol and generate thiyl radicals which, in turn may elicit a pro-oxidant like function. Auto-oxidation of NAC is also promoted by the presence of metals and ROS such as H_2O_2, and once a thiol has undergone auto-oxidation it is unable to act as an antioxidant (Wang *et al.*, 2006).

Another study reported that NAC was able to induce DNA damage in cultured cell lines via the generation of ROS, thus acting as a pro-oxidant (Kawanishi *et al.*, 2005).

It has also been reported that in non-pathological states (healthy individuals), NAC can decrease the reduced form of glutathione (GSH) in favour of the oxidized form (GSSG), indicative of a pro-oxidant property. Given this phenomena, it has been recommended that NAC

not be given in conditions characterized by an absence or relative absence of significant oxidative stress (Ercal & Gurer-Orhan, 2002).

2.6.9 Conclusion

Supplementation with antioxidants (e. g. NAC), seems to be a viable approach in protecting against diseases in which oxidative stress and free radicals are implicated. Unfortunately, like many other antioxidants, NAC is able to act both as an antioxidant and pro-oxidant, depending on various biological conditions (Lee & Lee, 2006). Supplementation with NAC therefore needs to be carefully monitored and assessed in order to prevent the conversion of this protective antioxidant to a potentially damaging pro-oxidant. Since prevention against the later development of TD in patients receiving chronic neuroleptic teament for schizophrenia is a primary goal amongst clinicians, in the current study, careful consideration was afforded the question not only whether NAC could effectively reduce the symptoms of TD, as has recently been described (Sadan *et al.*, 2005), but also to consider its use in patients not suffering from TD but who are at risk due to their chronic use of a neuroleptic agent. Thus, its potential to act as either an antioxidant or pro-oxidant suggests that while NAC may react as a pro-oxidant in the absence of oxidative stress, this effect may be reversed under conditions of oxidative stress and as such may act as an antioxidant and hence improve the symptoms of the disorder.

Chapter 3 Materials and Methods

3.1 INTRODUCTION

With regard to diseases in which oxidative stress is increased, two types of intervention strategies may be considered: The first strategy is preventive, which consists of maintaining or restoring the normal antioxidant capacity in apparently healthy people *prior* to development of the particular disorder, and would include fortification, dietary supplements, modification of food composition etc. The second intervention can be seen as therapeutic, i.e. delivering antioxidant nutrient supplements in conditions caused or worsened by free radicals and/or when antioxidant levels have been depleted, in order to quench these radicals before further damage can be caused. In the aforementioned preventive strategy, oxidative stress and its associated tissue damage has not yet been established. Consequently, this approach holds great promise for neurodegenerative diseases in which prodromal oxidative stress is implicated in the neurodevelopment of the disorder with resulting permanent tissue/neuronal damage following directly from increased presence and action of these free radicals.

However, as has been emphasised in the afore going chapter, antioxidants may act as a two-edged sword depending on the redox milieu in which the particular antioxidant is applied. The primary aim therefore of this study was to evaluate the efficacy of the antioxidant, NAC, to prevent the bio-behavioural changes evoked by chronic haloperidol treatment using an animal model of TD, a neurological disorder evoked by long-term neuroleptic administration and causally linked to increased oxidative stress, particularly in the striatum. Moreover, the study also sought to establish the behavioural and neurological effects of NAC alone in the *absence* of neuropathology associated with oxidative stress.

3.2 MATERIALS

Haloperidol; glacial acetic acid; 2,6-di-tert-butyl-4-metylphenol (BHT); nitro blue diformazan (NBD); 1,1,3,3-tetramethoxypropane (TEP); 2-thiobarbituric acid (TBA); nitro blue tetrazolium (NBT); iodoacetic acid, 5-sulfosalicylic acid, ammonium bicarbonate, GSH, GSSG, bradford reagent; bovine serum albumin (BSA) and trichloroacetic acid (TCA) were obtained from Sigma Chemicals. St. Louis, USA. Butan-1-ol; ethanol, methanol, formic acid and concentrated

ammonia were obtained from Merck Chemicals. Darmstadt, Germany. Gabapentin was obtained from Godecke A.G. Freiburg, Germany. Acetonitrile was obtained from HiPerSolve, BDH, Poole, England.

3.3 METHODS

3.3.1 Animals

The study protocol was approved by the Ethics Committee (Medical) (Evaluation Subcommittee for Experimental Animals) of the North West University, Potchefstroom Campus (Ethics Number: 05D19). Male Sprague-Dawley rats, initially weighing 160-190g were used in the study. The rats were kept in the animal research centre of the North West University, Potchefstroom Campus. They were housed in identical cages, 6 rats per cage, and under constant temperature (21 ± 5°C), humidity (50 ± 10%) and light conditions (12 hour light/dark cycle, with lights on at 06:00). Free access to food and water was allowed.

3.3.2 Drug Treatment

3.3.2.1 NAC Dosing

In various studies in which NAC was used to asses its antioxidant properties, the drug was administered orally, either by mixing it with drinking water or food (Cocco *et al.*, 2005; Patriarca *et al.*, 2005; Ronis *et al.*, 2005; Sadan *et al.*, 2005). However, these methods of administering NAC are dependant on numerous factors which may influence the amount of actual NAC intake. Since the current study aimed to evaluate the differential effects of different doses of NAC, it was essential to ensure that accurate and controlled amounts of NAC were administered to each animal. Therefore NAC was administered by gastic gavage using a 1 ml syringe with a stainless steel bird needle (18 g/80 mm). Moreover, this method of administering NAC was also selected given that haloperidol would be administered via intra-peritoneal injection (§ 3.3.2.2). Administering both agents via the intra-peritoneal route would have caused undue discomfort for the animal and possibly injection stress.

As mentioned earlier (§ 2.6.2) NAC is readily absorbed after oral administration, although it is also extensively metabolized to various other compounds, reducing the bioavailability thereof to only 4 – 10%. However, much of NACs antioxidant ability has been attributed to the formation of these other compounds (Kelly, 2005). Studies showing the effect of NAC on brain oxidative status (refer to § 2.6.2) clearly indicates that NAC or its metabolites penetrates the blood brain barrier and offers protection against oxidative stress.

Three different dosages of NAC were selected to constitute a low, medium and high dose. This was done since dual anti-and pro-oxidative actions of antioxidants have been noted to be dose dependent (as described in § 2.4.1), and such an approach would thus assist in assessing whether any apparent pharmacological effects elicited by NAC displayed dosage dependency. Following a thorough search of the literature, no previous studies using direct gastric administration of NAC could be found. However, doses given orally included 200 mg/kg (Koros *et al.*, 2007), 1.5g/kg (Song *et al.*, 2005), 1000 mg/kg/day (Sadan *et al.*, 2005), as well as a diet containing 100 mg NAC per 100g food (Yang *et al.*, 2006). Given these doses and after personal communication with the University of Melbourne (Prof. Michael Berk), it was decided that the following doses would be applied in the dose ranging study, namely 10 mg/day, 100 mg/day and 300 mg/day to constitute a low, intermediate and high dose of NAC.

The NAC was dissolved in water, and rats received 10 mg NAC / 0.5ml water, 100 mg NAC / 0.5ml water or 300 mg NAC / 0.5ml water each day for 21 days. The solutions were prepared fresh each day. When the weight of each rat was factored in, the doses of NAC were as follows: 10 mg/day NAC = 50 mg/kg/day NAC; 100 mg/day = 500mg/kg/day; 300 mg/day = 1500 mg/kg/day.

3.3.2.2 Neuroleptic Dosing

Haloperidol was administered to the animals via intra-peritoneal injection (i.p.) at a dose of 1.5 mg/kg per day for 21 days. This regime has been previously used when establishing the rodent model of TD (Kelley *et al.*, 1997; Sumiyoshi *et al.*, 1997; Chakos *et al.*, 1998), and was subsequently also chosen for the current study.

Haloperidol was dissolved in a minimal amount of glacial acetic acid, and then diluted with double distilled water to a concentration of 1 mg/ml. The solution was buffered with NaOH (pH=7.0). The stock solution was freshly prepared every week, and stored in bottles covered with aluminium foil (haloperidol is light sensitive).

3.3.3 Study Design

Three different treatment regimes were designed:

3.3.3.1 Establishment of a rat model of TD

The first regime was set up in order to establish an animal model of TD and to further evaluate the effect of haloperidol administration on oxidative stress markers, especially since haloperidol in widely known to increase oxidative stress in the rat brain, see § 2.1.3. The group assignments in this phase of the study are presented in Table 3-1:

Table 3-1: Group assignments for the establishment of a rat model of TD

Group	Treatment	Duration
1	Water p.o + Haloperidol Vehicle (Control)	21 days
2	Water p.o + Haloperidol 1.5 mg/kg	21 days

- **Group 1:** The control group remained drug free and received only water p.o (by means of direct gastric administration) and a daily i. p. injection of haloperidol vehicle (glacial acetic acid / water solution, buffered with NaOH; pH=7.2), for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 2:** This group received only water p.o (NAC vehicle) and a daily i. p. injection of haloperidol (1.5mg/kg/d ip), for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

3.3.3.2 The effects of NAC in a non-pathological state

In the next regime, different doses of NAC were administered to animals in the absence of haloperidol administration in order to evaluate the effect of NAC in the absence of oxidative stress, i.e. in healthy animals. This was done in order to evaluate the effect of NAC as a possible preventative agent. The group assignments in this phase of the study are presented in Table 3-2:

Table 3-2: Group assignments for the evaluation of the effects of NAC in a non-pathological state

Group	Treatment	Duration
3	Water p. o.	21 days
4	NAC 10 mg/day p. o.	21 days
5	NAC 100 mg/day p. o.	21 days
6	NAC 300 mg/day p. o.	21 days

- **Group 3:** The control group remained drug free and received only water p.o. for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 4:** This group received 10 mg/day. NAC p.o.(by means of direct gastric administration) for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 5:** This group received 100 mg/day (by means of direct gastric administration) NAC p.o. for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 6:** This group received 300 mg/day (by means of direct gastric administration) NAC p.o. for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

3.3.3.3 The effects of NAC in a pathological state

The final regime was set up to evaluate the effect of the above-mentioned doses of NAC on haloperidol-induced behavioural and neurochemical changes as part of an established TD model. This was done in order to establish whether NAC is able to reverse the effects of haloperidol on behavioural and neurochemical markers of oxidative stress.

Table 3-3: Group assignments for the evaluation of the effects of NAC in a pathological state

Group	Treatment	Duration
7	NAC 10 mg/day p.o + Haloperidol 1.5 mg/kg	21 days
8	NAC 100 mg/day p.o + Haloperidol 1.5 mg/kg	21 days
9	NAC 300 mg/day p.o + Haloperidol 1.5 mg/kg	21 days

- **Group 7:** This group received 10 mg/day NAC p.o. and a daily i. p. injection of haloperidol – 1.5mg/kg/day, for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 8:** This group received 100 mg/day NAC p.o. and a daily i. p. injection of haloperidol – 1.5 mg/kg/day, for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

- **Group 9:** This group received 300 mg/day NAC p.o. and a daily i. p. injection of haloperidol – 1.5mg/kg/day, for 21 days. VCMs were rated on days 0, 7, 14, 17, 19 and 21. After the final rating session on day 21, animals were sacrificed by decapitation.

3.3.4 Behavioural Evaluation

Various animal models have been developed to study the behavioural and pathophysiological processes underlying TD, and the vacuous chewing movement (VCM) model has been widely used for this purpose. Long-term treatment with neuroleptics has been reported to induce spontaneous oral movements in rats (Levin *et al.*, 1987), collectively referred to as VCMs. "VCMs are usually characterized by purposeless mouth openings in the vertical plane, with or without tongue protrusion" (Andreassen & Jorgensen, 2000).

The rat model resembles human TD in several ways (the orofacial type) but is considered an analogous model since it reproduces only some of the essential features of the clinical syndrome (Casey, 1999). Just as in humans, rats that are chronically treated with typical neuroleptics develop abnormal, spontaneous and involuntary movements of the orofacial area (Levin *et al.*, 1987). Atypical neuroleptics possess a significantly lower potential for inducing TD (Barak *et al.*, 1995; Wittenberg *et al.*, 1996), which is also reproduced by the production of significantly less VCMs in rats (Nel, 2002). In humans, a subpopulation seems to be more at risk to develop the condition; the same applies to rats, since not all of the animals develop these abnormal oro-facial movements, as was also found in the current study. Advancing age seems to be a major risk factor for the development of TD in humans (Goldberg, 2002; Miller *et al.*, 2005), and an increase in VCMs related to aging has also been found in rats (Harvey & Nel, 2003). TD symptoms have been shown to increase with cumulative neuroleptic exposure (Miller *et al.*, 2005) and the same is true for VCMs in rats (Turrone *et al.*, 2005). Persistent high levels of VCMs have been found in rats treated with neuroleptics, after drug-withdrawal (Andreassen *et al.*, 1999) and is in accordance with studies showing persistent TD symptoms even after cessation of neuroleptic treatment in TD patients (Jeste *et al.*, 1979).

3.3.4.1 Vacuous Chewing Movement Rating

The method for VCM rating followed was based on that described earlier (Harvey and Bester, 2000) which was validated for our laboratory conditions by Bester (1999), with minor modifications. VCM rating sessions were held between 8 a.m. and 1 p.m. in the same room in which the animals were housed. Rats were placed in a raised Perspex cage (34 x 18 x 24 cm), and the raters were able to observe the animals at all times. The same raters were used throughout the study. In addition to normal behaviour such as grooming and rearing, the following were recorded as VCMs: single chews, chew bursts (unseparated chews), tongue

protrusions and jaw tremors. VCMs were determined over a 2 minute period after a habituation period of 5 minutes. This behavioural assessment was carried out on days 0, 7, 14, 17, 19 and 21.

3.3.5 Neurochemical Assays

Animals were sacrificed by decapitation. The brains were then immediately dissected on an ice-cooled slab. The striata were removed, and immediately fixed in liquid nitrogen. The striata were then stored in the -72°C freezer, until the day of assay.

3.3.5.1 Preparation of Brain Homogenate (for superoxide and lipid peroxidation assays)

Preparation of PBS:

To prepare 2.5 litres of a 100x strength solution:

Weigh 200g NaCl, 5g KCl, 22.5g Na_2HPO_4 and 5g H_2PO_4.Add to 2.5 litres of double distilled water.

On the day of the assay, dilute 1 part PBS (100x) to 9 parts with double distilled water before use.

Preparation of brain homogenate:

On the day of assay, the striata were removed from -72°C storage and allowed to thaw on ice. The striata were weighed and then homogenized to prepare a 10% tissue homogenate with PBS, using a Teflon homogenizer.

Protein Determination

Protein determination of brain homogenate carried out on the day of superoxide and lipid peroxidation assays, was based on the Bradford method (Bradford, 1976).

- The Bradford reagent was gently shaken in the bottle; a sufficient amount was withdrawn and allowed to reach room temperature in a dark environment.
- Protein standards were prepared by dissolving 5 mg bovine serum albumin (BSA) in 1 ml double-distilled water (to produce a 5 mg/ml solution) before making a series of 100 µl dilutions as indicated in Table 3-4:

Table 3-4: Protein concentration dilutions

Protein concentration	Dilution in test tubes	
	Volume of 5 mg/ml BSA	Volume of PBS buffer
0 mg/ml	0 µl	100 µl
0.5 mg/ml	10 µl	90 µl
1.0 mg/ml	20 µl	80 µl
1.75 mg/ml	35 µl	65 µl
2.5 mg/ml	50 µl	50 µl
3.5 mg/ml	70 µl	30 µl
5.0 mg/ml	100 µl	0 µl

- 3 × 5 µl of each dilution, as well as the brain homogenate, was added to separate wells of a 96-well plate, i.e. all in triplicate.
- 250 µl of Bradford reagent was added to each well, and immediately shaken on the mixing facility of the plate reader for 30 seconds. The plate was incubated for 15 minutes at room temperature.
- The absorbance in each well was determined in a 96-well plate reader using a 560 nm filter. The protein concentration of the brain homogenate was then calculated from the plotted net absorbance against protein concentration of the standards.

3.3.5.2 The Measurement of Lipid Peroxidation Using the Thiobarbituric Acid (TBA) Assay (Butanol Extraction).

3.3.5.2.1 Background

The thiobarbituric acid (TBA) assay is currently the most widely used assay in use as an index of lipid peroxidation (Gutteridge & Halliwell, 1990), and is based on the reactivity of malondialdehyde (MDA), a colourless end product of lipid peroxidation, with TBA (Garcia *et al.*, 2005). The method used for the evaluation of lipid peroxidation in the current study was carried out as described previously (Ottino & Duncan, 1997). At low pH and high temperatures MDA

reacts with TBA in a nucleophilic addition reaction, generating a red, fluorescent 1:2 MDA:TBA product (Ohkawa *et al.*, 1979) that is extracted with butanol and the absorbance read at 532nm (Garcia *et al.*, 2005). Tetramethoxy-propane is used as a standard and expressed as nmol MDA (Ohkawa *et al.*, 1979). Final results are expressed as pmol/mg protein.

