Metallothionein is Implicated in Zn^{2+} Neuroprotection of Cultured Rat Dopaminergic Neurons

By

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Abstract

Metallothioneins are a group of low molecular weight, metal-binding proteins which can be induced or up-regulated upon exposure to metals such as Zn^{2+}, Cu^{2+}, and Cd^{2+}. These proteins, known for their high cysteine content, are believed to possess antioxidant roles and free radical scavenging abilities, and may act as a cofactor with Zn^{2+} to protect cells of mammalian systems. Zn^{2+} is an essential transition metal in biological systems and an important neuromodulator within the central nervous system. While current research suggests that Zn^{2+} is toxic to neurons both *in vivo* and *in vitro*, and does not play a protective role in PC12 cell survival, our laboratory has recently uncovered a survival enhancing role for this essential metal ion in cultured dopaminergic neurons. Here, dissected embryonic day 15 rat midbrain neurons were cultured and treated with Zn^{2+}. In a separate experiment, we cultured the embryonic midbrain neurons with Zn^{2+}, BDNF, and a combination of the two. 14 to 21 day Zn^{2+} and control treated midbrain cultures were also subject to immunofluorescence double labelling experiments to determine whether metallothionein levels are up-regulated following Zn^{2+} treatment. Lastly, metallothionein was combined with dopamine and 6-hydroxydopamine, and their abilities to form covalent adducts *in vitro* were determined via SDS PAGE and Western blotting. These experiments investigate Zn^{2+}'s ability to exude neuroprotective effects, and also to determine if metallothionein protein can be up-regulated upon Zn^{2+} treatment in dissected embryonic rat midbrain cultures and act as a mediator of Zn^{2+}'s protective effects. Here, we were able to demonstrate that Zn^{2+} does in fact exhibit a protective role for dopaminergic neurons, and that Zn^{2+} treatment induces metallothionein protein levels to increase in midbrain cultures. We also illustrate via

arylation studies that metallothionein can form covalent adducts with both dopamine and 6-OHDA, indicating that metallothionein may scavenge dopamine and dopamine metabolites, thereby protecting dopaminergic neurons in culture. These studies hold significant physiological and pathological implications for understanding the underlying mechanisms of neurodegenerative diseases, such as Parkinson's disease.

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Statement of Authorship

Gail Lawrance assisted with photographs of cultured dopaminergic neurons and performed the immunoprecipitation experiments. All other figures, experiments, analysis and written text are my own work except for colleague input for text and figure editing purposes.

Table of Contents

List of Figures

Abbreviations

AA	ascorbic acid
ABC	Avidin biotinylated horseradish peroxidase complex
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
BBB	blood brain barrier
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CATNAP	catecholamine arylated protein
CMF BSS	Ca^{2+} and Mg^{2+} free Gey's Balanced Salt Solution
CNS	central nervous system
CRP-40	catecholamine-regulated protein 40
DA	dopamine
DAB	diaminobenzidine
DOPAC	3,4-dihydroxyphenylacetic acid
DPM	disintegrations per minute
ED15	embryonic day 15
FADD	fas associated death domain
FCS	fetal calf serum
GSH	glutathione
ICE	interleukin-1β converting enzyme
IFDL	immunofluorescence double labelling
IHC	immunohistochemistry
IP	immunoprecipitation
MEM	minimal essential media
MT	metallothionein
MTs	metallothioneins
MT-1,2,3	metallothionein-1,2,3
NGF	nerve growth factor
NT-3	neurotrophin-3
p53	tumor suppressor gene 53
p75	common-low affinity neurotrophin receptor 75
PBS	phosphate-buffered saline
PC12	rat pheochromocytoma cell line 12
PD	Parkinson's disease
PDL	poly-D-lysine
PKC	protein kinase C
PMSF	phenyl-methyl-sulphonylfluoride
ROS	reactive oxygen species
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean

SOD	superoxide dismutase
TBS	tris-buffered saline
TBST	tris-buffered saline + Tween 20
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
Trk	receptor tyrosine kinase
$Zn^{2+}/ZnCl_2$	zinc
ZnT-1,2,3	zinc transporter-1,2,3
6-OHDA	6-hydroxydopamine
·OH	hydroxyl radical

Introduction

This thesis focuses on metal ions (specifically zinc, Zn^{2+}) and the role they play in neurological disease states, such as Parkinson's disease (PD). This study also investigates metallothionein (MT), a protein that strongly binds Zn^{2+} which has recently been implicated as an antioxidant and a potent free radical scavenger in the nervous system. Here, the roles that Zn^{2+} and MT play in protecting neurons that degenerate in PD were explored. Recent evidence has suggested protective roles for Zn^{2+}-bound metallothionein (Zn^{2+}-MT) in neurological diseases (Asanuma *et al.*, 2002; Mendez-Alvarez *et al.* 2002), and even potential therapeutic interventions via MT treatment (Penkowa & Hidalgo, 2000, 2001). This study investigates the ability of Zn^{2+} to protect rodent dopamine (DA)ergic neurons in culture, and also whether MT protein can be up regulated after Zn^{2+} administration, and further to determine the role MT plays in forming covalent adducts with DA and whether this protein may be involved in scavenging toxic DA oxidation products. Understanding the mechanisms of MT activity in the oxidative metabolism of DA (and its modification by Zn^{2+}) may be important to the study of neurological diseases such as PD, where oxidative stress is implicated. Concepts will be introduced in the following order: metal ions and free radicals in the nervous system, Zn^{2+} in the nervous system, cell death (apoptosis), DA-induced cell death, a comparison of DAergic neurons vs. PC12 cells, followed by an introduction to a ubiquitous family of proteins called MT, Parkinson's disease and the role Zn^{2+} and MT play in neurodegenerative diseases.