3.3.5.2.2 Validation of the TBA assay

For the validation of the TBA assay fresh brain homogenate of healthy animals were used. The method used is described in § 3.3.5.2.5 and the calibration curve in § 3.3.5.2.4. The only difference is that three different temperatures were used, namely 60, 80 and 95°C. A temperature of 60°C was not a sufficiently high temperature and no reaction was observed. A temperature of 95°C proved to be too hot and the protein was compromised. However, a temperature of 80°C provided sufficient heat for the reaction to take place without any negative effects on the protein. Figure 3-1 presents the results of an example of a calibration curve at 80°C:

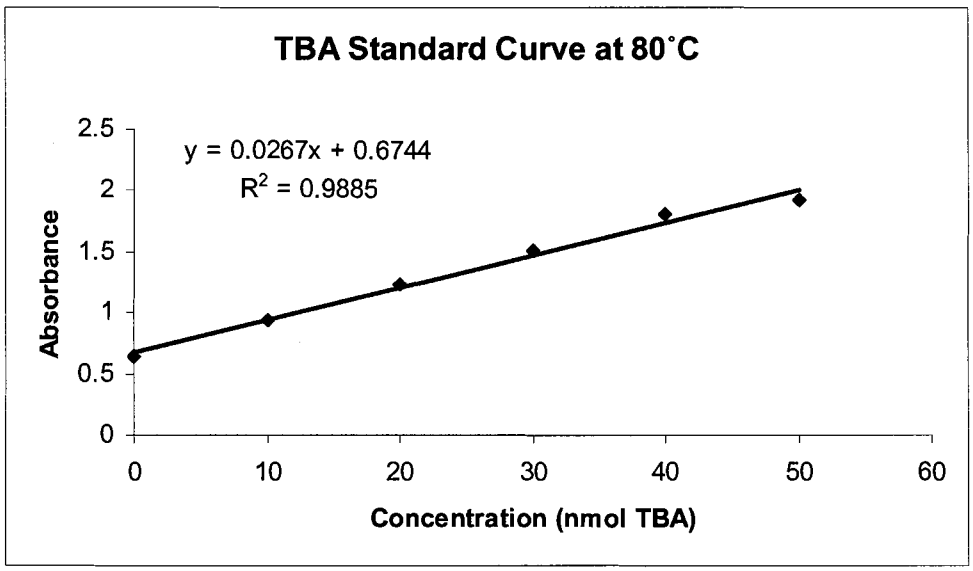

Figure 3-1: Validation of the TBA assay: An example of a typical calibration curve at 80°C.

The next part of the validation process used different concentrations of brain homogenate. A 5%, 10% and 15% homogenate was used, and such results are presented in Figure 3-2:

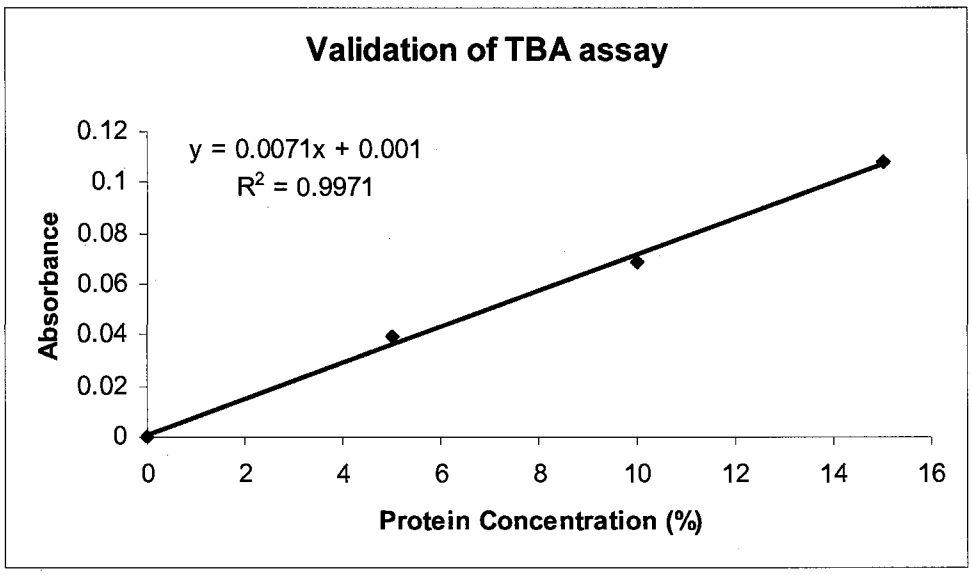

Figure 3-2: Validation of the TBA assay: Absorbance increases linearly with increasing protein concentrations.

It is evident in Figure 3-2 that increasing protein concentrations is associated with a linear increase in absorbance with no substrate inhibition at any of the studied protein concentrations. A 10% protein concentration was chosen for this assay throughout the study.

3.3.5.2.3 Reagents:

- Tetramethoxypropane (TEP)

Remove 82µl TEP and add to 10ml PBS. Remove 10µl from this solution and add to 10ml PBS.

- Trichloraceticacid (TCA)

Add 10mg TCA to 100ml water to yield a 10% solution.

- 2,6-Di-tert-butyl-4-methylphenol (BHT)

Add 0.05g to 100ml methanol to yield a 0.5 g/L solution.

- Thiobarbituric acid (TBA)

Add 0.033g to 10ml water to yield a 0.33% solution. The solution is light sensitive, therefore cover with aluminium foil.

3.3.5.2.4 Calibration Curve

Table 3-5: Dilutions for the calibration curve

Concentration	Dilution in test tubes	
	Volume TEP	Volume of PBS buffer
0 nmol/L	0.0 ml	1.0 ml
10 nmol/L	0.2 ml	0.8 ml
20 nmol/L	0.4 ml	0.6 ml
30 nmol/L	0.6 ml	0.4 ml
40 nmol/L	0.8 ml	0.2 ml
50 nmol/L	1.0 ml	0.0 ml

- The dilutions as shown in Table 3-5.
- Test tubes were vortexed. 0.5 ml BHT and 1ml TCA were added to test tubes and vortexed.
- Remove 0.5ml, left with 2ml.
- 5 ml TBA was added to test tubes and vortexed.
- Test tubes were incubated at 80°C for 1hour.
- After 1 hour, test tubes were allowed to cool on ice.
- 2ml Butanol was added to test tubes and vortexed.
- The test tubes was the centrifuged at 2000 x g for 5 minutes at room temperature.
- 250 µl of the supernatant (in triplicate), was added to separate wells of a 96-well plate and the absorbance read at a wavelength of 530nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer.

3.3.5.2.5 Determination of lipid peroxidation in brain homogenate.

- 1ml of brain homogenate was added to each test tube and vortexed.
- 0.5 ml BHT and 1 ml TCA was added to each test tube and vortexed. BHT is a chain-breaking antioxidant that was included to suppress peroxidation that occurs during the assay itself (Lykkesfeldt, 2001).
- Test tubes were then incubated at 80°C for 15 minutes in order to release protein bound to MDA.
- Test tubes were cooled on ice and then centrifuged at 2000 x g for 20 minutes at 4°C in order to remove insoluble proteins.
- 2 ml of the supernatant was then added to new test tubes and 0.5 ml TBA added, after which the test tubes were vortexed.
- Test tubes were incubated at 80°C for 1 hour.
- After 1 hour the test tubes were cooled on ice and 2 ml butanol added and vortexed.
- The test tubes was the centrifuged at 2000 x g for 10 minutes at room temperature.
- 250 µl of the supernatant (in triplicate), was added to separate wells of a 96-well plate and the absorbance read at a wavelength of 530nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer.
- The concentration of MDA in the brain homogenate was then calculated from the plotted net absorbance against MDA concentration of the standards.

3.3.5.3 Superoxide Radical Formation Assay

3.3.5.3.1 Background

A method that was previously described (Ottino & Duncan, 1997) was used for the evaluation of striatal superoxide radical formation. The assay is based on the ability of superoxide radicals to reduce nitro blue tetrazolium (NBT; yellow) to nitro blue diformazan (NBD); (Choi *et al.*, 2006), of which the absorbance is then measured at 560 nm and converted to µmol diformazan using a standard curve generated from NBD. Final results are expressed as µmol/mg protein (Pillay *et al.*, 2003).

3.3.5.3.2 Validation of the NBT assay

For the validation of the NBT assay fresh brain homogenate of healthy animals were used. The method used is described in § 3.3.5.3.5 and the calibration curve in §.3.3.5.3.4. Different protein concentrations (5%; 8% and 12%) were used as well as different concentrations of substrate, NBT (0.025; 0.03; 0.035; 0.04 µmol). The results of this validation are described in Figure 3-3.

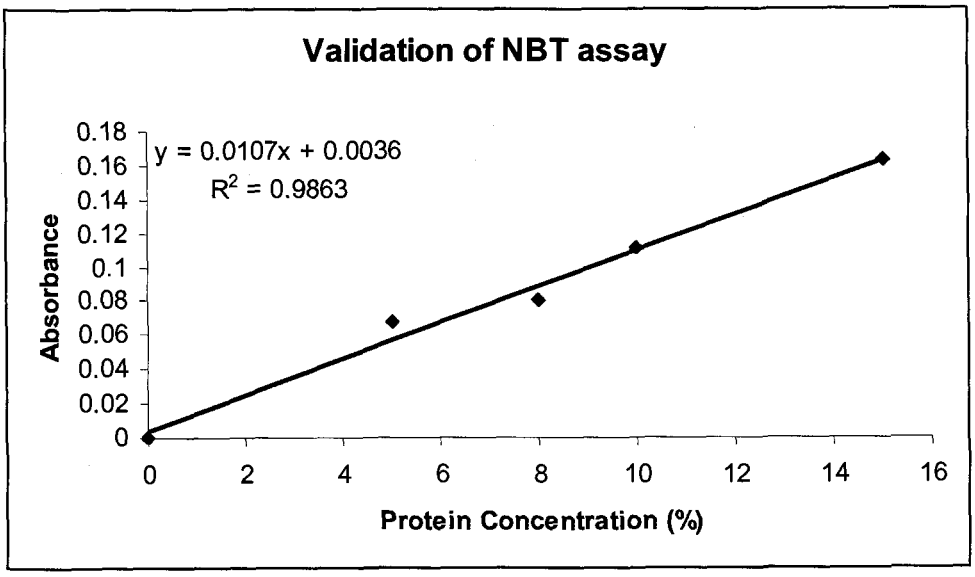

Figure 3-3: Validation of the NBT assay. Absorbance increased linearly with increasing protein concentrations.

As is evident in Figure 3-3, increased protein concentrations were associated with a linear increase in absorbance. A 10% protein concentration was chosen for this assay throughout the remainder of the study.

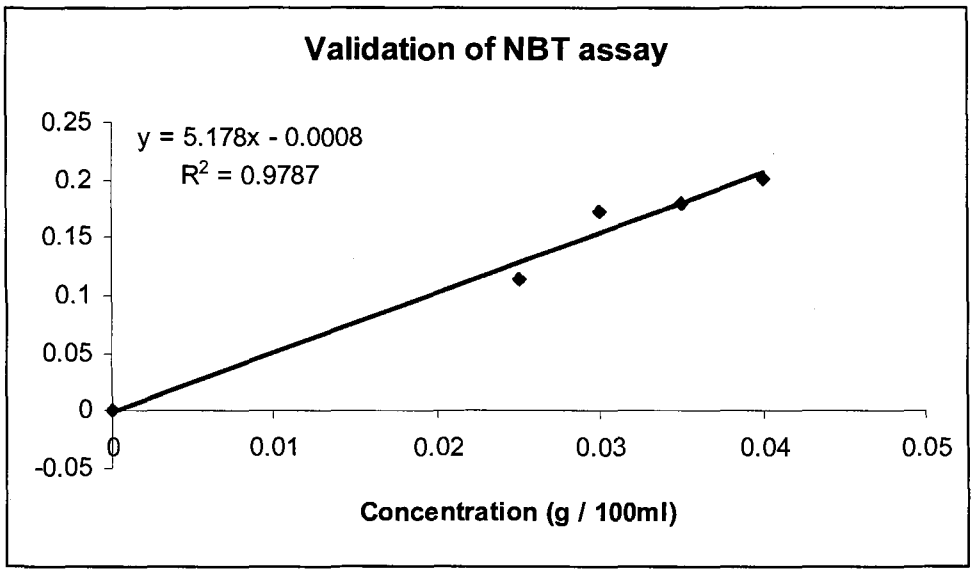

Figure 3-4: Validation of the NBT assay. Absorbance increased linearly with increasing NBT concentrations.

As can be seen from Figure 3-4, absorbance increased linearly with increasing NBT concentrations. The reaction remained linear for the entire concentration range used here. 0.03% NBT solution was chosen for all subsequent assays during the study.

3.3.5.3.3 Reagents:

- Nitro blue Diformazan (NBD):

400 μM/L concentration needed. Therefore, add 0.03g NBD and made up to 100ml with glacial acetic acid.

- Nitro blue Tetrazolium (NBT):

Add 0.005g NBT to 0.1ml ethanol and 4.9ml water.

3.3.5.3.4 Calibration Curve

Table 3-6: Dilutions for calibration curve.

Concentration NBD	Dilution in test tubes	
	Volume NBD	Volume GAA
0 µM	0.0 ml	2.0 ml
100 µM	0.5 ml	1.5 ml
200 µM	1.0 ml	1.0 ml
300 µM	1.5 ml	0.5 ml
400 µM	2.0 ml	0.0 ml

Note: NBD denotes nitro blue diformazan and GAA denotes glacial acetic acid

- Standards were prepared as in Table 3-6.
- 250 µl of standards (in triplicate) was added to separate wells of a 96-well plate and the absorbance read at a wavelength of 560nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer.

3.3.5.3.5 Determination of superoxide radical formation in brain homogenate

- 1ml of brain homogenate was added to each test tube and vortexed.
- Add 0.4 ml NBT to each test tube and vortex.
- Centrifuge test tubes for 10 minutes at 3000 x g at room temperature.
- Liquid was thrown out and the pellet resuspended with 2 ml GAA and vortexed.
- Test tubes were then centrifuged again for 5 minutes at 3000x g at room temperature.
- 250 µl of the supernatant (in triplicate), was added to separate wells of a 96-well plate and the absorbance read at a wavelength of 560nm using a Spectronic 20 (Bausch and Lomb) spectrophotometer.

3.3.6 Liquid chromatography / mass spectrometry (LC/MS) determination of reduced (GSH) and oxidized (GSSG) glutathione

GSH and GSSG in rat striata were analysed utilising the chromatographic method of Bouligand (Bouligand *et al.*, 2006). In the assay derivatization of thiols is achieved with iodoacetic acid after which proteins are precipitated using sulfosalicylic acid. Thereafter, both reduced (GSH) and oxidized (GSSG) glutathione is simultaneously measured. The quantity of GSH and GSSG present in the striata of the rats, as obtained directly from this assay, was expressed in terms of nmol/mg brain tissue, whereafter the GSH:GSSG ratios were calculated.

3.3.6.1 Chromatographic Conditions

3.3.6.1.1 Analytical instrument

For this study, an HP1100 series HPLC with a binary gradient pump, auto-sampler and vacuum degasser coupled to an Applied Biosystems API 2000 triple quadrupole mass spectrometer was used to determine striatal levels of GSSG and GSH. Analyst 1.4 data acquisition and analysis software was used for analysis of the data.

3.3.6.1.2 Column

Gemini C18 column, 150 x 2 mm, 5µm (Phenomenex, Torrance, CA)

3.3.6.1.3 Mobile Phase:

A: 0.1% formic acid in water

B: 0.1% formic acid in acetonitrile

A flow rate of 250 µl/min was used, with an injection volume of 10 µl.

3.3.6.1.4 Gradient

Table 3-7: Gradient

Time (minutes)	% A	% B
0	100	0
2	100	0
6	0	100
10	0	100
10.5	100	0
20	100	0

3.3.6.2 Mass Spectrometer (MS) Settings

For the purposes of this study, the MS was set at the following: Atmospheric pressure electron ionisation (Turbo ion spray source), positive ion mode, multiple reaction monitoring (MRM) scan. The following ion pairs were used as quantifier and qualifier respectively:

GSH: 366.1/134.2, 366.1/83.9

GSSG: 613.21/231.0, 613.21/177.1

Gabapentin (internal standard) 172.04/137.2

Declustering potential 131 V

Focusing potential 200 V

Entrance potential 3 V

Curtain gas 20

Ion spray voltage 5000 V

Temperature 250 °C

Ion source gas 1 20

Ion source gas 2 20

Reduced glutathione was derivatised to form the carboxymethyl derivative prior to determination to prevent oxidation to GSSG during sample preparation. The molecular mass of the carboxymethyl derivative is 365.2 compared to the molecular mass of 307.3 of reduced glutathione.

3.3.6.3 Sample Preparation

The striatum was accurately weighed and placed into a 1.5 ml eppendorf tube. 300 µl of the derivatisation solution was added together with 25 µl internal standard (200 ng/ml of gabapentin), vortexed and sonicated for 10 seconds. This was left on ice for 15 minutes. 150 µl of sulfosalicylic acid solution (10% w/v) was then added to precipitate proteins, followed by vortexing for about 15 seconds before being centrifuged for 2 minutes at 10 000 rpm. The supernatant was then placed in 200 µl inserts and analysed.

3.3.6.4 Derivatisation Solution

The derivatisation solution consisted of 10mM iodoacetic acid in 10mM ammonium bicarbonate and 0.5% concentrated ammonia solution (pH 9.5).

3.3.6.5 Statistical Analysis of Data

For behavioural data (i. e. measurement of VCMs) observations were determined individually for each rat. For analysis of superoxide radicals and lipid peroxidation one brain sample was used for both assays. For the glutathione assay, one brain sample was used for each assay.

Graph Pad Prism® (Version 4.01 for Windows®) and StatSoft, Inc: Statistica Data Analysis Software System® (Version 7) was utilized for statistical analysis of data. For comparison of two values, the nonparametric Student's *t* test (two-tailed) was implemented. For multiple comparisons the one-way ANOVA comparison was performed followed by either the Dunnett's post-hoc test (for comparing experimental groups to the control) or the Tukey–Kramer post-hoc test (for comparing experimental groups to each other). For all reported statistical probability values, $p<0.05$ was regarded as statistically significant.

Chapter 4 Results

4.1 INTRODUCTION

Various clinical (Galecki *et al.*, 2005) and pre-clinical (Sadan *et al.*, 2005) studies have provided evidence for the involvement of oxidative stress in TD, while there are some studies that have proposed the possible value of using antioxidants in the treatment of TD (Adler *et al.*, 1998). Despite a clear rationale for using antioxidants as preventatives in patients on long-term neuroleptic treatment, this has not proven to be irrefutably effective (Mahadik *et al.*, 2001). The objectives of this study are centered around the possible use of the anti-oxidant NAC in the treatment of TD using an animal model of TD that has been previously validated in our laboratory (Bester & Harvey, 2000; Harvey & Bester, 2000; Harvey & Nel, 2003; Nel & Harvey, 2003). Moreover, in lieu of recent evidence of a pro-oxidant action for anti-oxidant (see § 2.4.1) this question will also be addressed in a non-pathological model where NAC was administered over a set dosage range to healthy animals, and in this manner address a concern regarding the use of NAC as a preventative treatment in the absence of oxidative stress. Dose ranging studies for both NAC alone and in the TD model were employed since antioxidants may show dose-dependent effects (refer to section 2.4.1). Striatal oxidative stress was assessed by determining striatal levels of lipid peroxidation and superoxide as well as oxidized (GSSG) versus reduced (GSH) glutathione concentrations. Orofacial dyskinesia were used as a behavioral expression of striatal toxicity (Marchese *et al.*, 2002; Kelley & Roberts, 2004).

Rats initially weighing 160-190 g were monitored under standard housing conditions as described in § 3.3.1.

VCMs were rated and recorded as described in § 3.3.4. After 21 days of treatment rats were sacrificed by decapitation the striata dissected out and fixed in liquid N_2 and stored at -80° C (§ 3.3.1).

On the day of assay, tissue were removed from the freezer and thawed on ice.

The objectives of the study can be summarized as follow, with the results to follow presented in a likewise sequential manner:

- Establishing the rat model of TD, with regard to its effects on behaviour and markers of oxidative stress in the striatum. Oxidative stress has been strongly implicated in the pathology of TD, therefore the current study aimed to investigate whether the free radical, superoxide may be implicated in a rodent model of this disorder. The effect of chronic haloperidol administration on lipid peroxidation, a widely used measure of oxidative stress and free radical induced damage, as well as striatal superoxide levels, was determined. Finally, striatal levels of oxidized (GSSG) versus reduced (GSH) glutathione were determined.

- Evaluating the effect of chronic NAC administration on behavioural and neurochemical markers of oxidative stress in healthy, non-pathological rats. In this study the potential value of NAC as a preventative agent was evaluated. As with most other antioxidants, NAC has the ability to act as both an antioxidant and pro-oxidant under various conditions such as different doses and the presence or absence of oxidative stress. For this reason differential NAC doses was used in order to more closely evaluate the effects of NAC alone. As above, the effect of NAC administration on lipid peroxidation, superoxide levels and striatal levels of oxidized (GSSG) versus reduced (GSH) glutathione were determined.

- Evaluating the effect of NAC on behavioural and striatal neurochemical markers of oxidative stress in an animal model of TD. In this study NAC and haloperidol was co-administered. The neurotoxic effects of haloperidol have been shown to be related to increased free radicals and oxidative stress. In accordance with the proposed free radical hypothesis of TD, by attenuating these free radicals it may be able to attenuate the associated behavioural and neurological effects of haloperidol treatment. To this end NAC, a well known antioxidant and glutathione precursor was co-administered with haloperidol in order to quench the potentially harmful free radicals generated by haloperidol administration. Since NAC may have reciprocal actions under varying conditions of dose and the presence or absence of oxidative stress, different NAC doses was used in order to more closely evaluate the effects of NAC treatment on the effects of haloperidol. Consequently, the effect of NAC administration on haloperidol-associated effects on lipid peroxidation,

superoxide levels and striatal levels of oxidized (GSSG) versus reduced (GSH) glutathione were determined.

The average weight of the rats in the current study was 230 g. Rats receiving 10 mg/day NAC thus received an average of 43 mg/kg/day NAC while the 100 mg/day and 300 mg/day dosage groups received an average of 434 mg/kg/day and 1304 mg/kg/day respectively.

4.2 ESTABLISHING THE TD MODEL: AN *IN VIVO* ANIMAL MODEL OF STRIATAL OXIDATIVE STRESS

In this series of experiments, the ability of chronic haloperidol treatment to evoke striatal oxidative stress in rats was verified by behavioural and neurochemical analyses. This sought to establish an animal model of TD for later use in the NAC studies as an *in vivo* animal model of striatal oxidative a stress.

4.2.1 Behavioural Effects

As depicted in Figure 4-1, chronic haloperidol treatment produces significantly more VCMs compared to control (3.60 ± 0.22 versus 7.64 ± 0.35; $p<0.0001$). These results are expressed as the average of the cumulative values of the 5 rating sessions per animal on days 7, 14, 17, 19 and 21, amounting to 120 readings per group consisting of 24 animals each.

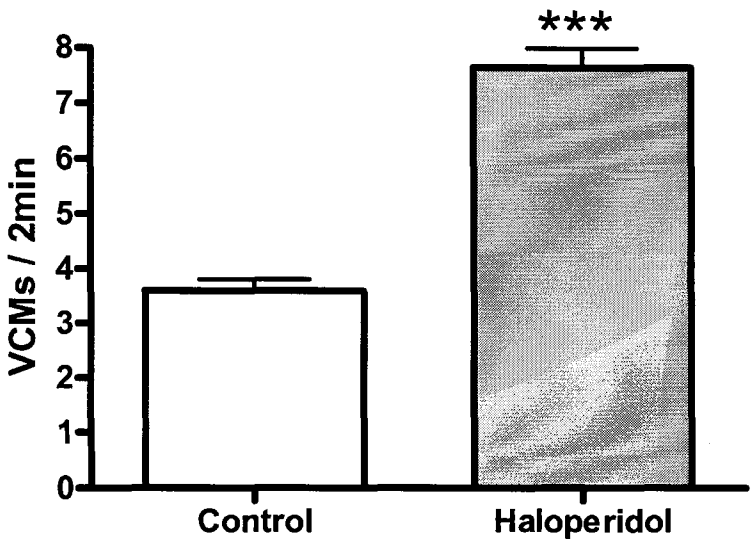

Figure 4-1: The effect of chronic haloperidol treatment (1.5 mg/kg/) and vehicle for 3 weeks on VCMs. The results are expressed as the mean of 5 individual rating sessions on days 7, 14, 17, 19 and 21 on 24 rats in each session. *** $p<0.0001$ (Student *t*-test); n = 120 for all groups.

In order to more accurately analyse the development of striatal toxicity over time compared to control, VCMs for the two groups (24 animals/group) were scored at 6 time points during treatment (baseline or zero time now included). These results are presented in Figure 4-2.

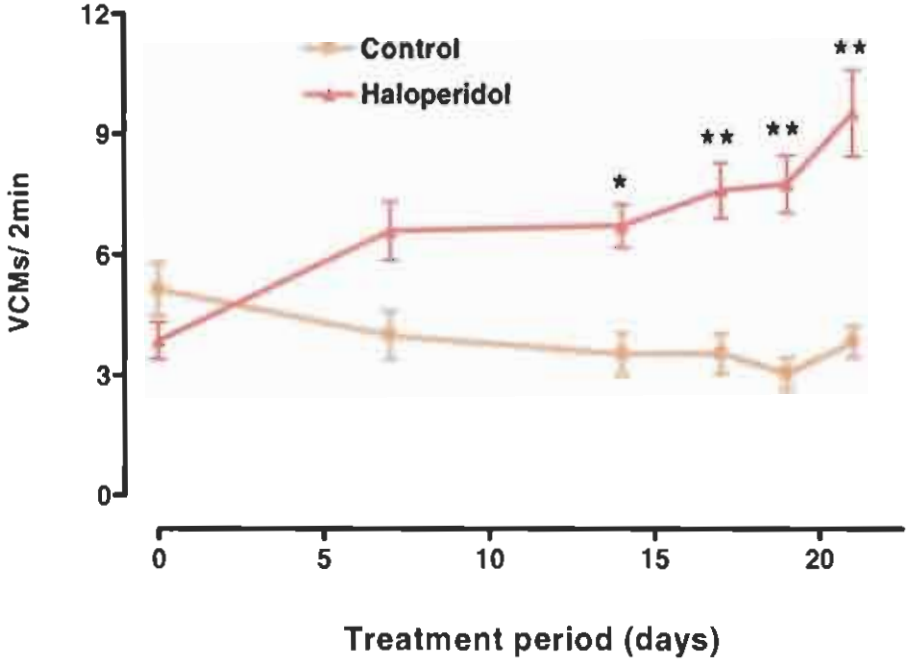

Figure 4-2: The time-dependant effect of chronic haloperidol treatment (1.5 mg/kg/day) and vehicle on VCMs during a treatment period of 3 weeks in rats. The results are expressed as the average number of VCMs during a 2 minute rating session (± SEM) on days 0, 7, 14, 17, 19 and 21. * $p<0.05$; ** $p<0.001$ (One-way ANOVA followed by a Dunnett's post-test). n = 24 for all groups.

One way ANOVA analysis of these data revealed significant differences across the groups [$F(4,715) = 21.03$; ($p<0.0001$)]. Subsequent post-hoc analyses with the Dunnett's test indicated the following: Haloperidol treatment produced a numerical increase in VCMs in the first week of the treatment period compared to control animals. This increase reached statistical significance during the second and third weeks of treatment (3.53 ± 0.53 versus 6.7 ± 0.53; $p<0.05$ on day 14; 3.55 ± 0.49 versus 7.60 ± 0.70; $p<0.01$ on day 17; 3.06 ± 0.39 versus 7.76 ± 0.71; $p<0.01$ on day 19; 3.8 ± 0.40 versus 9.53 ± 1.07; $p<0.01$ on day 21).

4.2.2 Neurochemical Effects

4.2.2.1 Alterations in Superoxide Radical Formation

As depicted in Figure 4-3, chronic haloperidol treatment produced a significant increase in the concentration of superoxide radicals in the striatum (9.23 ± 0.60 nmol/mg protein versus 16.56 ± 1.06 nmol/mg protein, $p<0.0001$).

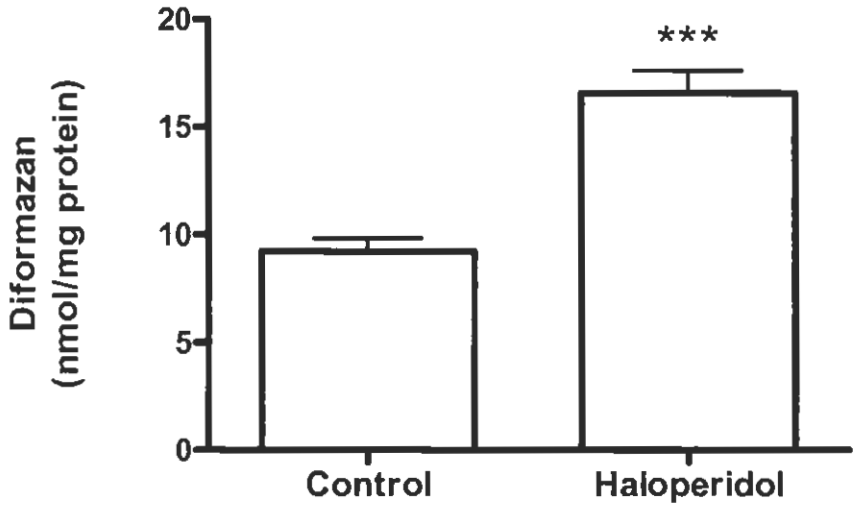

Figure 4-3: The effect of chronic haloperidol treatment (1.5 mg/kg/day and vehicle for 3 weeks on superoxide radical formation in the striatum of rats. The results are expressed as nmol/mg protein. *** $p<0.0001$ (Student *t*-test). n = 12 for all groups.

Increased oxidative stress is associated with compromised cellular function and damage; hence the next series of experiments were aimed at investigating the effects of haloperidol treatment on lipid peroxidation, a marker widely used to assess oxidative stress induced damage.

4.2.2.2 Alterations in Lipid Peroxidation

As is evident in Figure 4-4, chronic haloperidol treatment produced a significant increase in lipid peroxidation in the striatum of rats compared to control (1.14 ± 0.06 pmol/mg protein versus 1.82 ± 0.07 pmol/mg protein; $p<0.0001$).

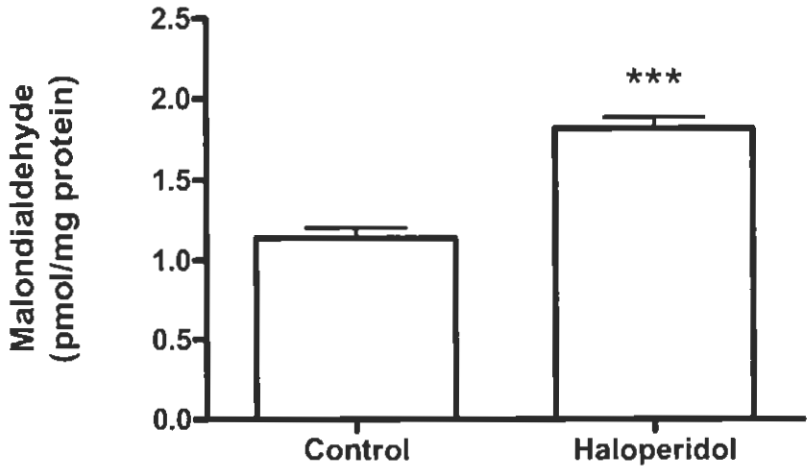

Figure 4-4: The effect of chronic haloperidol treatment (1.5 mg/kg/day and vehicle for 3 weeks on lipid peroxidation in the striatum of rats. The results are expressed as pmol/mg protein. *** $p<0.0001$ (Student *t*-test). n = 12 for all groups.

4.2.2.3 Changes in the Glutathione Redox Balance

The afore-mentioned data clearly describe a condition of raised superoxide levels and increased lipid peroxidation. The next series of experiments was aimed at confirming an alteration in cellular redox state by determining GSSG/GSH balance.

As illustrated in Figure 4-5, treatment with haloperidol did not induce any significant alteration in the GSSH/GSH ratio compared to control (0.12 ± 0.01 versus 0.13 ± 0.01; $p>0.05$). The concentration of GSH and GSSG in control and haloperidol treated rats were 0.629 ± 0.04 nmol/mg brain tissue (ranging from 0.350 to 0.788 nmol/mg brain tissue) and 0.025 ± 0.001 nmol/mg brain tissue (ranging from 0.017 to 0.033 nmol/mg brain tissue), respectively.

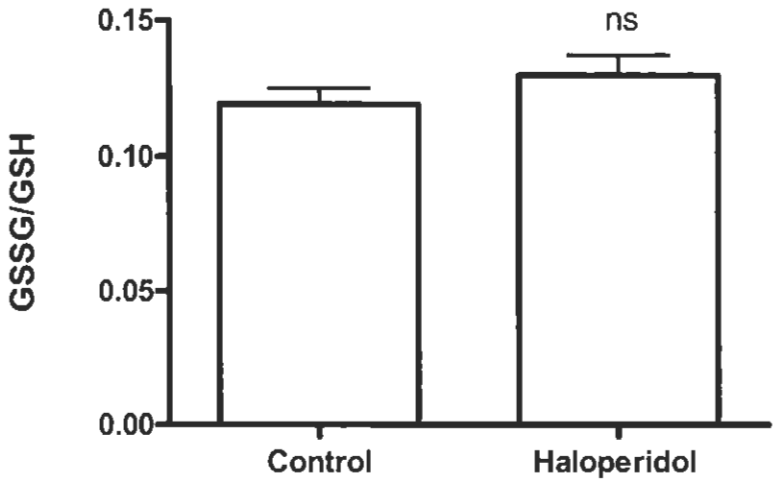

Figure 4-5: The effect of chronic haloperidol treatment (1.5 mg/kg/day) and vehicle for 3 weeks on the redox balance of GSH. ns $p>0.05$; (Student *t*-test). n = 12 for all groups.

The data described in the afore going series of experiments has now established a rodent model of striatal oxidative stress, viz. a rat model of TD, presenting with distinct evidence, at both behavioural and neurochemical levels, for increased oxidative stress. The next objective was to study the effects of differential doses of NAC on behavioural and striatal neurochemical markers of oxidative stress in healthy animals. This was done in order to assess the possible benefit that NAC may hold as a preventative strategy, ie in non-pathological states. With regard to clinical TD, this would be an equivalent scenario of placing schizophrenic patients on antioxidant therapy before commencing neuroleptic treatment in order to prevent later drug-induced striatal damage and the development of TD.