Metal Ions and Free Radicals in the Nervous System

Over the past decade, there has been a tremendous amount of research in the field of metalloneurochemistry, which is the study of metal ion function in the brain and nervous system (Sayre *et al.*, 1999; Bush, 2000; Burdette & Lippard, 2003). Consequently, an increasing number of investigators are studying the role of specific ions in the neuropathology of neurodegenerative disorders such as PD, Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS). Cu^{2+} and Fe^{3+} appear to be the key redox-active transition metals under current investigation regarding these neurodegenerative disease states. Zn^{2+} is an essential transition metal ion with unique properties compared to the others discussed above due to the fact that it is redox-inactive. Zn^{2+} has a full *d* orbital, and therefore is a redox stable metal. However, it is still implicated in many disease states and is a key player in several of the neurodegenerative diseases.

It is commonly regarded that metal ions are essential to many biological reactions and act as cofactors for numerous enzymes (Sayre *et al.*, 1999). If there are deficiencies in these metals, many different disturbances can occur due to the disruption of homeostasis which may cause damage to the central nervous system (CNS) and other organ function. However, it is more commonly regarded that excessive build-up of these metals in the nervous system can become cytotoxic, which lends the most relevance to the neurodegenerative processes. When redox-active metals build up in the nervous system, there can be a disruption of metal homeostasis which may lead to oxidative stress and consequently, an increase in free radical production such as hydroxyl radical (·OH). ·OH is considered the most harmful component of the reactive oxygen species (ROS)

pathway, because it can inflict impairment to biomacromolecules at diffusion-controlled rates (Sayre *et al.*, 1999). Along with free radical generation, there are other roles that transition metals may hold to evoke neurotoxic damage. Accompanying the generation of ROS, redox-reactive transition metals, along with redox-inert metals (such as Zn^{2+}), may contribute to the major neurological diseases via deleterious effects on protein and peptide structure (Sayre *et al.*, 1999; Bush, 2000). It is understood that many metal regulatory transport systems are energy dependent, and controlled by the blood-brain barrier (BBB). Therefore, two common hypotheses are that, i) damage to the BBB or ii) an energy compromise could lead to unbalanced metal ion levels, possibly instigating protein aggregation and neurodegeneration within the CNS (Bush, 2000).

Clearly, growing evidence supports the theory that many transition metals may be either responsible for, or contribute to, the neurodegenerative disease process, via protein aggregation or oxidative damage in neural tissue. However, each disease holds its own specific pathways of metal-induced damage, with specific protein targets that are vulnerable to the damaging effects (Bush, 2000). For example, in AD, Cu^{2+} and Fe^{3+} are known to induce β-amyloid aggregation, and considerable *in vitro* work has indicated abnormal levels of both of these metals, along with Zn^{2+}, present in β-amyloid deposits (the hallmark neuropathology of AD) (Lovell *et al.*, 1998; Atwood *et al.*, 1999). The exact pathways of neurodegenerative damage that occur in AD are complex and under great debate, but it is clear that these three metal ions can abnormally accumulate and elicit oxidative damage, which can induce β-amyloid deposition in patients with AD. In ALS, it is believed that a mutation of Cu/Zn superoxide dismutase 1 (SOD1), an enzyme which normally acts as an antioxidant within the CNS, can become toxic, allowing

aggregates of SOD1 to damage motor neurons and glia via oxidative stress mechanisms (Bruijn *et al.*, 1998; Kong & Xu, 1998). It has also been suggested that elevated levels of Zn^{2+} and Cu^{2+} may play a significant role in the pathogenesis of the disease (Wiedau-Pazos *et al.*, 1996; Azzouz *et al.*, 2000). Lastly, in PD, current evidence has demonstrated that accumulation of Al^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+} can generate free radicals which may lead to the death of DAergic neurons of the basal ganglia (Dexter *et al.*, 1989, 1991; Riederer *et al.*, 1989; Sziráki *et al.*, 1998). Accumulation of Fe^{3+} deposits within the basal ganglia have been identified in post-mortem PD brains, and it appears that this metal may be responsible for increased formation of the highly toxic ·OH, along with damage to the mitochondrial electron-transport chain which can lead to neuronal death (Dexter *et al.*, 1991; Kienzl *et al.*, 1995; Hirsch & Faucheux, 1998; Floor, 2000).

Zinc in the Nervous System

Zinc (Zn^{2+}) is an important transition metal in biological systems and is critical for the growth and survival of many cells. Zn^{2+} may act as a catalytic, structural or regulatory element of many proteins, or as a signalling messenger that can alter many different membrane channels and neurotransmitter receptors (Choi & Koh, 1998). The nervous system houses large amounts of Zn^{2+}, and the element resides in many different areas of the brain. It is a key modulator within the CNS and is often present as a cofactor bound to proteins involved with gene regulation and metalloproteins (O'Halloran, 1993; Choi & Koh, 1998). Many Zn^{2+}-metalloproteins function to protect cells of the nervous system, providing structural stability, prevention of apoptosis and antioxidant roles such