4.3 ESTABLISHING THE EFFECTS OF CHRONIC NAC ADMINISTRATION IN HEALTHY ANIMALS

In this series of studies, the effect of various doses of the antioxidant, NAC alone on various bio-behavioural markers of oxidative stress in healthy rats was determined.

4.3.1 Behavioural Effects

Looking firstly at total VCMs over the 3 week treatment period (Figure 4-6), one-way ANOVA analysis of the data revealed significant differences across the groups

[F(3,476) = 31.19; (p<0.0001)]. Subsequent post-hoc analysis with Dunnetts test indicates that spontaneous (basal) VCMs are evident at a low level in healthy animals (control). Doses of 10 and 100 mg/day of NAC did not significantly alter VCMs compared to control (3.42 ± 0.30 versus 2.09 ± 0.18; p>0.05 and 3.42 ± 0.30 versus 4.15 ± 0.36; p>0.05 respectively), whereas treatment with NAC at a dose of 300 mg/day over 3 weeks significantly increased the occurrence of VCMs compared to control (3.42 ± 0.30 versus 7.56 ± 0.67; p<0.01).

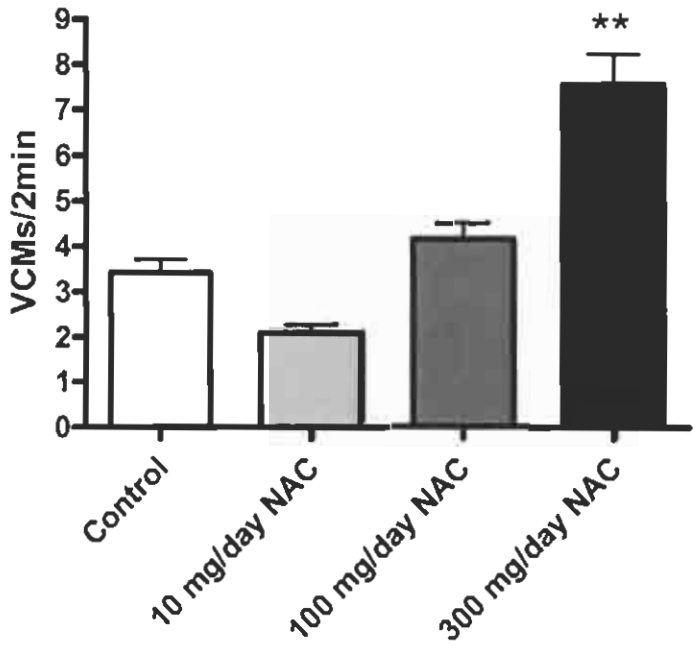

Figure 4-6: The effect of increasing doses of NAC (10 mg/day, 100 mg/day, 300 mg/day) versus control (vehicle) on the number of VCMs in healthy animals. Results are expressed as the mean of 5 individual rating sessions on days 7, 14, 17, 19 and 21 on 24 rats in each session. ** p<0.01 (One-way ANOVA followed by a Dunnett's post test). n = 120 for all groups.

These results are expressed as the average of the cumulative values of the 5 rating sessions per animal on days 7, 14, 17, 19 and 21, amounting to 120 readings per group consisting of 24 animals each.

In order to more accurately analyse the development of striatal toxicity over time compared to control, VCMs of the 24 animals were scored at 6 time points (days 0, 7, 14, 17, 19 and 21) during the 3 week treatment period with increasing doses of NAC (Figure 4-7) and analysed accordingly.

One-way ANOVA of these data revealed significant differences across the groups [F=(3,476) = 31.19; p<0.0001]. Subsequent post-hoc analysis of the data with Dunnetts test (Figure 4-7) on days 7, 14, 17, 19 and 21 revealed that , while the 10 mg/day and 100 mg/day NAC doses did not produce significant effects compared to control, a profound worsening of VCMs were produced by a 300 mg/day dose of NAC within the first week (day 7; 4.64 ± 0.70 versus 8.19 ± 1.67; p<0.05;) and continued to increase in the second week (day 14; 2.60 ± 0.38 versus 9.10 ± 1.94; p<0.001) of treatment. However, on day 17 the VCMs started to numerically decrease, but remained significantly more compared to control on day 17 (3.13 ± 0.66 versus 6.98 ± 1.37; p<0.01), day 19 (2.99 ± 0.47 versus 7.02 ± 1.43; p<0.01) and day 21 (3.73 ± 0.93 versus 6.50 ± 0.95; p<0.05).

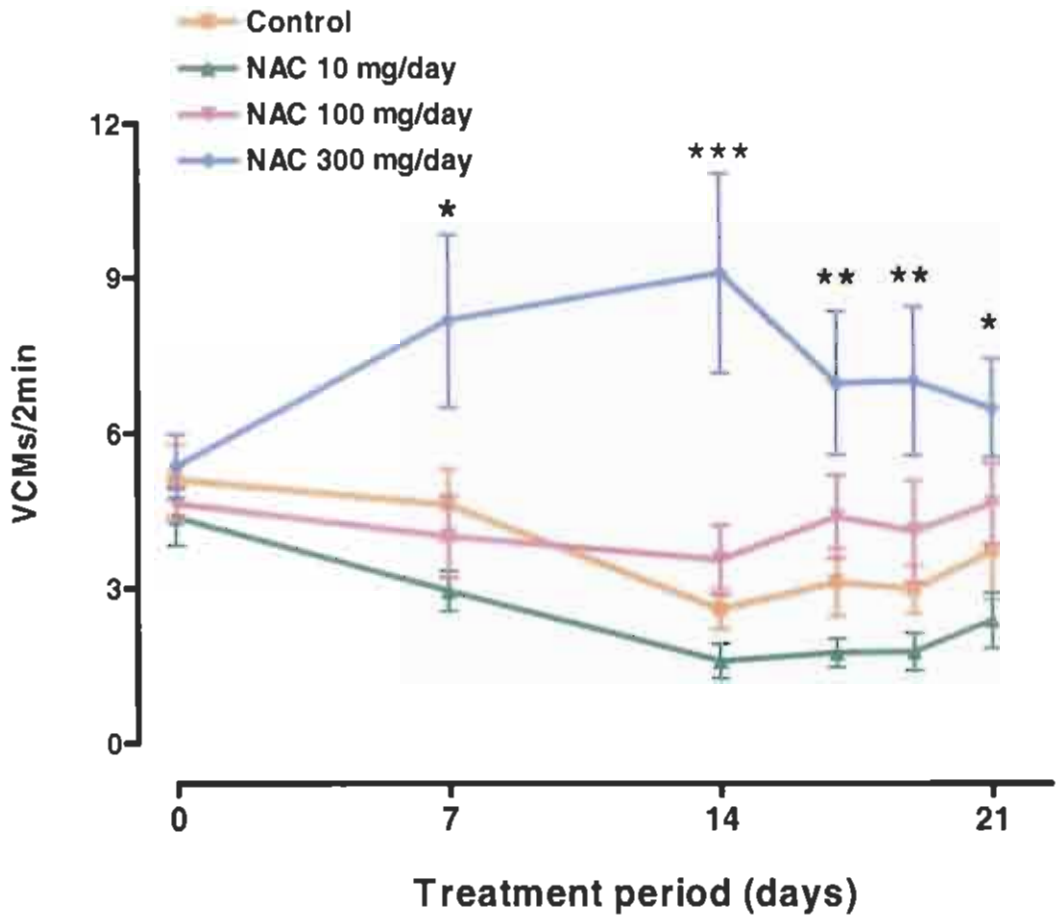

Figure 4-7: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day) versus control (vehicle) on the number of VCMs at different time intervals in healthy animals during a treatment period of 3 weeks. The results are expressed as the average number of VCMs during a 2 minute rating session (± SEM) on days 0, 7, 14, 17, 19 and

21. * p<0.05; ** p<0.01; *** p<0.001 (One way ANOVA followed by a Dunnett's post-test). n = 24 for all groups.

4.3.2 Neurochemical Effects

4.3.2.1 Alterations in Superoxide Radical Formation

One-way ANOVA of these data revealed that there were significant differences across the groups [F(3,44) = 32.42; (p<0.0001)]. As depicted in Figure 4-8, post-hoc analysis revealed that superoxide radical formation was not affected by 10 mg/day (11.32 ± 0.98 nmol/mg protein versus 10.22 ± 0.85 nmol/mg protein; p>0.05) or 100 mg/day (11.20 ± 0.98 nmol/mg protein versus 7.40 ± 0.80 nmol/mg protein; p>0.05) doses of NAC compared to control. In contrast, a 300 mg/day dose of NAC significantly increased striatal levels of superoxide compared to control (11.20 ± 0.98 nmol/mg protein versus 30.12 ± 3.32 nmol/mg protein; p<0.001).

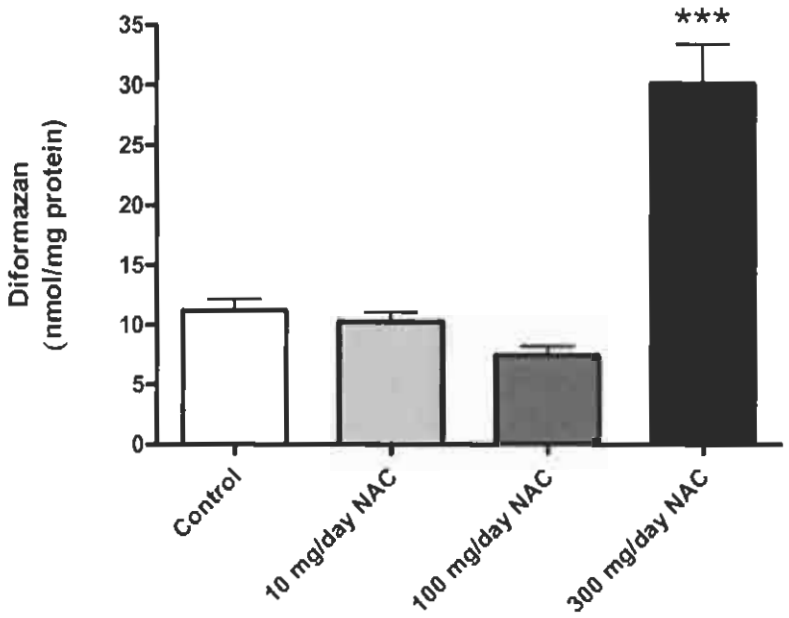

Figure 4-8: The effect of treatment with increasing doses of NAC (10 mg/day, 100 mg/day, 300 mg/day) versus control (vehicle) on superoxide radical formation in the striatum of rats. The results are expressed as nmol/mg protein. *** p<0.001 (One-way ANOVA followed by a Dunnett's post-test). n = 12 for all groups.

Given these interesting data with 300mg/day NAC on superoxide levels, the next series of experiments were aimed at investigating the effects of various doses of

NAC treatment on lipid peroxidation, a marker widely used to assess oxidative stress induced damage.

4.3.2.2 Alterations in Lipid Peroxidation

One-way ANOVA of these data revealed that there were significant differences across the groups [$F(3,44) = 4.73$; ($p=0.006$)]. Subsequent post-hoc analysis of these data with a Dunnett's test, as can be seen in Figure 4-9, indicated that doses of 10 mg/day (2.02 ± 0.20 pmol/mg protein versus 1.72 ± 0.15 pmol/mg protein; $p>0.05$) or 100 mg/day (2.02 ± 0.20 pmol/mg protein versus 1.67 ± 0.17 pmol/mg protein; $p>0.05$) of NAC were unable to produce a significant alteration in lipid peroxidation compared to control, whereas treatment with 300mg/day NAC significantly decreased lipid peroxidation compared to control (2.02 ± 0.20 versus 1.19 ± 0.093; $p<0.01$).

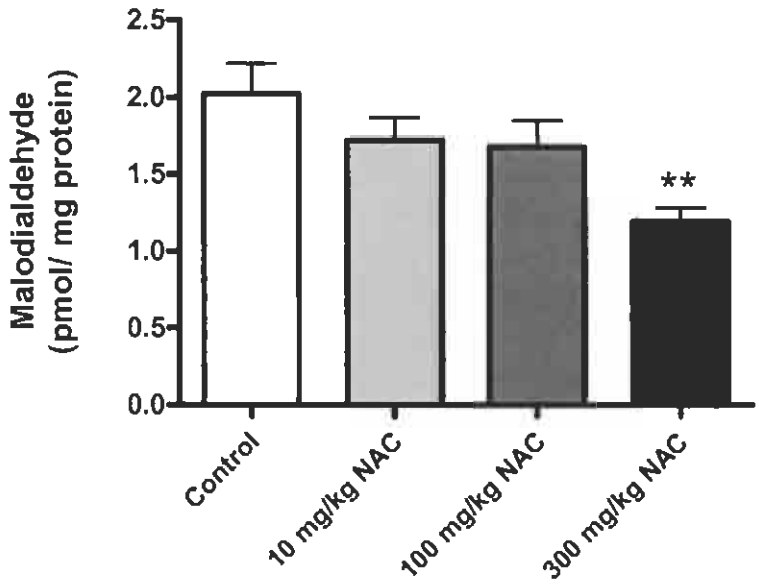

Figure 4-9: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day), versus control (vehicle) on lipid peroxidation in the striatum of rats. The results are expressed in terms of pmol/mg protein. ** $p<0.01$ (One-way ANOVA followed by a Dunnett's post-test). n = 12 for all groups.

The afore-mentioned data clearly describe a condition of raised superoxide levels but decreased lipid peroxidation in the striatum of rats treated only with 300 mg/day NAC. The next series of experiments was aimed at confirming whether alterations in

cellular redox state were evident following NAC treatment by determining GSSG/GSH balance.

4.3.2.3 Changes in the Glutathione Redox Balance

One-way ANOVA of these data revealed that there were significant differences across the groups [F(3,43)=4.06; (p=0.013)]. As depicted in Figure 4-10, post-hoc analysis of the data with a Dunnett's test indicates that chronic treatment with NAC at a dose of 10 mg/day as well as a dose of 100 mg/day did not produce any significant changes in the GSSG/GSH ratio compared to control. In contrast, a NAC dose of 300 mg/day produced a significant increase in the GSSG/GSH ratio, in favour of the oxidized form of glutathione (0.08 ± 0.0079 versus 0.11 ± 0.0037; p<0.05).

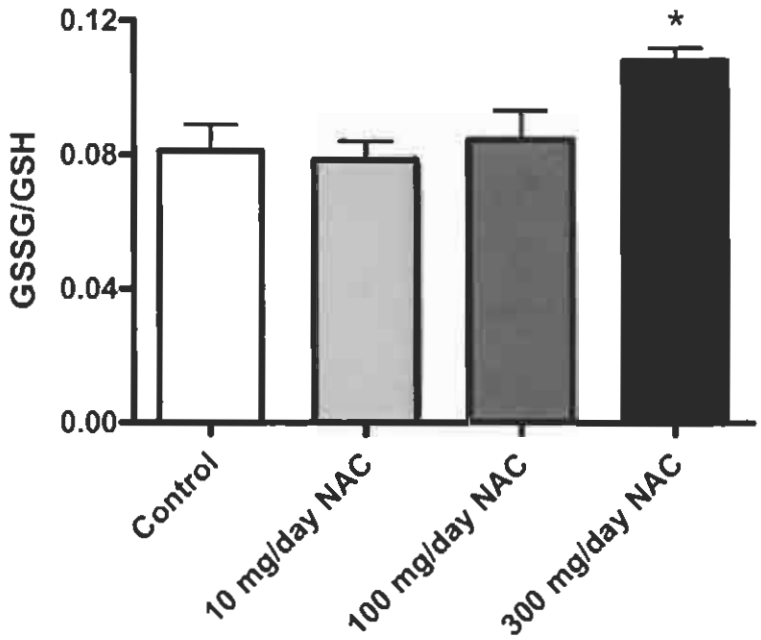

Figure 4-10: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day), versus control (vehicle) on the redox balance of glutathione in the striatum of rats. * p<0.05 (One-way ANOVA followed by a Dunnett's post-test). n = 12 for all groups.

The results in this section established the bio-behavioural effects of chronic NAC treatment in healthy animals. The next step was to evaluate the efficacy of NAC to possibly reverse changes in oxidative stress in an *in vivo* animal model of striatal oxidative stress, ie the TD model.

4.4 THE EFFECT OF CHRONIC NAC ADMINISTRATION IN AN ANIMAL MODEL OF STRIATAL OXIDATIVE STRESS, THE TD MODEL

In Section 4.2 the ability of chronic haloperidol treatment to evoke striatal oxidative stress in rats was verified by behavioural and neurochemical analyses. In Section 4.3, the effects of the antioxidant, NAC, alone in healthy animals was likewise studied. In this series of studies, the effect of various doses of NAC on the bio-behavioural effects evoked by chronic administration of haloperidol was determined.

4.4.1 Behavioural Effects

Looking at total VCM over the 3 week treatment period (presented in Figure 4-11), one-way ANOVA suggested that there were significant differences across the groups [$F(4,595) = 21.03$; ($p<0.0001$)]. Post-hoc analysis with Dunnetts test revealed that chronic treatment with haloperidol induced a significant increase in the number of VCMs compared to control (3.60 ± 0.22 versus 7.64 ± 0.35; $p<0.01$). In addition, when haloperidol was co-administered with NAC 10 mg/day (3.60 ± 0.22 versus 6.46 ± 0.32; $p<0.01$), NAC 100 mg/day (3.60 ± 0.22 versus 5.91 ± 0.32; $p<0.01$) and NAC 300 mg/day (3.60 ± 0.22 versus 7.26 ± 0.47; $p<0.01$), VCMs remained significantly greater when compared to control. However, when compared to haloperidol treatment using a Tukey-Kramer multiple comparison test, a dose of 100 mg/day NAC was found to significantly reduce haloperidol-induced VCMs in the rats (7.64 ± 0.35 versus 5.91 ± 0.32; $p<0.01$).

These results are expressed as the average of the cumulative values of the 5 rating sessions per animal on days 7, 14, 17, 19 and 21, amounting to 120 readings per group consisting of 24 animals each.

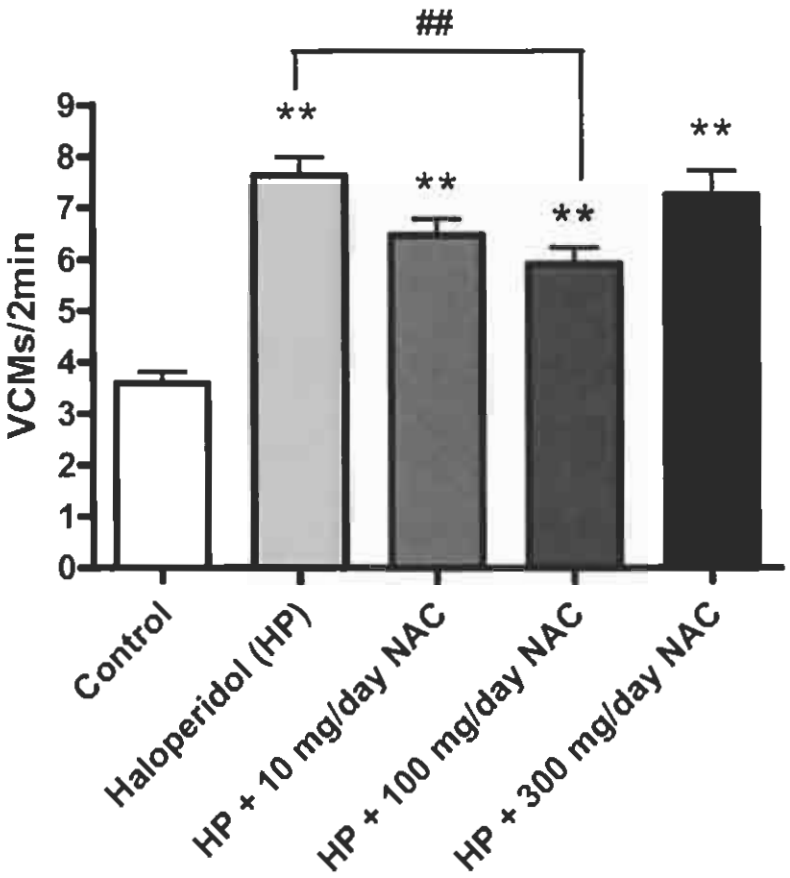

Figure 4-11: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day), versus control (vehicle) on VCM's in haloperidol treated animals. Results are expressed as the mean of 5 individual rating sessions on days 7, 14, 17, 19 and 21 on 24 rats in each session. ** $p<0.01$ (One-way ANOVA followed by a Dunnett's post-test); ## $p<0.01$ vs haloperidol (One-way ANOVA followed by a Tukey post-test). n = 120 for all groups.

However, when the behavioural data are more accurately expressed per day of treatment on 6 separate days over the 21 day treatment period with the inclusion of a zero time point (Figure 4-12), one-way ANOVA analysis revealed significant differences across the groups [$F(4.595) = 21.03$; ($p<0.0001$)]. Post-hoc analysis with Dunnetts test illustrates that chronic treatment with 300 mg/day NAC in combination with haloperidol produced significant differences on days 14, 17 and 19 compared to control (3.533 ± 0.530 versus 8.201 ± 1.285; $p<0.01$; 3.550 ± 0.494 versus 8.383 ± 1.060; $p<0.01$; and 3.058 ± 0.0393 versus 7.163 ± 1.030; $p<0.01$, respectively). However, by day 21 this difference was no longer significant.

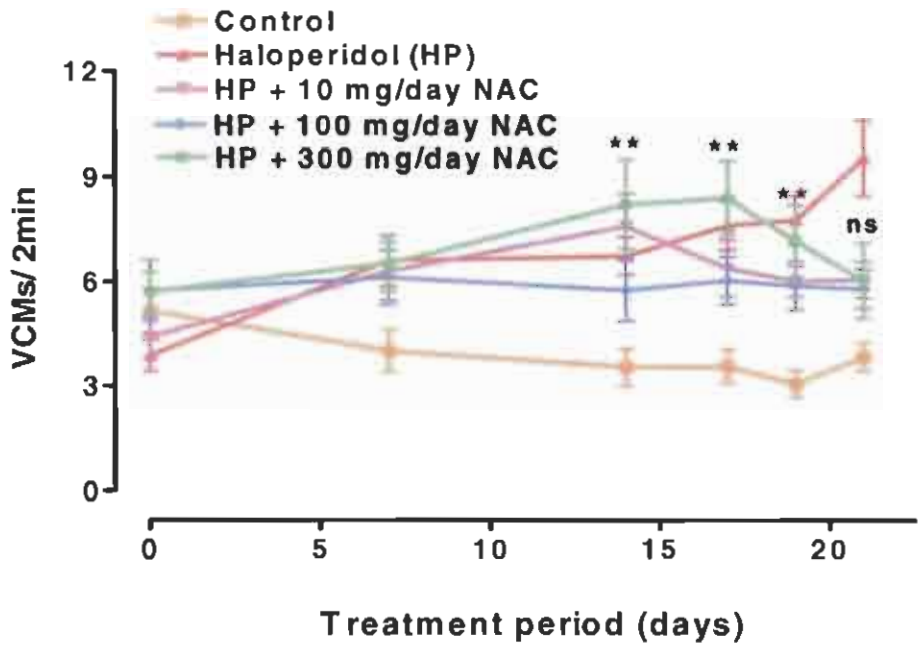

Figure 4-12: The effect of increasing doses of NAC (10 mg/day, 100 mg/day; 300 mg/day), versus control (vehicle) on VCMs at different time intervals in chronic haloperidol (1.5 mg/kg/day) treated animals during a treatment period of 3 weeks. The results are expressed as the average number of VCMs during a 2 minute rating session (± SEM) on days 0, 7, 14, 17, 19 and 21. ** $p<0.01$ (One-way ANOVA followed by a Dunnett's post-test). n = 24 for all groups.

4.4.2 Neurochemical Effects

4.4.2.1 Alterations in Superoxide Radical Formation

One way ANOVA of these data revealed that there were significant differences across the groups [$F(4,55) = 23.35$; ($p<0.0001$)]. As presented in Figure 4-13, subsequent post-hoc analysis with Dunnetts test indicated that superoxide radical formation was significantly increased in haloperidol treated rats compared to control (9.23 ± 0.60 nmol/mg protein versus 16.56 ± 1.06 nmol/mg protein; $p<0.01$). When 10, 100 and 100 mg/day doses of NAC were combined with haloperidol, superoxide radical formation was not statistically different compared to control rats (9.23 ± 0.60 versus 7.31 ± 0.21; $p>0.05$; 9.23 ± 0.60 versus 11.43 ± 0.46; $p>0.05$ and 9.23 ± 0.60 versus 10.25 ± 0.92; $p>0.05$, respectively), suggesting complete reversal of haloperidol effects. Indeed, when haloperidol was co-administered with 10 mg//kg/day NAC (16.56 ± 1.06 versus 7.31 ± 0.21; $p<0.001$), 100 mg/day NAC

(16.56 ± 1.06 versus 11.43 ± 0.46; p<0.001) and 300 mg/day NAC (16.56 ± 1.06 versus 10.25 ± 0.92; p<0.001), post-hoc analysis with the Tukey-Kramer test confirm that significant decreases in superoxide radical formation were observed compared to haloperidol treated animals.

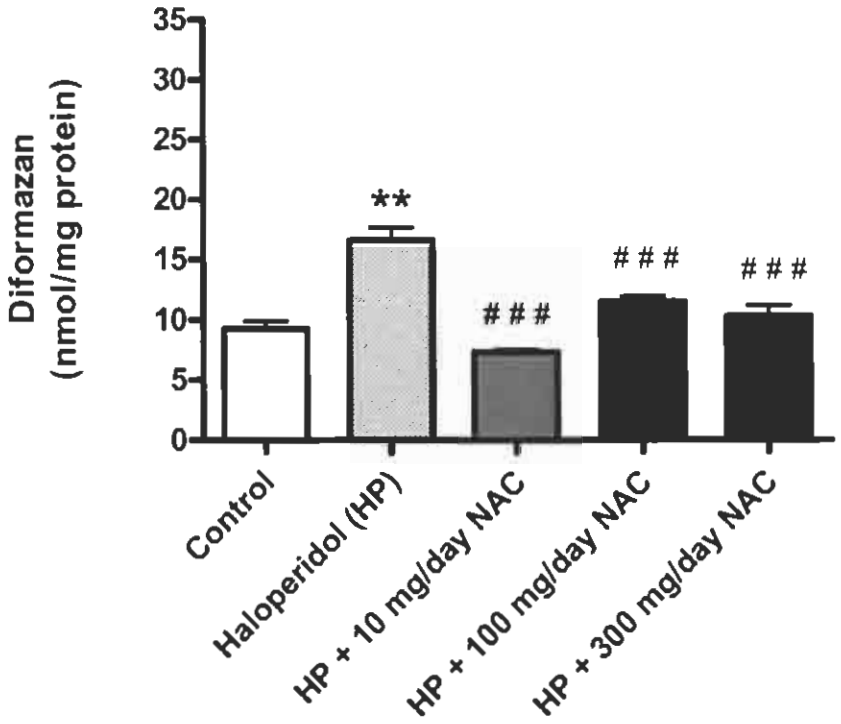

Figure 4-13: The effect of treatment with increasing doses of NAC (10 mg/day 100 mg/day; 300 mg/day), versus control (vehicle) on superoxide radical formation in the striatum of rats treated with haloperidol (1.5 mg/kg/day) for 3 weeks. The results are expressed as nmol/mg protein. ** p<0.01 (One-way ANOVA followed by a Dunnett's post-test); ### p<0.001 vs haloperidol (One-way ANOVA followed by a Tukey post-test). n = 12 for all groups.

Given that NAC was able to reverse haloperidol-induced increases in superoxide, the next series of experiments were aimed at investigating whether a similar inhibitory response could be realised with respect to haloperidol-induced effects on lipid peroxidation, a marker widely used to assess oxidative stress induced damage.

4.4.2.2 Alterations in Lipid Peroxidation

One-way ANOVA of the data revealed that there were significant differences across the groups [F(4,55) = 5.20; (p<0.0013)]. Subsequent post-hoc analysis with Dunnetts

test, as depicted in Figure 4-14, revealed that haloperidol treatment significantly increased lipid peroxidation in the striatum compared to control (1.14 ± 0.06 versus 1.82 ± 0.07; $p<0.01$). Similarly, treatment with haloperidol plus NAC at a dose of 10 mg/day significantly increased lipid peroxidation in the striatum compared to control (1.14 ± 0.06 versus 1.86 ± 0.25; $p<0.01$), suggesting NAC at this dose was unable to modify lipid peroxidation induced by chronic haloperidol. However, when haloperidol was co-administered with NAC doses of 100 mg/day (1.14 ± 0.06 versus 1.33 ± 0.13; $p>0.05$) and 300 mg/day (1.14 ± 0.06 versus 1.47 ± 0.09; $p>0.05$), lipid peroxidation returned to basal (control) levels (no significant difference versus control).

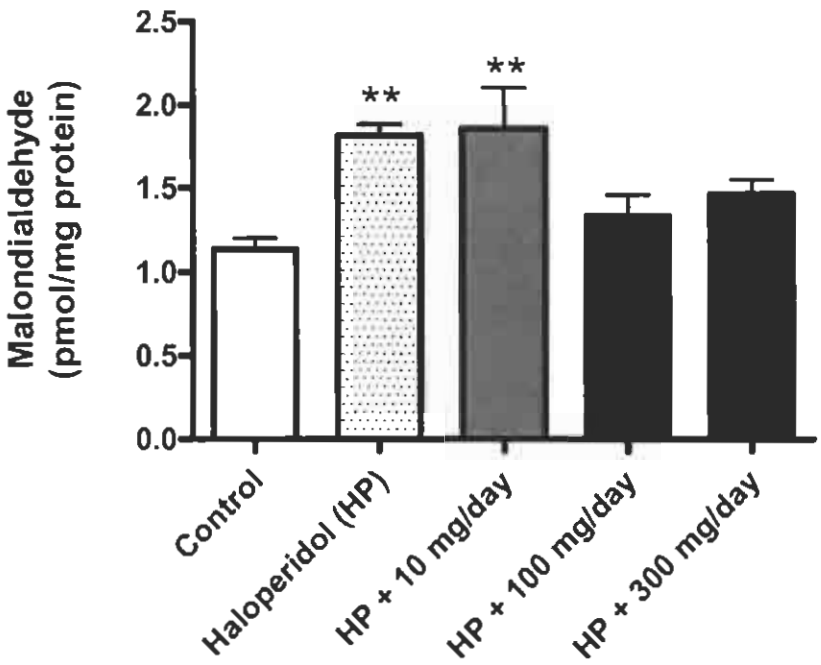

Figure 4-14: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day), versus control (vehicle) on lipid peroxidation in the striatum of rats treated with haloperidol (1.5 mg/kg/day) for 3 weeks.. The results are expressed in terms of pmol/mg protein. ** $p<0.01$ (One-way ANOVA followed by a Dunnett's post-test). n = 12 for all groups.

The above data suggest that selected (higher) doses of NAC are able to reverse haloperidol-induced striatal oxidative stress, as measured by striatal superoxide formation and lipid peroxidation. The next series of experiments was aimed at confirming whether alterations in cellular redox state were evident following these selected doses of NAC by determining GSSG/GSH balance.

4.4.2.3 Changes in the Glutathione Redox Balance

One-way ANOVA analysis of the data revealed that there were significant differences across the groups [$F(4,49) = 16.17$; ($p<0.0001$)]. Subsequent post-hoc analysis with Dunnetts test, as evident in Figure 4-15, indicate that chronic treatment with haloperidol alone (0.12 ± 0.01 versus 0.13 ± 0.01; $p>0.05$), as well as haloperidol in combination with 10 mg/day NAC (0.12 ± 0.01 versus 0.49 ± 0.03; $p>0.05$) did not produce significant effects on the GSSG:GSH ratio compared to control. However, combinations of haloperidol with higher NAC doses of 100 mg//kg/day (0.12 ± 0.01 versus 1.89 ± 0.43; $p<0.01$) and 300 mg/day (0.12 ± 0.01 versus 1.74 ± 0.26; $p<0.01$) produced significant increases in the GSSG/GSH ratio, in favour of the oxidized form (GSSG) compared to control.

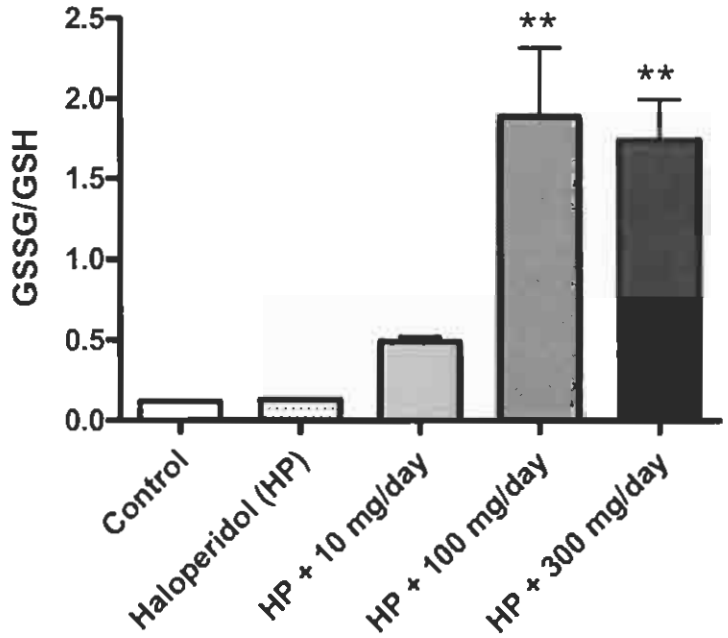

Figure 4-15: The effect of increasing doses of NAC (10 mg/day; 100 mg/day; 300 mg/day), versus control (vehicle) on the redox balance of glutathione in the striatum of rats treated with haloperidol (1.5 mg/kg/day) for 3 weeks. * $p<0.01$ (One-way ANOVA followed by a Dunnett's post-test). n = 12 for all groups.

The latter group of studies has confirmed that haloperidol-associated striatal oxidative stress can be attenuated by selected (higher) dosages of NAC.

In conclusion, the study as a whole has provided distinct evidence that the neurochemical and behavioural effects following chronic haloperidol treatment in rats

is associated with the presence of pronounced striatal oxidative stress, akin to that that has been suggested in TD. The study has also provided novel insight into the neurological and behavioural effects of NAC alone as well as its ability to address the above-mentioned pathology in a rodent model of TD. These data, and its implications with respect to the neurobiology and psychopharmacology of TD, are discussed in the chapter to follow.