as free radical scavenging (Erickson *et al.*, 1997; Frederickson *et al.*, 2000). Zn^{2+} interacts robustly with many different elements, such as nitrogen, oxygen and sulphur moieties, but unlike other metal ions such as Cu^{2+} or Fe^{3+}, Zn^{2+} is redox inert, meaning that under physiological conditions it is not reactive and does not promote the formation of toxic free radicals (Berg & Shi, 1996). The average intracellular concentration of Zn^{2+} in the CNS is approximately 150μM (Wallwork, 1987; Frederickson, 1989), while the extracellular concentrations or concentrations in terminal boutons of hippocampal mossy fibres can be as high as 300μM (Frederickson *et al.*, 1983; Assaf & Chung, 1984). Zn^{2+} is often located within the nervous system as one of four different components: it can be bound to membranes or receptors, bound to intracellular proteins, it may exist as free Zn^{2+} within the cytoplasm, or within synaptic vesicles of nerve terminals (Cuajungco & Lees, 1997). The possibility that Zn^{2+} exists as free ionic pools in the CNS is currently unresolved. Zn^{2+} can modulate the activities of many different neurotransmitters and excitatory ions, such as glutamate and Ca^{2+}, and may affect the actions of many different receptors such as GABA, NMDA, AMPA and possibly kainate. Zn^{2+} can also enter postsynaptic neurons and activate protein kinase C (PKC) (Weiss *et al.*, 1989).

Recently, three mammalian Zn^{2+} transporters (ZnT1-3) were discovered and characterized (Palmiter & Findley, 1995; Palmiter *et al.*, 1996a,b). ZnT-1 is a non-energy dependent transporter localized within the plasma membrane and is expressed within the CNS and other organs, while ZnT-2 is suggested to be a component protein of vesicular intracellular compartments which facilitates Zn^{2+} accumulation (Palmiter & Findley, 1995; Palmiter *et al.*, 1996b). ZnT-2 has low affinity for Zn^{2+} and is scarcely present in the brain. Lastly, ZnT-3 was cloned in the mid-1990's and exists in all Zn^{2+}

containing vesicles within the brain, and may be involved in intracellular Zn^{2+} uptake in presynaptic neurons (Palmiter *et al.*, 1996b). Since Zn^{2+} is a stable ion that lacks redox activity, it is generally thought to be non-toxic compared to other transition metals such as Cu^{2+} and Fe^{3+}. Previous studies in our lab as well as others have demonstrated that Zn^{2+} may offer a neuroprotective role in the CNS, by protecting neurons and other cells of the nervous system from apoptosis and other forms of cellular damage (Bray & Bettger, 1990; Ebadi *et al.*, 1996a; Frederickson *et al.*, 2000).

Conversely, it is well documented that the protective effects of Zn^{2+} can be over-ridden at higher concentrations in some cells, notably as a result of injury to the CNS (Kim *et al.*, 1999; Sheline *et al.*, 2000). Studies have indicated that necrotic cell death may result if very high (>500μM) concentrations of extracellular Zn^{2+} is administered, however, lower toxic concentrations (300μM or less) tend to induce programmed cell death (apoptosis) in cortical neurons (Lobner *et al.*, 2000). Zn^{2+} overload can be toxic to many populations of neurons, initiating insults such as disturbances in energy metabolism, increases in oxidative stress, activation of apoptosis cascades, excitotoxicity-induced necrosis, focal ischemic brain damage and seizure-induced neuronal death (Cuajungco & Lees, 1997; Erickson *et al.*, 1997 for review). The exact mechanism of Zn^{2+}-induced neuronal death is complex and not completely resolved, but may be initiated by excitotoxic co-release with glutamate, voltage-gated Ca^{2+} or AMPA channels, or the Ca^{2+}/Zn^{2+} exchanger to induce activation of lipases, endonucleases and proteases (Choi 1990, 1992; Choi & Koh, 1998). Nevertheless, it is commonly regarded that abnormal metabolism of Zn^{2+} in cells can have deleterious effects (Cuajungco & Lees, 1997; Lee *et al.*, 1999; Frederickson *et al.*, 2000). Furthermore, the changes in Zn^{2+}

metabolism that occur during oxidative stress may be important in neurological diseases where oxidative stress is implicated, such as AD, PD and ALS.

Cell Death in the Nervous System

During nervous system genesis, cells undergo many different stages of development, and a very important component to the neurogenic cycle is cell death. Neurons specifically undergo several distinct stages, including induction, differentiation, proliferation, migration, and formation of axonal pathways and synaptic connections (Sastry & Rao, 2000). It has been reported that somewhere between 20 to 80% of all neurons produced during neurogenesis are eliminated by programmed cell death before they reach maturity (Oppenheim, 1991; Deshmukh & Johnson, 1997). Cells that make up the nervous system can degenerate for a myriad of reasons, and over the past few decades, different classifications for cell death have been established. Cell death is a healthy, normal component of differentiation, development and maintenance for multicellular organisms (Columbano, 1995).

In 1972, Kerr *et al.* were first to thoroughly describe two distinct types of cell death: necrosis and apoptosis. Necrosis results from cell injury, and involves the swelling of mitochondria and endoplasmic reticulum, which leads to depletion of energy (ATP) stores, the loss of membrane integrity, along with cell lysis and inflammation. Necrosis often results when cells die from a severe or sudden injury, such as anoxia, hypothermia, physical trauma or chemical damage (Cohen, 1996). Conversely, apoptosis, or programmed cell death as it is known (Figure 1), is a "cell suicide"