Chapter 5 Discussion

5.1 INTRODUCTION

TD is a serious motor side-effect associated with prolonged treatment with neuroleptics drugs and characteristically involves involuntary movements in the orofacial region but may also include choreic movements of the trunk or extremities (Mitchell *et al.*, 2002). Various hypotheses have been put forward in order to explain the development of this devastating disorder (refer to § 2.1.3). However in recent years evidence in support of the free radical hypothesis has been mounting and TD is now believed to be related to an increase in free radical-induced damage associated with long-term neuroleptic use. Currently no effective cure exists for the treatment of TD although antioxidants have been suggested to hold some promise. While this approach has been more often centered around the treatment of TD, the concept of using antioxidants as a preventative measure in patients currently receiving neuroleptic treatment is an attractive option (Sachdev *et al.*, 1999), especially given that once established, TD is particularly resistant to treatment and in some cases may become irreversible (Jeste *et al.*, 1979).

The objectives of the current study were two-fold, namely to evaluate the behavioural and neurochemical effects of chronic NAC administration at various dosages in a non-pathological state (in healthy rats), as well as its effects in a pathological state characterised by increased striatal oxidative stress *in vivo*. To achieve this, a rat model of TD was used. VCMs were rated as a behavioural measure of striatal toxicity. The neurochemical assays that were performed include the striatal measurement of the levels of superoxide and lipid peroxidation as well as the assessment of oxidized versus reduced glutathione.

This chapter will discuss the results as presented in Chapter 4 as follows:

- The effects of chronic haloperidol treatment on the induction of VCMs and markers of striatal oxidative stress in rats in order to establish a rat model of TD in which oxidative stress may be implicated;

- The effects of increasing doses of NAC in a non-pathological state on VCMs and neurochemical markers of striatal oxidative stress in rats; and

- The effects of increasing doses of NAC in an animal model of TD on VCMs and markers of striatal oxidative stress in rats.

Various clinical (Lohr *et al.*, 1990; Zhang *et al.*, 2003) and pre-clinical (Behl *et al.*, 1996; Sagara, 1998; Parikh *et al.*, 2003) studies have suggested that TD is a result of excessive oxidative stress in especially the striatum, leading to possible irreversible damage to dopamine neurons and the ensuing motor manifestations that is typical of TD. Moreover, preliminary data support the use of antioxidants in reversing and/or treating this disorder (Adler *et al.*, 1998; Abilio *et al.*, 2003; Naidu *et al.*, 2003b). The antioxidant NAC is a potent free radical scavenger and glutathione precursor that may be useful in this regard.

Before being able to test the effects of NAC, it was necessary to first establish a rodent model of TD at the behavioural level, but also to confirm the existence of a pro-oxidative state in the striatum of animals exposed to chronic neuroleptic treatment to validate the hypothesis that free radicals are indeed implicated in neuroleptic toxicity. Various animal models have shown that chronic haloperidol administration in rats is associated with bizarre oral-buccal and facial motor disturbances (Levin et al., 1987; Andreassen & Jorgensen, 1994; Egan et al., 1995; Burger et al., 2005) that are correlated with increased levels of ROS (Adler et al., 1998; Brown et al., 1998; Arnaiz et al., 1999; Naidu & Kulkarni, 2002). Therefore this hypothesis required the validation of an animal model of striatal oxidative stress that should incorporate both behavioural and neurobiological abnormalities, and has strong validity for a specific human neuropsychiatric illness that presents with oxidative stress as one of its primary aetiological determinants. The VCM rat model of TD was used to this end.

5.2 THE EFFECT OF CHRONIC HALOPERIDOL TREATMENT ON BEHAVIOUR AND MARKERS OF OXIDATIVE STRESS

5.2.1 Behavioural Effects

The results of the current study, as well as earlier studies describing the VCM model of TD (Harvey & Bester, 2000; Harvey & Nel, 2003; Burger *et al.*, 2005), confirm that healthy (control) rats indulge in a very low level of purposeless chewing activities (as evident in Figure 4-1 and Figure 4-6), which have also been shown to increase with age (Steinpreis *et al.*, 1997). While these spontaneous VCMs are considered normal in healthy rats, their presence has never been fully explained. Nevertheless, it has been suggested that VCMs may be used as an analgous behavioural expression of striatal oxidative stress and/or damage and suitable for use as

readout for the VCM model of TD. Indeed, VCMs are a robust behavioural change used to assess the motor toxicity of neuroleptics (Kelley & Roberts, 2004).

As is evident Figure 4-1, there is a significant increase in the occurrence of VCMs following a chronic, 3 week, treatment period with haloperidol. This is in accordance results from previous studies (Naidu & Kulkarni, 2002; Naidu *et al.*, 2003a; Naidu *et al.*, 2003b; Burger *et al.*, 2005) as well as earlier studies conducted in our laboratory (Bester & Harvey, 2000; Harvey & Bester, 2000; Harvey & Nel, 2003; Nel & Harvey, 2003). These findings establish the behavioural aspect of the TD model. As depicted in Figure 4-2, haloperidol induces a time-dependant increase in the number of VCMs. This is in accordance with other reports in the literature that VCMs increase with time following exposure to a neuroleptic agent (Harvey & Nel, 2003; Bishnoi *et al.*, 2006). As has been mentioned earlier, an increase in the number of VCMs is regarded as a behavioural measure of neuroleptic-induced striatal toxicity (Marchese *et al.*, 2002; Kelley & Roberts, 2004), for which a number of hypotheses have been proposed, including the dopamine supersensitivity- (Margolese *et al.*, 2005), GABA- (Fibiger & Lloyd, 1984), excitotoxicity- (McGeer & McGeer, 1976) and free radical hypotheses (Lohr *et al.*, 2003). Based on previous studies in our laboratory that have established a close association between haloperidol-induced TD in the VCM model and the reactive nitrogen species (RNS), nitric oxide (Bester & Harvey, 2000; Harvey & Bester, 2000; Harvey & Nel, 2003; Nel & Harvey, 2003), this study has chosen to focus further on the free radical hypothesis, but now examining the role of ROS, particularly superoxide, as well as increased lipid peroxidation and altered cellular redox state, in the VCM model of TD.

5.2.2 Neurochemical Effects

5.2.2.1 Alterations in Superoxide Radical Formation

Not only did chronic haloperidol evoke an increase in VCMs, but, as is evident in Figure 4-3, this drug also engendered a significant accumulation in superoxide in striatal tissue, suggesting a causal relation between striatal oxidative stress and dysfunctional motor behaviour. These results are also in accordance with previous studies that have demonstrated an increase in superoxide radicals in the striatum of rats following long-term neuroleptic treatment (Polydoro *et al.*, 2004). The free radical hypothesis of TD postulates that the disorder results from chronic D_2 receptor blockade by neuroleptic drugs, which are potent blockers of DA receptors (Lidsky *et al.*, 1997). By blocking these receptors a secondary increase in dopamine synthesis or release is induced in an attempt to override the hypodopaminergia induced by post-synaptic D_2 receptor blockade (See, 1991). The subsequent reactive upregulation of DA degradation by MAO leads to the formation of hydrogen peroxide as a by-product. Furthermore, haloperidol treatment of

rats and the subsequent DA upregulation may be associated with an increase in the auto-oxidation of this monoamine, leading to increased formation of superoxide radicals (Figure 4-3) (Loeffler *et al.*, 1998) as well as quinones and semiquinones (Ben-Shachar *et al.*, 2004). In agreement with this, patients suffering from TD have been found to present with increased circulating levels of SOD, which may imply that free radicals are increased in TD and that the increase in SOD is induced as a protective response to increased oxidative stress (Zhang *et al.*, 2003). The activity of the antioxidant enzyme CAT has also been shown to be higher in patients with TD symptoms, even though lipid peroxidation was found to be increased in these groups (Galecki *et al.*, 2005). The increase in the protective mechanisms such as SOD against oxidative stress suggest that free radicals are elevated in TD, and that the body upregulates protective enzymes in order to compensate for this increase. However, the presence of increased lipid peroxidation indicates that these enzymes are not able to sufficiently decrease free radicals. In fact, a paper recently published during the time that the current study was underway similarly concluded that chronic haloperidol treatment was associated with significant dyskinesia and increases protein oxidation and lipid peroxidation in rat brain (Sadan et al, 2005). The mechanism whereby haloperidol may increase superoxide accumulation has been proposed to be due to formation of an HPP^+ metabolyte and the subsequent inhibition of the mitochondrial electron transport chain (Burkhardt *et al.*, 1993). Another proposal involves the suppression of nitric oxide synthase and attentuation of NO release (Bester & Harvey, 2000; Harvey & Bester, 2000; Harvey & Nel, 2003; Nel & Harvey, 2003). NO occupies an important protective role in the brain by acting as a natural free radical "sink" for excessive superoxide production and is also able to protect against lipid peroxidation (Wink *et al.*, 1995).

Since increased oxidative stress is associated with compromised cellular function and damage (Valko *et al.*, 2007), any overt and sustained elevation in superoxide production following chronic haloperidol treatment is very likely to evoke damage to membrane lipids and to compromise cellular integrity. Considering the raised levels of ROS in the striatum noted following chronic haloperidol treatment, as well as the behavioural evidence for striatal dysfunction, levels of lipid peroxidation in the striatum were assessed.

5.2.2.2 Alterations in Lipid Peroxidation

In normal subjects, the endogenous antioxidant defences balance the production of ROS, except for approximately 1% of these ROS that are not effectively scavenged and which are able to induce some degree peroxidative damage such as lipid peroxidation (Berger, 2005). It was thus not altogether surprising to find basal levels of lipid peroxidation in non-haloperidol-treated rats.

The results depicted in Figure 4-4 confirm that chronic haloperidol treatment engenders a significant increase in striatal lipid peroxidation in rats. Increased free radical and ROS formation are associated with an increase in lipid peroxidation (Valko *et al.*, 2007) and it can be suggested that the cause of TD may be related to toxic effects of free radicals on striatal motor pathways.

The abovementioned results indicate that chronic haloperidol treatment is associated with an increase in oxidative stress, the accumulation of superoxide and a resulting increase in lipid peroxidation in the striatum. This may underlie the basis for striatal toxicity and motor dysfunction, as indicated in the increased VCMs evoked by haloperidol in this study and elsewhere (Behl *et al.*, 1996; Sadan *et al.*, 2005). Haloperidol has been shown to be toxic to neurons (Lezoualc'h *et al.*, 1996; Post *et al.*, 2002), and that its toxic effects are mediated through the production of free radicals and ROS, which in turn has the ability to cause lipid peroxidation (Behl *et al.*, 1996). Marked changes in superoxide and its resulting detrimental effects on cellular membrane, as described here, are very likely to be reflected in changes in redox state of the cells.

5.2.2.3 Changes in the Glutathione Redox Balance

The abovementioned results suggest increases in oxidative stress and free radical formation following haloperidol treatment. Since the GSSG:GSH ratio can be increased under these circumstances, this ratio was evaluated. However, as is evident in Figure 4-5, haloperidol treatment did not induce a significant change in this ratio. While this is indeed surprising, it is not altogether unexpected, as outlined below.

The metabolism of DA is associated with the formation of several toxic quinones, while various phase II enzymes are responsible for the detoxification of these toxic metabolites. One such enzyme is quinone oxidoreductase 1 (NQO1), also referred to as DT-diaphorase, which catalyzes the reduction of these quinones including their respective quinonoid derivatives into stable hydroquinones (Okada *et al.*, 2005). Hydroquinones are thiol reactive oxidative agents that have been reported to stimulate the production of GSH (Hultberg *et al.*, 1999). The chronic blockade of DA receptors by neuroleptic drugs can promote an increase in the release or synthesis of DA leading to a corresponding increase DA metabolism with an associated increase in quinone and subsequent hydroquinone formation which are thiol reactive agents (Hultberg *et al.*, 2001). Thiol reactive agents may cause complexes with GSH which could lead to increased transport of glutathione out of the cell, thus reducing intracellular GSH and in this way up-regulating the synthesis of GSH through y-glutamylcysteine synthase, an enzyme subject to feedback regulation (Hultberg *et al.*, 2001). Cells challenged with GSH depleting drugs or sub-lethal oxidative stress has been shown to increase the GSH synthesis through the up-regulation of this enzyme as well (Hultberg *et al.*, 2001). Haloperidol administration could

therefore induce y-glutamylcysteine synthase by its oxidative properties or through its increased formation of hydroquinones as explained above. Induction of this enzyme might explain the observation that GSH was not depleted as was expected.

Another possible explanation could be that the treatment period of haloperidol may also have not been sufficient to induce a decrease in GSH levels. However, increasing concentrations of oxidative agents can eventually lead to a situation were increased GSH production will not be able to deal with the oxidative stress which can eventually lead to decreased levels of intracellular GSH (Hultberg *et al.*, 2001). Continued administration of haloperidol would probably have led to decreased GSH over time. This observation may imply that the GSSG:GSH ratio is not altered by short-term haloperidol treatment. Indeed, some studies have opted to explore haloperidol-treatment periods of 3-12 months or even longer (Harvey & Bester, 2000; Rosengarten *et al.*, 2006). The latter treatment periods have been associated with an increase in the GSSG:GSH ratio in favour of GSSG (Vairetti *et al.*, 1999; Vairetti *et al.*, 2002). While this is a point of consideration, the 3 week haloperidol treatment model used here offers a rapid turn-around of results, is less time consuming on man-hours and remains extensively used to explore the behavioural neurobiology of TD (Naidu *et al.*, 2002; Nel & Harvey, 2003; Naidu *et al.*, 2003a; Naidu *et al.*, 2003b).

The results that have been described above therefore confirm that haloperidol is capable of inducing a significant increase in lipid peroxidation in the striatum, and that a state of striatal oxidative stress plays an important causal role in the current animal model and very likely in TD. While this study has implicated the striatum in the neuroanatomy of TD, other brain regions may also be involved, but are beyond the scope of this work.

5.3 THE EFFECT OF NAC ON BEHAVIOUR AND MARKERS OF OXIDATIVE STRESS IN A NON-PATHOLOGICAL STATE

Increasing evidence suggest a value for anti-oxidants in degenerative disorders, including the use of various antioxidants in Alzheimer's disease (Zana *et al.*, 2007). This study has focussed on the potential therapeutic value of NAC. As mentioned previously two intervention strategies may be considered, for this part of the study it was paramount to establish the role of NAC as a possible preventative agent. In order to assess the value of NAC in such a strategy the *in vivo* actions of NAC alone on markers of oxidative stress in the rat brain and its effect on behaviour in the *absence* of pathology, needed to be evaluated. This study has specifically chosen the striatum as it represents one of the important brain region in regulating movement. Using the

VCM model of striatal toxicity (discussed in § 3.3.4), this study set about to evaluate the dose dependant effect of NAC on behavioural and neurochemical markers of oxidative stress in the striatum or healthy rats after a chronic, 3 week treatment. After the weight of each rat was factored in, the doses of NAC administered were as follows: 10 mg/day NAC = 50 mg/kg/day NAC; 100 mg/day = 500mg/kg/day; 300 mg/day = 1500 mg/kg/day, and should be borne in mind when discussing the data.

NAC displays antioxidant activity over a wide dosage range that is dependant on the biological system in which it is administered, for example the presence of oxidative stress (De La Fuente *et al.*, 2002), the presence or absence of transition metal ions (Sagrista *et al.*, 2002). NAC can act as an antioxidant via two mechanisms, firstly by acting as a free radical scavenger, directly scavenging H_2O_2, HOCl, $^{\cdot}OH$, (Wang *et al.*, 2006) and superoxide (Benrahmoune *et al.*, 2000), and secondly, by acting as a precursor of GSH, and thereby increasing GSH levels (Wang *et al.*, 2006). Cells with a GSH deficiency are more sensitive to the effects of oxidative stress, while increased GSH synthesis supports cell survival under oxidative challenge (Ghizoni *et al.*, 2006), so that increased GSH synthesis produced by NAC administration may prove to be protective.

5.3.1 Behavioural Effects

As depicted in Figure 4-6, neither 10 mg/day nor 100 mg/day doses of NAC had any significant effect on the number of VCMs of healthy rats compared to control. Since NAC acts as an antioxidant in the presence of oxidative stress, and that pathological oxidative stress was not induced in the current experiment, it was not expected that NAC would modify the basal number of VCMs displayed in healthy animals. Neverthess, whether NAC at these doses actually exerted an antioxidant effect in the striatum would only become apparent in the neurochemical data presented hereafter.

What is of great interest is that, in contrast to the insignificant effects of 10 mg/day and 100 mg/day doses of NAC on VCMs, the highest dose (300 mg/day) of NAC induced a significant increase in the number of VCMs compared to control. These behavioural effects speak of a pronounced degree of striatal motor toxicity, which was somewhat unexpected. However, such a paradoxical response may possibly be associated with a pro-oxidative action of NAC at this dose. Indeed, several studies in the literature report that antioxidants may, under specific circumstances, exhibit pro-oxidant activity (see § 2.4 and 2.4). The behavioural data presented here would therefore suggest that NAC may act as a pro-oxidant at high doses in non-pathological states (i.e. healthy animals), were ROS is not abnormally increased. It is widely believed that if a moderate amount of an essential nutrient (or antioxidant) is beneficial, then

more of the supplement should be better. However, various studies have reported the contrary, and many of these substances have been shown to be counter-productive at high doses (Berger, 2005). To illustrate this better, data from the behavioural study depicting the time-dependant effect of chronic NAC administration were expressed per day of treatment across the treatment period (Figure 4-7). Important additional detail regarding the effects of NAC, especially at high dose, is revealed by this data.