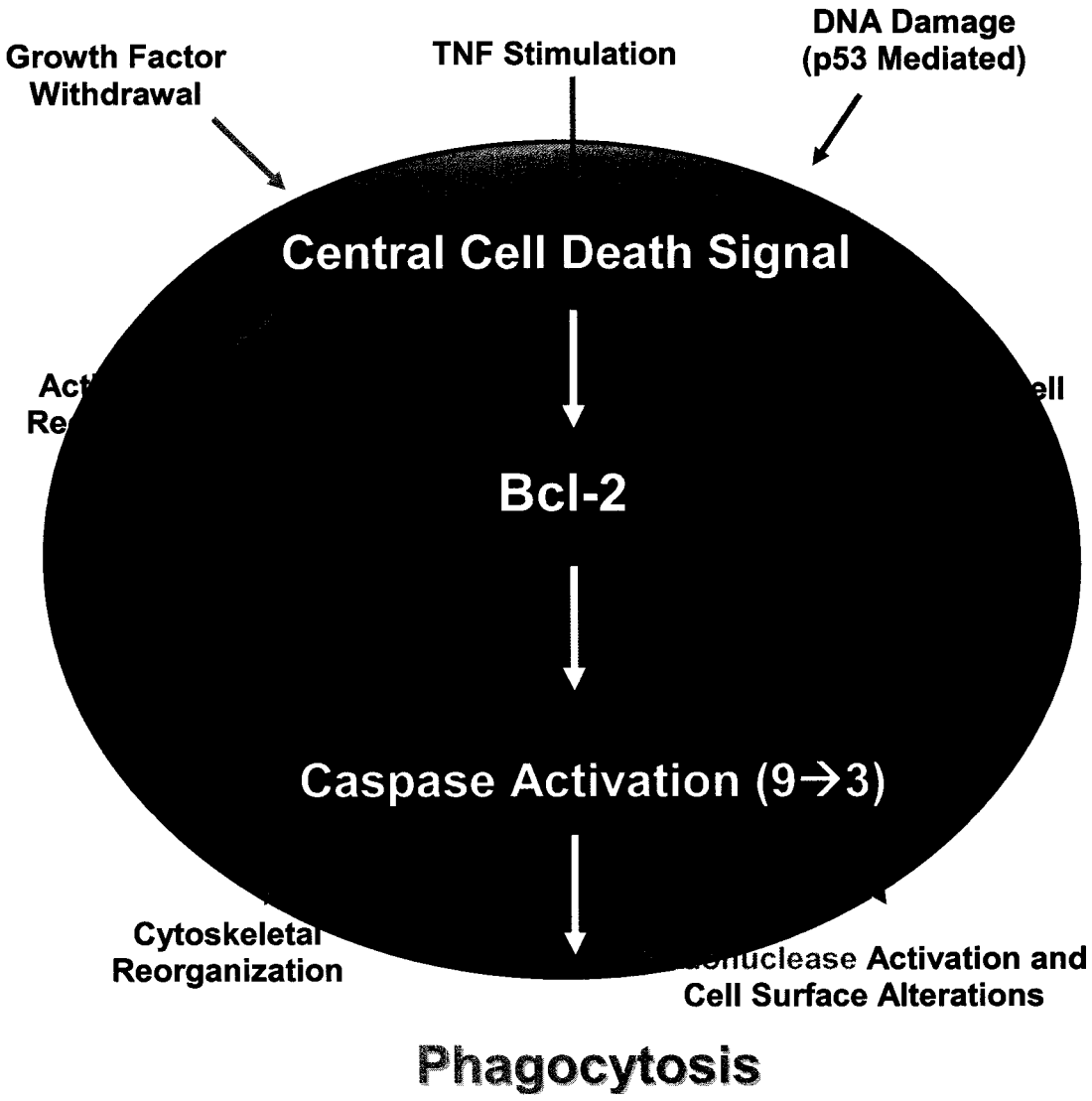


Figure 1. Proposed schematic for the regulation of apoptosis (programmed cell death) (adapted from Thompson, 1995).

mechanism that begins as a cascade of events where cells themselves induce death via activation of endogenous proteases. This cell suicide mission can cause nuclear and cytoplasmic condensation, plasma membrane blebbing, intrasomal DNA cleavage, chromatin condensation and fragmentation, along with phagocytosis (Cohen, 1996; Holtzman & Deshmukh, 1997; Yuan & Yankner, 2000). Apoptosis is critical to the health of many organisms, and is required to maintain normal functioning of the nervous and immune systems. Although the hallmark feature of apoptosis is often DNA cleavage or fragmentation (Wyllie *et al.*, 1980), it has been recently reported (e.g. within the past decade) that this type of damage may not be an essential marker of programmed cell death (Barbieri *et al.*, 1992; Xu *et al.*, 1996; Cuajungco & Lees, 1997).

Much of the current research regarding apoptosis in mammalian cells originated from elegant experiments conducted on the nematode *C. elegans*, which is an excellent model and miniature version of the mammalian apoptotic pathway. There are three main cell death genes that were discovered in *C. elegans*: ced3, ced4, and ced9. ced3 and 4 are pro-apoptotic, and ced9 is anti-apoptotic. The mammalian homologue to ced3 is interleukin-1-β-converting enzyme (ICE), while ced9 corresponds to bcl-2 (Yuan & Horvitz, 1992; Yuan *et al.*, 1993; Hengartner & Horvitz, 1994; Vaux *et al.*, 1992; Deshmukh & Johnson, 1997). ICE is known today as a member of the caspase family. Caspases are a novel family of intracellular cysteine proteases that developed their name due to their ability to cleave the target protein following a C-terminus aspartic acid residue. Normally, caspases exist in cells as inactive proenzymes, but proteolytic processing at a few specific sites can enable them to destroy cell machinery and trigger cell destruction, especially within the cell nucleus (Hengartener, 1998; Thornberry &

Lazebnik, 1998 for review). It is not entirely clear how caspases kill cells of the nervous system, but it is generally understood that they can function either by direct cell structure disassembly, or by influencing upstream regulatory events. A hallmark feature of caspases and their involvement in apoptosis includes the inactivation or deregulation of proteins implicated in DNA repair, mRNA splicing, and DNA replication (Rheaume *et al.*, 1997; Cryns & Yuan, 1998).