As depicted in Figure 4-7, and in correlation with the previous data, 10 mg/day and 100 mg/day doses of NAC showed minimal change across the treatment period compared to control on the 6 days of evaluation. However, an initial administration of 300 mg/day NAC induced an almost immediate increase in the number of VCMs that became more pronounced especially in the first and second week of treatment. This increase in VCM's is similar to that induced by the atypical neuroleptic, haloperidol (Harvey and Bester, 2000) or the dopamine depleter, reserpine (Raghavendra *et al.*, 2001), suggesting that the severity of this behavioural change approaches or equals that of the traditional drugs well-known to cause oro-facial bucco-lingual disturbances in humans and animals. However, thereafter VCMs appear to diminish over time with a reduction becoming apparent within the third week of treatment, yet remaining significantly higher than control. This reduction in VCMs may be due to a natural adaptive process initiated by increased oxidative stress, i.e. NAC as a pro-oxidant, which in turn, can activate certain protective mechanisms that are only activated after an extended period of time (2 weeks in the rat). Various studies have reported that chronic, mild forms of oxidative stress can induce an adaptation to oxidative stress and thereby prepare the host to better cope with physio-pathological conditions that are mediated by ROS (Hernandez *et al.*, 1995; Leon *et al.*, 1998; Barber *et al.*, 1999). Indeed, studies in cells and humans have found an increase in protective SOD following a pro-oxidative challenge (Yao *et al.*, 1998; Kim & Park, 2004). So, while severe oxidative stress can cause damage, mild oxidative stress can up-regulate the expression of antioxidant enzymes and so protect against oxidative stress induced damage. While a mechanism for the improvement in VCMs over time is speculative at this moment, it may become an important behavioural correlate for the later studies on striatal oxidative markers presented and discussed below.

5.3.2 Neurochemical Effects

5.3.2.1 Effects on Superoxide Radical Formation

Superoxide radicals are formed throughout the body in several oxidative metabolic pathways (§ 2.2.2 and 2.2.2.1), such that the presence of basal levels of this particular free radical is not peculiar (Arnaiz *et al.*, 1999; Polydoro *et al.*, 2004). However, these levels are tightly controlled

by various cellular mechanisms. Under both normal and pathologic conditions, O_2 is transformed into ROS, such as H_2O_2, O_2- and $^{\bullet}OH$ (Gloire *et al.*, 2006). These oxygen-centred radicals, which are produced under normal aerobic metabolism, are mainly produced by leukocytes and by the respiratory mitochondrial chain and are essential for cell signalling, as well as for bacterial defence (Berger, 2005). In the daily consumption of oxygen, a normal 60 kg woman would produce 160-320 nmol of superoxide each day from mitochondrial respiratory alone, while an 80 kg male would produce between 215 and 430 nmol daily (Cadenas & Davies, 2000). When the regulatory mechanisms of superoxide are disabled, or the production of superoxide far exceeds the scavenging action of antioxidants, a pro-oxidative state is evoked with deleterious consequences for health.

NAC has been reported to be an effective scavenger of numerous free radicals and ROS, such as H_2O_2, HOCl, ·OH, (Wang *et al.*, 2006) and superoxide (Benrahmoune *et al.*, 2000) while it also possesses the ability to reduce peroxynitrite (ONOO−) (Failli *et al.*, 2002). In correlation with the behavioural data, chronic NAC administration at 10 mg/day and 100 mg/day doses did not markedly alter the basal superoxide radical content in the rat striatum (Figure 4-8). This apparent lack of scavenging effect of NAC on superoxide may, however, not be unusual, since earlier reports have indicated that NAC is an ineffective scavenger of superoxide (Failli *et al.*, 2002; Schneider *et al.*, 2005). However, it must be borne in mind that this is under basal conditions, and that any scavenging effect by NAC may be a function of ambient O2•− levels such that NAC may be more effective under conditions of pathologically raised superoxide levels (which is presented in § 5.4). To illustrate this point, an earlier study determined the reaction rate constants of O2•− with N-acetylcysteine to be 68 ± 6 M−1 s−1 and to display second order kinetics (Benrahmoune *et al.*, 2000). In this reaction, the reaction rate is directly proportional to the square of the concentration of one of the reactants (Glasstone & Lewis, 1960) such that in states of increased O2•− radical formation, as in pathological conditions, the reaction rate will increase with the square of the increase in O2•− and more O2•− will effectively be scavenged by NAC. With regard to this, administration of NAC has indeed been shown to decrease superoxide radical formation in pathological induced states, as with acute paraquat intoxication (Yeh *et al.*, 2006).

However, as is evident in Figure 4-8, a different profile emerges at 300 mg/day NAC where the antioxidant evoked a significant increase in superoxide radical production after chronic administration, and pointing towards the possible pro-oxidant effect of NAC at this dose. This data correlates with evidence for striatal toxicity highlighted in the behavioural studies described earlier, namely the significantly increased number of VCMs at this dose. Antioxidants such as ascorbate have been shown to exhibit pro-oxidative properties at high dosages (Hininger *et al.*, 2005), while high doses have also been reported to increase oxidative stress parameters,

including that of superoxide (De La Fuente *et al.*, 2002). Thus, even though NAC is an antioxidant, it is able to act as a pro-oxidant at high doses as demonstrated by the current study and by others (Oikawa *et al.*, 1999; De La Fuente *et al.*, 2002). In the afore mentioned study (De La *et al.*, 2002) adult and aged mice were treated with NAC at doses of 0.1% and 0.3%. The ingestion of the 0.3% NAC by adult mice resulted in an increase in oxidative stress parameters. The latter study demonstrated that the ability of NAC to become prooxidative is not only dependant on dose but also on other factors such as the age of the animal. Moreover, NAC has been reported to increase the generation of free radicals in the presence of H_2O_2 *in vitro* (Sagrista *et al.*, 2002) and there is a growing body of evidence suggesting that compounds that are antioxidants at some concentrations may become pro-oxidant at other concentrations (refer to § 2.4.1). NAC has also been found to exert prooxidative action in certain cell lines while others are not affected (Oikawa *et al.*, 1999). In suggesting the pro-oxidant actions of NAC alone, the behavioural data therefore correlates well with the data from the superoxide radical assay, with doses of 300 mg/day NAC (and probably higher, although not tested) being more likely to evoke this response. This would suggest that superoxide radicals may be causally related to the incidence of VCMs and supports the free radical theory regarding neuroleptic induced VCMs.

The current data is therefore in agreement with the aforementioned behavioural findings that a dose of 300 mg/day NAC displays pro-oxidant properties under normal (non-pathological) conditions via the generation of superoxide radicals. Of importance is that this has taken place in the striatum, a brain region that is associated with high levels of oxidative metabolism (Hermida-Ameijeiras *et al.*, 2004) and, as a result, is more prone to damage by oxidative stress (Pereyra-Munoz *et al.*, 2006). At both the behavioural and biochemical levels, this work has therefore provided strong evidence that NAC induces striatal toxicity at a high dose in rats. Gross non-physiological and sustained increases in superoxide will invariably have a deleterious effect on surrounding tissue, in this case the striatum. This was the focus of the next investigation.

5.3.2.2 Effect on Lipid Peroxidation

Sustained and excessively high levels of ROS are known to damage cell membranes, particularly through peroxidation of the lipid content of cell membranes, resulting in altered cellular function and even cell death (Valko *et al.*, 2007). Given the aforementioned evidences, especially in the high-dose group where NAC treatment resulted in an increase in superoxide content in the striatum of rats, there was an expectancy of increased lipid peroxidation in striatal tissue extracts from these animals. However, as is evident in Figure 4-9, data provided by the lipid peroxidation studies were not entirely congruent with an interpretation that NAC acts as a pro-oxidant at a high dose resulting in an increase in membrane damage, at least not at face

value. In true paradoxical fashion, 300 mg/day NAC significantly decreased basal levels of lipid peroxidation, with no change noted at 10 and 100 mg/day doses. Despite this apparent unexpected turn of events, the data are nonetheless convincing that the most pronounced changes in oxidative markers occur at the high doses of NAC used in this particular study.

Oxidation of lipids, nucleic acids or proteins has been suggested to be involved in aging in general. Under normal physiological conditions, about 1% of the daily produced ROS is not effectively scavenged by the endogenous antioxidant defence mechanisms. This 1% is able to produce peroxidative damage to surrounding tissues (Berger, 2005), so that basal levels of tissue lipid peroxidation can be expected. Lipid peroxidation is a unique mode of oxidative injury that can be triggered by a variety of radicals as well as by the catalytical decomposition of preformed lipid hydroperoxides in tissues (Lykkesfeldt, 2007). This peroxidative injury causes not only structural and functional derangement of the phospholipid bilayer of cells, but also several damaging aldehydic end-products, such as MDA (Lykkesfeldt, 2007), can cause further damage to protein and DNA. These end-products of lipid peroxidation will further increase any primary oxidant-induced damage via ROS.

As presented in Figure 4-9, and in correlation with the behavioural data as well as the data on superoxide formation, both the 10 mg/day and 100 mg/day NAC doses did not significantly alter basal levels of lipid peroxidation in the rat striatum compared to control. However, in direct paradox to the effects on behavioural and superoxide radical formation, a 300 mg/day dose of NAC significantly decreased lipid peroxidation in the striatum. While an increase in superoxide radicals would be expected to give rise to an increase in lipid peroxidation, it becomes clear that a different mechanism is at play under the current circumstances. It is known that in states of excessive oxidative stress, the protective enzyme superoxide dismutase (SOD) is rapidly induced (Ahmad, 1995) as a protective mechanism against continued oxidative damage. It is thus reasonable to suggest that the excessive production of superoxide following a 300 mg/day dose of NAC results in a reactive induction of SOD. Unfortunately, SOD activity was not studied in the current study to corroborate this suggestion. Thus, the increase in oxidative stress, provoked by the increase in superoxide radicals, may induce certain oxidative protective mechanisms thereby inducing a tolerance against free radicals and the associated oxidative stress, resulting in the observed decrease in lipid peroxidation. This latter response may be regarded as a normal physiological response in a healthy subject, and so may also explain the gradual reduction in VCMs seen in the 3rd week of the NAC 300 mg/day treatment group (Figure 4-7). In this regard, superoxide radicals have been described to possess an ability to both initiate and terminate lipid peroxidation (Nelson *et al.*, 1994), albeit this being described in the reperfused heart. Therefore, the increase in superoxide radicals may have the ability to terminate lipid peroxidation chain reactions which as such may offer an explanation for the decrease in lipid peroxidation levels observed in the high-dose NAC group.

As mentioned earlier, NAC presents with two possible ways of lowering ROS in tissue, one being its radical-scavenging actions which have been the focus up till now, and the second being its ability to bolster levels of glutathione (GSH) (Wang *et al.*, 2006). The next part of the study thus set about to study the effect of the above-mentioned doses of NAC on the levels of GSH in the striatum.

5.3.2.3 Changes in the Glutathione Redox Balance

The ratio of the oxidised (GSSG) versus reduced (GSH) form of glutathione was determined using LC-MS. Since reduced GSH strongly modulates the redox state (ratio of oxidizing to reducing equivalents) of the cell, a role which is critical for cell survival, higher levels of GSH via exogenous administration of a precursor such as NAC, may bolster the ability of GSH to lower redox tension in cells since the ratio of GSSG:GSH serves as a sensitive index of oxidative stress (Bains & Shaw, 1997).

As can be seen in Figure 4-10, and in correlation with the previous data in the current study, neither 10 mg/day NAC nor 100 mg/day doses of NAC had any notable effect on the GSSH:GSH ratio compared to control. It was expected that the administration of NAC would increase the levels of GSH. The inability of NAC to increase GSH levels has also been reported in previous studies (Lalitha *et al.*, 1990; Kheir-Eldin *et al.*, 2001; Karageorgos *et al.*, 2006). However, at a dose of 300 mg/day, NAC evoked a significant increase in the GSSG:GSH ratio in favour of the oxidative form (i.e. GSSG). An increase in the oxidized form of glutathione implies an increased consumption of GSH and is indicative of an increased presence of ROS in the immediate environment. This once again confirms the stimulation of ROS with a 300 mg/day dose of NAC administration. However, it should also be borne in mind that, while these data imply that 300 mg/day NAC stimulates ROS, the goal of NAC supplementation is advocated to increase GSH formation with the aim of countering oxidative conditions. Thus, these findings seem to be counter intuitive. However, previous studies (Hultberg et al., 2001) have reported that the administration of NAC (200-4000 ul/L in HeLa cell cultures) is capable of effecting a decrease in intracellular GSH concentrations. The authors suggested that NAC is an easily oxidized reductant, capable of generating oxygen radicals which might explain the consumption of GSH (Hultberg *et al.*, 2001). While one cannot exclude the presence of other ROS on the data provided, the evidence for increased generation of ROS is very likely directly attributable to the increase in superoxide radicals induced by 300 mg/day NAC, as shown in Figure 4-8.

Having now established the behavioural and biochemical effects of chronic NAC under healthy (non-pathological) conditions, the next aim of the study was to access how NAC would perform under conditions of oxidative stress, and particularly whether it is able to reverse behavioural

and neurochemical changes evoked under pro-oxidative conditions *in vivo*, in this case in the rat striatum.

5.4 THE EFFECT OF NAC ON MARKERS OF OXIDATIVE STRESS IN AN ANIMAL MODEL OF TD

Considering the objectives of this study, with the possible exception of the GSSG:GSH data, the data describing the pro-oxidant effects of haloperidol (see § 5.2) have provided convincing evidence for the establishment of an *in vivo* animal model of striatal oxidative stress. This model was then used to investigate the biobehavioural effects of NAC under conditions of increased oxidative stress, and at the same time was used to investigate the possible therapeutic value of NAC in the treatment of neurological conditions characterised by increased oxidative stress, in this case TD. After the weight of each rat was factored in, the doses of NAC administered were as follows: 10 mg/day NAC = 50 mg/kg/day NAC; 100 mg/day = 500mg/kg/day; 300 mg/day = 1500 mg/kg/day, and should be borne in mind when discussing the data.

Even though NAC has been shown to have no biobehavioural effects in healthy animals at doses of 10 mg/day and 100 mg/day, but evoked pro-oxidative effects at 300 mg/day dose, one cannot assume that the same will be true under pathological conditions, especially with substantial evidence suggesting that antioxidants behave differently dependant on the redox environment in which they are administered, including the precense or absence of metals, (Oikawa *et al.*, 1999) different cells lines (Oikawa *et al.*, 1999), dose (Choi *et al.*, 2006; Sahu *et al.*, 2006) and even the age (De La Fuente *et al.*, 2002) of the animals. Therefore the administration of high dose haloperidol that is associated with the induction of experimental TD in rats, and corroborated by increased markers of striatal oxidative stress, together with NAC may change the anti- or pro-oxidant character of the latter and certainly warrants investigation. As a result, the next part of the study investigated the effect of co-administering haloperidol with increasing doses of NAC on VCM behaviour and striatal markers of oxidative stress associated with the TD model.

5.4.1 Behavioural Effects

Using an animal model of TD, characterised by behavioural and neurochemical changes indicative of striatal oxidative stress, viz. increased striatal superoxide and lipid peroxidation as well as increased VCMs (Figure 4-1 to Figure 4-4), the significant increase in VCMs induced by haloperidol treatment was found to be unaffected by concurrent treatment with all doses of NAC

compared to control, including 10 mg/day, 100 mg/day and 300 mg/day doses, as is evident in Figure 4-11. This was a dissappointing finding. Indeed, all comparisons remained significantly higher than control, although there were trends to attenuation of haloperidol effects. In fact, when compared to haloperidol treatment and not control, it was found that co-administration of NAC 100 mg/day, but not 10 mg/day or 300 mg/day, did demonstrate a significant decrease in the number of VCMs, suggesting that NAC at the doses applied in this particular study did have some abrogating effects on the behavioural effects following chronic haloperidol treatment. This can be more readily observed in Figure 4-12, where the time-dependent responses to treatment are depicted. Recently, in a similar designed study (Sadan *et al.*, 2005), which was published during the undertaking of the current study, 1000 mg/kg/day NAC-amide significantly attenuated haloperidol-associated VCMs which was associated with a significant decrease in lipid peroxidation, back to normal levels. It is important to note that the afore mentioned authors used NAC-amide and not standard NAC as was used in the current study. In the current study the same effects were observed with the 100 mg/day NAC dose (500 mg/kg daily; refer to § 3.3.2) as was observed by these authors (Sadan *et al.*, 2005) with regard to the behavioural and lipid peroxidation data (refer to § 2.6.5.2). This dose (100 mg/day) was able to significantly decrease VCMs when compared to the haloperidol treated group and was also able to return lipid peroxidation levels back to basal values (Figure 4-14). The 300 mg/day NAC dose (1500 mg/kg daily) was not able to significantly decrease VCMs and may indicate that 300 mg/day is not an optimal dose. The current study and the study conducted by Sadan et al. (2005), differs from each other mostly in that the current study set out to evaluate the effect of increasing doses of the antioxidant NAC (in contrast to NAC-amide) as well the effect thereof in healthy animals (Sadan studied NAC only in the TD model) in order to evaluate NAC's value as an prophylactic agent.

This attenuation of the behavioural effects of haloperidol by a 100 mg/day dose of NAC is of great interest, especially since haloperidol-associated increased lipid peroxidation was also reduced by this dosage (Figure 4-14). Also of interest is that haloperidol in combination with NAC at a dose of 300 mg/day demonstrated a significant increase in the number of VCMs on days 14, 17 and 19 compared to control, although by day 21 this increase was no longer significant (see Figure 4-12). The data would therefore suggest that high doses of NAC has a bimodal effect on the number of VCMs, with VCMs initially increasing (between day 0 and 19) and then later decreasing (between day 19 and 21) without concomitant lipid peroxidation (Figure 4-14).

Previous studies conducted in our laboratory have implicated NO and NOS in the pathology of haloperidol-induced VCMs. These studies showed that haloperidol suppresses NOS activity and that nitregic activity underlies both ageing and haloperidol-induced VCMs (Harvey & Nel, 2003), while the nNOS-inhibitor, methylene blue, resulted in the worsening of haloperidol-induced

VCMs (Bester & Harvey, 2000). NAC is a scavenger of NO (Rodriguez-Martin *et al.*, 2002) and this scavenging effect, resulting in decreased NO levels may explain why NAC administration failed to attenuate haloperidol-induced VCMs and in fact resulted in the exacerbation of VCMs in normal rats (Figure 4-6 and Figure 4-7), even though lipid peroxidation was significantly reduced in both cases. It is also possible that these VCMs may be more indicative of acute EPS than of TD since they appear to diminish with time (Figure 4-12).

These observations, particularly that of the 100 mg/day NAC dose, may suggest some promise for the administration of NAC to protect against haloperidol-induced toxicity. However, the effect of NAC (especially 10 and 300 mg/day doses) on VCMs may require a longer investigation period in order to determine whether these VCMs are only transient and do eventually diminish with time.

5.4.2 Neurochemical Effects

5.4.2.1 Effects on Superoxide Radical Formation

In keeping with its *in vivo* modelling of a pro-oxidative state, the haloperidol-TD model was associated with a significant increase in striatal levels of superoxide, as is evident in Figure 4-13. Co-administration of NAC at all doses fully reversed haloperidol-induced free radical production. Of major interest, however, was that while NAC when administered alone at low doses did not affect basal levels of superoxide, high doses of NAC actually increased these levels (see Figure 4-8). However, when co-administered with haloperidol, NAC is able to act as an antioxidant and to diminish the formation of superoxide radical following haloperidol treatment at both low doses and, most interesting, also at high dose (refer to § 2.6.7 for NAC's ability to act as an antioxidant). These data strongly suggest that NAC should be able to attenuate cellular damage induced by superoxide following chronic treatment with haloperidol. Under this premise, the effects of concomitant NAC administration on cellular levels of lipid peroxidation in striatal tissue of rats treated chronically with haloperidol were studied next. Since the TD model was associated with evidence for lipid peroxidation under these conditions of raised striatal oxidative stress, a successful response to antioxidant therapy would be the reversal or prevention of oxidation-induced cellular damage. The important question then is whether NAC is able to reverse lipid peroxidation evident in the TD model?

5.4.2.2 Effects on Lipid Peroxidation

Indeed, as was highlighted earlier in Figure 4-4, chronic haloperidol administration in the TD model evoked a significant increase in lipid peroxidation the striatum of rats. As is evident in Figure 4-14, while the 10 mg/day NAC dose was unable to effectively reverse the effect of

haloperidol on lipid peroxidation, the administration of 100 mg/day and 300 mg/day doses of NAC were both able to abolish lipid peroxidation in such a manner that these dosage groups did not differ significantly from control. Despite these very distinct effects on haloperidol-induced lipid peroxidation, the 100 mg/day NAC dose narrowly missed significance against the haloperidol-treated group (Figure 4-14; $p>0.0697$). Clearly, the dose of NAC had a role to play, with NAC at 10 mg/day not being sufficient in its antioxidant abilities to protect against haloperidol toxicity. Even though NAC at 10 mg/day showed a significant decrease in superoxide radical production compared to the haloperidol-treated group, it was not able to decrease lipid peroxidation. This is an interesting paradox, but could possibly indicate that with regard to haloperidol-induced lipid peroxidation, it is not only superoxide but also other ROS that may play a role in the induction of lipid peroxidation. In fact, H_2O_2 has also been shown to be increased by haloperidol administration (Arnaiz *et al.*, 1999), such that any or all of the reactive species may have a contributory role in lipid peroxidation following haloperidol treatment. A dose of 10 mg/day NAC, while effective in scavenging superoxide, might have been insufficient to protect against other ROS, although the antioxidant effect of 100 mg/day of NAC and 300 mg/day of NAC have proved to be adequate.

NAC may protect against oxidative stress by two mechanisms, one being a direct anti-oxidant action, which has been demonstrated in the studies above, and the second involves the replenishment and bolstering of glutathione levels. The next series of experiments was aimed at studying the effect of haloperidol with and without concomitant NAC administration on reduced (GSSG) and oxidized (GSH) glutathione levels in rat striatum.

5.4.2.3 Changes in the Glutathione Redox Balance

The next experiment set out to determine the role of NAC as a glutathione precursor and whether it was able to alter the GSSG:GSH ratio in the striatum of rats treated chronically with haloperidol in combination with different doses of NAC (Figure 4-15).

As depicted in Figure 4-5 and as highlighted earlier (refer to § 5.2, specifically that pertaining to the GSSG:GSH data), haloperidol alone did not alter the GSSG:GSH ratio compared to control, a somewhat unexpected result given the very robust increases in superoxide and lipid peroxidation induced by the drug (see Figure 4-3 and Figure 4-4). A low dose of NAC (10 mg/day) similarly did not alter this ratio significantly. However, both 100 mg/day and 300 mg/day doses of NAC administered concurrently with haloperidol significantly increased the GSSG:GSH ratio in rat striatum, suggesting a shift in favour of the oxidized form of glutathione (GSSG) in the presence of high dose NAC. Thus, there is an increased consumption of GSH, indicative of increased ambient levels of ROS.

Even though 100 mg/day and 300 mg/day doses of NAC proved to be protective against haloperidol-induced toxicity by protecting against lipid peroxidation, the failure of NAC alone to increase GSH may suggest that the protective effects of NAC are not directly due to its ability to increase GSH synthesis, but rather attributed to its potent ROS-scavenging properties. This observation has been most convincing in the current work and has also recently been corroborated by others (Wang *et al.*, 2006). However, another possible explanation is that GSH was consumed through the scavenging of superoxide. Co-administration of both 100 mg/day and 300 mg/day doses of NAC with haloperidol was associated with a significant decrease in the levels of superoxide radical formation compared to haloperidol administration alone (Figure 4-13), which was also accompanied by an increase in the oxidized form of GSH (GSSG), as depicted in Figure 4-15. Thus it is logical to assume that GSH was consumed due to the scavenging of superoxide by GSH to form GSSG, which is the major product of GSH oxidation by superoxide (Jones *et al.*, 2002). Thus, although increased consumption of GSH is indicative of increased ambient levels of ROS following haloperidol treatment, increasing levels of GSH provided by NAC aids in the successful channeling of ROS to reacting with GSH to form GSSG and in this way assisting in limiting the deleterious effects of ROS formation and as a result limiting oxidative damage, as noted by reduced superoxide and lipid peroxidation at these dosages of NAC.

The current study supports the theory that the effects of antioxidant therapy is largely dependant on the biological system in which it has been administered and on the absence or presence of oxidative stress, as well as on the antioxidant dose (Wang *et al.*, 2006). It is precisely these characteristics of antioxidants that make them double-edged swords, potentially beneficial under certain circumstances while harmful under others, making it impossible to assume their possible therapeutic role in disease without proper investigation.

5.5 CONCLUDING REMARKS

This study can essentially be divided into 3 sections, namely the validation of an *in vivo* animal model of oxidative stress with valid criteria for TD, a dose-exploratory study of NAC alone on motor-behaviour and correlated with striatal markers of oxidative status, and finally an investigation into the potential therapeutic efficacy of NAC in reversing the behavioural and neurochemical pathology associated with an animal model of TD.

One of the most noteworthy observations from this study was that antioxidants, in this case NAC, may act differently (as an antioxidant or as a pro-oxidant) depending on their application in healthy animals or animals presenting with pathology associated with oxidative stress. The

ability of NAC to act in this manner was also demonstrated by the current study to be related to the dose at which it is administered.

Thus, while an intermediate dose NAC (100 mg/day) was effective in reversing the behavioural pathology associated with TD in the VCM model, on its own at a dose of 300 mg/day NAC worsened striatal dysfunction with distinct evidence for an aetiological role for increased superoxide generation. However, 300 mg/day NAC has the ability to decrease lipid peroxidation in both healthy statres, and under conditions of oxidative strress, although different mechanisms are very likely involved in these two scenarios. In healthy states, 300 mg/day NAC, acts as a pro-oxidant by increasing superoxide radical formation which in turn is able to activate a protective mechanism and so protect against oxidative stress and the ensuing lipid peroxidation. However, with regard to haloperidol-induced oxidative stress in the TD model, 300 mg/day NAC displays antioxidative properties as shown by the decrease in superoxide radicals, when compared to the haloperidol control, and a resultant abolishment of increased lipid peroxidation associated with the TD model.

It is however, important to note that none of the doses of NAC proved to be effective in reversing VCMs evident in the TD model, with high doses of NAC (300 mg/day) in both healthy rats and rats with TD having an apparent negative effect on motor function. However, both of these doses did show, after the initial worsening of oro-facial movements, a trend to decreasing VCMs especially during the 3rd week of treatment. It would seem that high doses of NAC may adversely effect motor regulation, despite beneficial effects on striatal markers of oxidative stress, the exact mechanisms of which require further study. The increase in VCMs induced by NAC was not associated with increased lipid peroxidation as was the case in haloperidol-induced VCMs, in which lipid peroxidation levels were significantly raised. This inability of NAC to induce cellular damage, as indicated by lipid peroxidation, may suggest that the VCMs induced are also only transient, especially since VCMs seem to decrease in the 3rd week, possibly as a result of a reactive protective mechanism following on the raised superoxide levels induced by NAC.

Haloperidol treatment did not have any effect on the GSSG:GSH ratio, which was unexpected since an increase in free radicals will lead to an increase in GSSG at the cost of GSH. However this lack of effect may be related to too short a treatment period and may later have become apparent. In contrast high doses of NAC in haloperidol treated animals caused a significant increase in GSSG levels. This could possibly indicate that GSH was consumed due to the scavenging of superoxide by GSH to form GSSG made possible by increased availability of GSH via NAC adminstration. GSSG is the major product of GSH oxidation by superoxide (Jones *et al.*, 2002).

The ability of NAC to act as either an antioxidant or pro-oxidant may be subject to the local redox environment of the cell or tissue under investigation (Wang *et al.*, 2006). This study is strongly supportive of this phenomenon. When NAC was administered to healthy rats, the animals were in a state of intracellular redox balance. At low doses NAC was able to act as an antioxidant against the natural presence or flux of free radicals and ROS that are a consequence of normal cellular respiration and that are effectively controlled by endogenous anti-oxidant mechanisms. However at high doses, NAC disrupted this state of redox homeostasis through its ability to now act as a pro-oxidant (Wang *et al.*, 2006) with negative effects on the body, as evinced by subtle striatal toxicity and resultant motor dysfunction. However, when NAC was administered to animals with evidence for high oxidative status, in this case animals with TD, that are characterised by a pre-existing imbalance in redox homeostasis (a pro-oxidative state), NAC now showed remarkable effectiveness as an anti-oxidant to reverse cellular markers of oxidative stress, namely elevated superoxide levels and lipid peroxidation, and to readjust the balance back to its previous balanced state of homeostasis. Some, but not marked, suppressive effects on VCMs were observed and can be considered a draw back to the current study. Considering the glutathione data, some degree of benefit may also reside in the ability of NAC to bolster the protective levels of reduced glutathione (GSH).

Chapter 6 Conclusion

Tardive dyskinesia (TD) is a serious and highly debilitating disorder associated with long-term neuroleptic use. It is a motor disorder of the oro-facial region (tongue, mouth and face) although in some cases a more complex form of the disorder may evolve, involving the trunk and limbs (Andreassen *et al.*, 1999). Various hypotheses have been suggested to explain this severely disfiguring syndrome but the precise pathology still remains unclear (Lohr *et al.*, 2003). The free radical hypothesis of TD has in recent years been receiving increased support, while much attention has been given to the potential value of antioxidants to effectively treat TD. However, data in support of antioxidants as effective treatment options are conflicting (Rotrosen *et al.*, 1996; Dorevitch *et al.*, 1997; Adler *et al.*, 1998; Sachdev *et al.*, 1999; Sachdev, 2000; Abilio *et al.*, 2003).

The current study set forth to ascertain whether the antioxidant and GSH precursor, NAC, could be effective as a treatment for TD. The dose-dependant effect of NAC was evaluated in an animal model of TD in which rats were treated chronically with haloperidol. The effect of NAC on its own, in healthy animals were also evaluated.

The data from the various studies may be summarised as follows (see Table 6-1 and Table 6-2):

Table 6-1: Summary of the results obtained in the non-pathological study

	10 mg/day NAC	100 mg/day NAC	300 mg/day NAC
VCMs	No change	No change	Increase
Superoxide	No change	No change	Increase
Lipid peroxidation	No change	No change	Increase
Oxidized glutathione (GSSG)	No change	No change	Increase

Note: In the table, all NAC-treated groups are compared to vehicle treated control groups.

Table 6-2: A summary of the results obtained using a rat model of TD. All groups are compared to control

Effect on:	Haloperidol + NAC vehicle	Haloperidol + 10 mg/day NAC	Haloperidol + 100 mg/day NAC	Haloperidol + 300 mg/day NAC
VCMs	Increase	Increase	Increase (Decrease when compared to haloperidol + NAC vehicle group)	Increase
Superoxide	Increase	No change	No change	No change
Lipid peroxidation	Increase	No change	No change	No change
Oxidized glutathione (GSSG)	No change	No change	Increase	Increase

Note: In the table, all treatment groups are compared to vehicle treated control groups.

This study has demonstrated the following:

- Chronic treatment of rats with haloperidol induces significant oro-facial dyskinesias which are associated with a significant increase in superoxide radical formation and lipid peroxidation in the striatum of rats, with no significant effect on the GSSG:GSH ratio.

- In the TD model, chronic treatment of rats with NAC did not demonstrate any attenuating effect on haloperidol-induced VCMs compared to control. However, all the NAC doses significantly decreased haloperidol-induced increases in striatal superoxide radical formation, with the 100 mg/day and 300 mg/day doses also significantly decreasing haloperidol-induced lipid peroxidation. This decrease in striatal lipid peroxidation was associated with an increase in GSSG, suggesting that GSH was converted to GSSG by a reactive species and that this effect mediated the decrease in lipid peroxidation. NAC is a GSH precursor and may have increased the levels of GSH thereby increasing the turnover of GSH to GSSH and offering a protective antioxidant effect.

- Normal rats indulge in a very low frequency VCMs with low and medium doses NAC alone having no effect on these abnormal oro-facial movements.

- A high dose of NAC alone increases VCMs in healthy rats. This is associated with an increase in superoxide levels in the striatum, indicating that increased superoxide may have a role to play in NAC associated abnormal VCMs and striatal toxicity.

- A high dose of NAC alone, while increasing VCMs and striatal superoxide radical formation, is able to reduce striatal lipid peroxidation, suggesting that high doses of NAC may be neuroprotective via the upregulation of antioxidant defence systems.

- The current study demonstrated that the antioxidant NAC may have some potential in protecting against haloperidol-induced neurotoxicity. However, the negative effect of NAC on motor behaviour warrants further investigation, particularly by using a longer treatment period.

- Preliminary evidence from this study would emphasise caution in advocating NAC, or any other antioxidant, as a possible preventative measure, or as a prophylactic treatment, against the development of TD in patients on long-term neuroleptic treatment. However, once patients have developed TD, these substances may have distinct clinical value in reducing the tardive motor side effect burden of neuroleptics, even possibly preventing TD from becoming entrenched and irreversible.

Limitations of the study and suggestions for future work:

- One of the major limitations of the current study is that NAC did not show marked reversal of haloperidol-induced VCM's, despite significantly reversing haloperidol-associated effects on striatal oxidative status. Since the behavioural aspect of the study attempts to draw a parrallel with a human movement disorder, namely TD, this lack of behavioural and neurochemical correlation is disappointing. The reasons for this are speculative at present and warrant further investigation.

- A longer treatment period is warranted in order to access the time-dependant effect of NAC over these longer periods.

- In order to ascertain whether protective enzymes are upregulated by increases in oxidative stress, as has been hypothesized in the current study, measurement of antioxidant enzymes such as CAT and particularly SOD would be an interesting focus of future studies.

- Further studies exploring the pro-oxidant character of NAC at high doses should also be considered in order to identify the exact mechanisms by which this supplement engenders this effect.

- The current study has demonstrated that superoxide radicals are increased following a high dose NAC administration. Measurement of other free radicals is also necessary to establish whether NAC affects any of these.

- Identification of the mechanism(s) responsible for the increase in superoxide radicals induced by high dose NAC should be explored.

- Investigation into the effects of NAC on the release and metabolism of neurotransmitters important in regulating movement, especially DA, 5HT, glu and GABA, needs to be carried out.

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