The apoptotic pathways are complex and controversial, and several have yet to be elucidated. What is certain is that there are several pathways *leading* to apoptosis, the induction phase, which is highly regulated and involves a series of proteins, receptors and enzyme cascades. There are multiple triggering mechanisms, such as Fas, cytokines (e.g. tumor necrosis factor [TNF]), growth factor withdrawal (e.g. NGF) and p53-mediated DNA damage which may activate death receptors such as FADD, TNFR1, TRAFs and TRADs. Activation of these receptor-associated proteins may then induce a "central cell death signal" leading to Bax, Bcl-2 and caspase activation, as well as endonuclease induction, cell surface alterations, cytoskeletal reorganization and eventual phagocytosis (Thompson, 1995). Apoptosis is crucial in molding the nervous system's final appearance and function and consequently, when apoptotic homeostasis is altered in mammalian systems, undesirable pathological insults can occur. Too little apoptosis can result in cancer or autoimmune disorders, and too much apoptosis may lead to chronic neurodegenerative disease. Many experimental conditions and agents can induce apoptosis within the nervous system, such as: amyloid β-peptide, oxidative stress, ischemia, hypoxia, nitric oxide accumulation, lipids such as ceramide and cholesterol, ultraviolet irradiation, Ca^{2+} ionophores, neurotoxicants such as MPP^{+} and cocaine, DNA

damaging agents and the neurotransmitters DA and glutamate (Sastry & Rao, 2000). Along with the above compounds and conditions, enhanced transcriptional factors such as c-Jun, c-Fos and cytochrome c have all been implicated as apoptotic inducers.

Cytochrome c's implication in apoptotic induction is twofold. It is part of the mitochondrial electron transport chain and appears to activate caspase cascades if Bcl-2 induces its release from the mitochondria to the cytosol. A variety of key events in apoptosis can take place in mitochondria. In vertebrate mitochondria, if electron transport and energy metabolism are altered, if there is a loss in mitochondrial transmembrane potential, if caspase-activating proteins such as cytochrome c are released, and if the cellular oxidation-reduction potential is modified apoptosis may result (Green & Reed, 1998).

It has been well documented that apoptosis is an important result of pathological insults such as stroke, myocardial infarction and heart failure, and it is likely that apoptosis plays a significant role in the pathogenesis of many neurodegenerative disorders, including AD, PD and ALS (Thompson, 1995 for review; Wyllie, 1997). Neuronal apoptosis has been presumed to be a mechanism typically occurring in these disease states, yet the actual role of apoptotic cell death in neurodegenerative disorders is a controversial subject due to difficulty regarding precise identification of apoptosis in human tissues (Thompson, 1995). It is difficult to investigate the process of apoptosis during the course of a disease. Furthermore, because in most tissues (including the brain) cells can undergo apoptosis and necrosis simultaneously, it is difficult to detect what type of death the cells endure, along with the possibility that cells under study may be dying simply due to normal programmed cell death.

A study by Mochizuki *et al.* (1996) reported evidence of neuronal apoptosis in the substantia nigra of Parkinson's disease brains. Consequently, another recent study investigated the role of apoptosis in ED14 rodent mesencephalic cultured DAergic neurons. Branton & Clarke (1999) utilized an *in vitro* model to demonstrate that significant numbers of DA neurons died via apoptosis. They also determined that there were increases in cellular debris levels the longer cells remained in culture, and that the debris was likely degraded material from apoptotic cells (Branton & Clarke, 1999).

Dopaminergic Neurons and PC12 Cells – How Similar Are They?

PC12 cells are a clonal catecholaminergic cell line derived from a rat pheochromocytoma tumor, first described by Greene and Tischler in 1976. In 1976, they demonstrated that PC12 cells respond to nerve growth factor (NGF) and have a near-diploid chromosome number of 40 (Greene & Tischler, 1976). There are many reasons why scientists today often utilize PC12 cells to study the mechanisms of catecholamine-secreting (e.g. DAergic) neuronal cells and neurodegenerative diseases, such as PD. Firstly, they are a homogeneous population of cells available in large numbers, and can develop a neuronal phenotype. Researchers can choose to leave the cells undifferentiated, or differentiate them with NGF. Secondly, PC12 cells are often used as a model to study DA neurons because they possess many features that are similar to such cells. PC12 cells have similar processes, organelles, signalling pathways, enzymes and cytoplasmic proteins compared to neurons in primary cell culture. Thirdly, they also synthesize and store neurotransmitters such as DA and norepinephrine, and apoptosis can be induced in these cells in a very similar manner as for DA neurons. Lastly, PC12 cells

may also take up DA, oxidized products of DA such as 6-OHDA, or other toxic metabolites which can generate oxidative stress, leading to cell damage and apoptosis (Offen *et al.*, 1997; Mayo *et al.*, 1999).

Conversely, research with actual DA neurons to study the pathophysiological implications for PD is not so simple. Obtaining a homogeneous sample of DAergic neurons from a dissected midbrain is difficult because it is extremely laborious to isolate only DA neurons, which make up only 0.5-2% of the midbrain cell population (Pardo *et al.*, 1997). Primary culture samples are rich in many different types of cells, including glia, astrocytes and neurons. It is the immunostaining of TH (tyrosine hydroxylase)-positive cells which determines the presence of DA neurons in culture. TH is an enzyme within the pathway of DA synthesis that is a specific marker for cultured DAergic neurons (Pardo *et al.*, 1997).

PC12 cells are often chosen for studies to investigate the mechanisms underlying neurodegenerative diseases such as PD, AD and ALS. Although PC12 cells are a good model for studying underlying mechanisms of neurological disease states, they may not be appropriate for studying the actual degenerative process in Parkinson's patients. Although there are plenty of similarities between these cell types, it must be recognized that PC12 cells do not behave exactly the same as DAergic neurons. Whenever possible, it is best to study mechanisms of PD in actual DA neurons.

BDNF (brain-derived neurotrophic factor) is a neurotrophin that has previously been shown to promote survival of ED15 rat embryonic neurons in culture, along with many other types of cells within the CNS (Hughes *et al.*, 1993; Shimohama *et al.*, 1993; Cheng & Mattson, 1994; Lewin & Bard, 1996 for review). The pleiotropic effects of

BDNF are mediated by two distinct receptor types, namely the tyrosine kinase receptor (Trk) family, and the common neurotrophin receptor p75 (Barbacid, 1994; Ibáñez, 1995). BDNF is metal ion-sensitive, and has been shown to protect neurons against several metabolic and excitotoxic insults (Cheng & Mattson, 1994) as well as conferring protection against the neurotoxic effects of 6-OHDA in DAergic neurons, specifically (Spina *et al.*, 1992).

Dopamine Induced Cell Death

Although there are several different types of neurological insults which may trigger apoptosis and other mechanisms of cell death, the ability of the neurotransmitter DA to auto-oxidize and induce apoptosis is receiving much attention (Hastings *et al.*, 1996; Hastings & Zigmond, 1997; Stokes *et al.*, 1999). The self-destruction of DA and inappropriate activation of apoptosis by either DA alone or its reactive oxidation products/metabolites (such as DA-radicals, 6-hydroxydopamine [6-OHDA], and DA-quinones) may directly initiate cell death or vulnerability in PD. Increasing evidence has suggested that the ROS derived from DA and DA metabolites are a major cause of the loss of DAergic cells in the brains of Parkinson's patients (Jenner & Olanow, 1996; Youdim & Riederer, 1997). If DA is synthesised in DAergic neurons faster than it can be eliminated (which tends to occur in early Parkinsonian stages), a build-up of DA or its toxic metabolites may be responsible for an oxidative stress on the cells due to an increase in free radical production that cannot be buffered by available antioxidants or free radical scavengers (Ebadi *et al.*, 1996b). Recent evidence has also demonstrated that DA auto-toxicity may stem from a defect in DA's regular ability to become sequestered

into synaptic vesicles, which may lead to oxyradical stress and neuronal degeneration (Larsen *et al.*, 2002). The mechanism of this endogenic process for cumulative production of toxic substances begins with the auto-oxidation of DA to semiquinones which leads to the formation of neuromelanin, a black, oxidized polymer which can further damage DA neurons (Graham, 1978; Stokes *et al.*, 1999 for review). The oxidation of DA to produce reactive quinone moieties is capable of covalently modifying and damaging cellular macromolecules, as well as increasing oxidative stress, which may lead to neuronal death. Reactive quinone formation can occur spontaneously, or it may be accelerated by metal ions, such as Mn^{2+} and Fe^{3+} (Stokes *et al.*, 1999). There is a large pool of reduced Fe^{3+} bound to neuromelanin in the striatum which may play an important role in the overproduction of cytotoxic ·OH, making neurons especially vulnerable to oxidative damage. Subsequently, excess reactive Fe^{3+} levels, or decreases in glutathione (GSH) may be responsible for converting hydrogen peroxide (H_2O_2) into harmful, highly reactive free radicals (such as ·OH) which may damage nigral cells directly (Ciccone, 1998). These above mechanisms, potentially the result of DA auto-toxicity and reactive quinone formation, may play a significant role in the pathophysiology associated with PD.

The above mechanism describes a non-enzymatic oxidation pathway, however, as noted above, toxic semiquinones may be formed via harmful enzyme-catalyzed reactions which occur when DA is metabolized to homovanillic acid and 3,4-dihydroxyphenylacetic acid (DOPAC) (Graham, 1978; Ebadi *et al.*, 1996b). These metabolites can generate excessive amounts of H_2O_2, leading to the production of ·OH,

possibly by the Fe^{3+}-mediated Fenton reaction which may lead to neuronal apoptosis (Hastings, 1995).

It is clear that DA auto-oxidation can create overproduction of free oxygen radicals and ·OH, and that these substances may exert chronic, damaging effects on DAergic neurons which could possibly contribute to the pathological process associated with PD and other neurodegenerative diseases. A recent study utilizing PC12 cells as a model of DAergic neurons demonstrated that thiol antioxidants (such as GSH) appear to hold the most protective capacity for rescuing cells from DA-induced apoptosis (Offen *et al.*, 1996). They investigated other antioxidants such as vitamins C and E and found that neither were able to protect DAergic neurons from DA-induced cell death. MT is a possible member of the thiol-related antioxidants, which holds great promise for the theory that Zn^{2+}-MT may be involved in protecting DA neurons from oxidative damage via endogenous free radical scavenging of the oxidation products or reactive metabolites of DA.

Metallothionein – The Wonder Protein?

In 1960 and 1961, two important papers were published by Kägi & Vallee which introduced the science community to a new protein, metallothionein (MT). This ubiquitous protein was first isolated from horse kidney, and appeared to possess high metal content and cysteine residues, along with the ability to bind cadmium (Cd^{2+}). Today, metallothioneins (MTs) (Figure 2) are known to comprise a family of metal ion-binding, low molecular weight proteins (6000 - 7000 Da) unique in properties such as inducibility upon exposure to metals (e.g. Cd^{2+}, Zn^{2+}, and Cu^{2+}), unusually high sulfur and cysteine content (approximately 30%), and heat stability (Aschner *et al.*, 1997; Miles

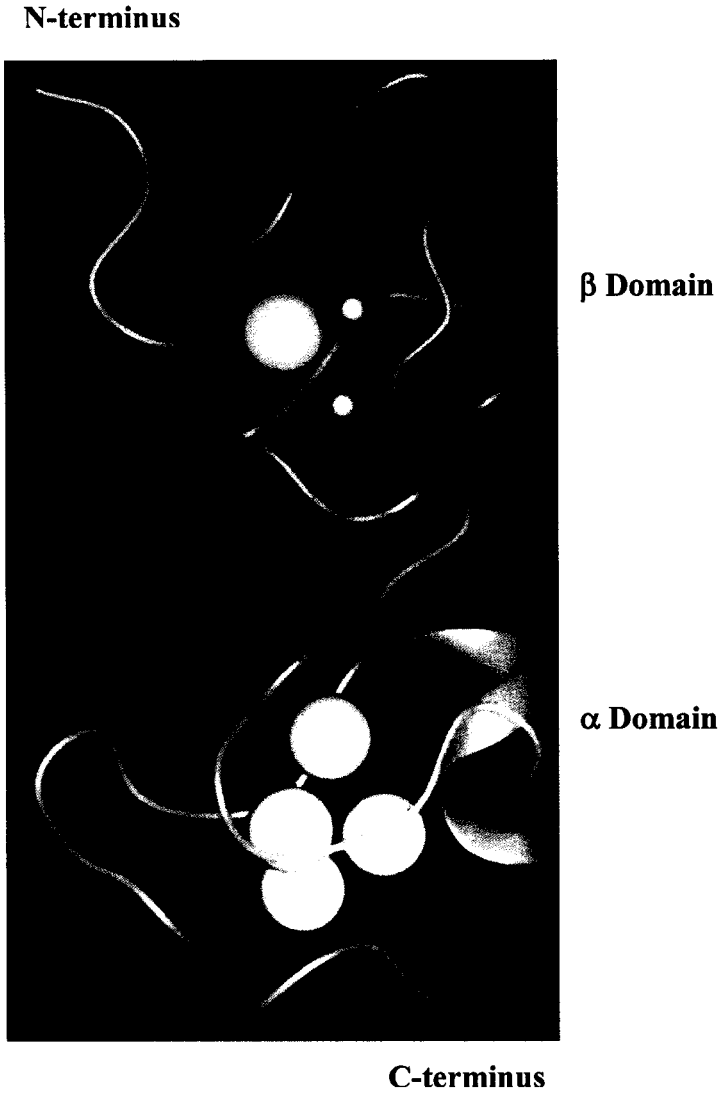


Figure 2. Primary structure of Metallothionein-2 derived from X-ray crystallography (adapted from Dr. Peter Kille, © Cardiff University). The protein is a dumbbell shape with an α domain consisting of 11 Cys residues able to bind 4 metal ions, and a β domain, consisting of 9 Cys residues capable of binding 3 metal ions. In this illustration, the yellow spheres represent Cd^{2+} while the red spheres represent Zn^{2+}.

et al., 2000). MT's are the only known biological compounds to contain Cd^{2+}, and human MT genes appear to be located primarily on chromosome 16, whereas in mice, the MT gene is located on chromosome 8 (Palmiter *et al.*, 1992). These proteins consist of two domains (an α-domain and β-domain) which have almost no contact with each other. The domains contain different numbers of cysteine ligands and are therefore capable of forming metal clusters of contrasting thermodynamic stability and kinetic lability. The stability arises from the specific folding of the peptide backbone which allows the sulfur atoms of cysteine to be provided to metal ions in such a manner that the coordination geometry of the metal ion is met (Otvos *et al.*, 1987; Kelly *et al.*, 1996; Dabrio *et al.*, 2002). The unique composition of MT lends credence to the idea that this protein may be able to form covalent adducts with other compounds and may be arylated (i.e. able to bind covalently to an aryl ring on a compound) by catecholamines like DA, and DA metabolites such as 6-0HDA.

The majority of MT research has been conducted on rodent models, specifically mice. In murine studies, four MT genes/isoforms have been described; MT-1 to MT- 4. However, it has been noted that there are many more genes/isoforms in humans, with current data demonstrating at least ten (Miles *et al.*, 2000; Coyle *et al.*, 2002). MT-1 and MT-2 are widely expressed in most tissues including the CNS, while MT-3 is primarily confined to the brain and MT-4 to the stratified squamous epithelium (Aschner *et al.*, 1997; Miles *et al.*, 2000; Hidalgo *et al.*, 2001, 2002). MT-1, MT-2 and MT-3 are composed of a single polypeptide chain of 61-68 amino acids, and 20 of these are cysteine residues, none of which are either aromatic amino acids or histidines. Amino acid sequences for MT are characterized by Cys-Xaa-Cys and Cys-Cys repeats (Hidalgo

et al., 2001). The most commonly studied mammalian MT's are MT-1 and MT-2. These two isoforms are single chain polypeptides composed of 61-62 amino acids that usually bind seven divalent metal ions (Zn^{2+} or Cd^{2+}), and up to twelve monovalent Cu ions via thiolate bonds (Kägi, 1991; Dabrio *et al.*, 2002). Each cluster is located in a separate protein domain designated α (residues 1-31) and β (residues 32-62) (Kägi, 1991). It has been noted that the metal composition of the specific isoforms depends on the organ and the previous exposure to metals. It is important to note that the sequence of MT-3 is rather different. The sequence itself has 68 amino acids, and all MT-3 sequences contain a conserved Cys(6)-Pro-Cys-Pro(9) motif which appears to be absent from all other members of the MT family (Sogawa *et al.*, 2001). MT-4 has 62 amino acids, however, very little else is known about this subtype at the current time.

It has been suggested that the principle physiological role for mammalian MT is to provide homeostasis of Cu^{2+} and Zn^{2+}, along with maintenance of the intracellular redox status (Bremner, 1987a,b; Miles *et al.*, 2000). However, MTs have very diverse functions in biological systems, and many physiological and biochemical actions of this protein remain to be elucidated. For example, by virtue of free sulfhydryl groups, these proteins have the ability to participate directly in redox chemistry, including the ability to scavenge free radicals, especially ·OH. It is the cysteine residues that are the prime target sites for reactions with free radicals and metal ion binding. In addition to the ability of MT to act as an antioxidant, it is classically considered to have a role in the detoxification of metal ions (including Cd^{2+} and Hg^{2+}, and occasionally Ag^{+} and Au^{2+}) by binding them. MT is constitutively expressed in many tissues under physiological conditions, and can be induced by various stresses, cytokines, growth factors, glucocorticoids, tumor

promoters, metal ions and hormones. It is also recognized to have a critical physiological role in the transport and storage of Zn^{2+} and Cu^{2+} ions (Maret 1995; Coyle *et al.*, 2002). MT is known to be one of the primary metalloproteins that strongly binds Zn^{2+}. Although the liver and kidney harbor the greatest amounts of MT, there are significant levels in many areas of the nervous system, and it is found within glia, astrocytes and neurons (both MT-1 and MT-2 have been localized within neurons of the basal ganglia). MTs are cytoprotective against the damaging effects of oxygen-derived free radicals, and clear evidence that MT, with its high cysteine content, is a significant neuroprotective factor for the CNS has been reported (Ebadi *et al.*, 1996a; Ebadi *et al.*, 1998; Aschner, 1998). Shiraga and colleagues (1993) demonstrated that both Zn^{2+} and MT levels are altered during oxidative stress. The observation that specific areas of the brain high in Fe^{3+} but low in MT levels, such as the striatum, are more vulnerable to oxidative stress (Rojas-Castaneda *et al.*, 1994) lends credence to the concept that MT is a crucial antioxidant and free radical scavenger. Earlier studies have demonstrated that MT-1 and MT-2 are up-regulated in several human neurodegenerative disorders, and that significant up-regulation of MT-1 and MT-2 follows both brain damage and metal ion induction (Hidalgo *et al.*, 1994; Ebadi *et al.*, 2002).

Parkinson's Disease

Parkinson's Disease (PD) is a progressive neurodegenerative disorder that results in the selective loss of nigrostriatal DAergic neurons in the basal ganglia. PD is often characterised as a 'striatal DA deficiency syndrome', and is one of the most common age-related movement disorders today, affecting approximately 1% of the population 65 years of age or older (Ebadi *et al.*, 2001). It is generally understood that DA and its

metabolites are consumed in the caudate nucleus, putamen, globus pallidus and pars compacta of the substantia nigra (Ebadi *et al.*, 1996b). The four main symptoms of PD are tremor at rest, postural abnormalities, muscle (cog-wheel) rigidity, and bradykinesia – a slowness or difficulty in initiating movement (Olanow & Tatton, 1999). PD is a disease of idiopathic origin, therefore the aetiology is still unknown. However, a growing body of evidence today suggests that although PD may be the final outcome of interactions among multiple factors, oxidative stress is likely the key mediator in the acceleration of DAergic neuronal death (Coyle & Puttfarcken, 1993; Ebadi *et al.*, 1996b for review; Jenner & Olanow, 1996; Olanow & Tatton, 1999 for review). Direct evidence for oxidative damage in PD has evolved from studies which report increased levels of malondialdehyde and lipid peroxides in PD substantia nigra (Dexter *et al.*, 1994).

Within the basal ganglia, there are direct and indirect pathways to facilitate or inhibit bodily movement, respectively. It is crucial to maintain homeostasis between the two pathways, in order for neurotransmitters to work properly and for normal movement. It is now commonly regarded that both D1 and D2 DA receptor activation are required for the proper control of movement, and that DA facilitates activity in the direct pathway while inhibiting activity in the indirect route (Smith *et al.*, 1998; Mengual *et al.*, 1999; Sil'kis, 2002). DA is the primary neurotransmitter in the substantia nigra, and it elicits direct actions on DAergic neurons in this midbrain region (Rabinovic & Hastings, 1998; Rabinovic *et al.*, 2000). In PD, metabolism of catecholamines, including DA oxidation, produces ROS and other free radicals, inducing an oxidative stress (Ebadi *et al.*, 1996b; Hastings *et al.*, 1996; Shen & Dryhurst, 1996; Hastings & Zigmond, 1997). For individuals with PD, the loss of DA appears to activate the indirect pathway, which

disrupts the homeostasis of the direct pathway and diminishes its actions, leading to bradykinesia and other abnormal bodily movement. In early Parkinsonism, there appears to be a compensatory increase in DA receptors to accommodate the initial loss of DA neurons (Rinne 1983, Rinne *et al.*, 1983; Hagglund *et al.*, 1987). This may lead to an increase in endogenous DA breakdown and the build-up of DA metabolites, inducing an increase in ROS and eventual apoptosis. A study by Ziv and colleagues (1996) concluded that the most likely route of cell death in PD patients is apoptosis, since experimental cell culture systems have detected characteristic markers of apoptotic degeneration resulting from endogenous DA toxicity.

In Parkinson's patients, GSH levels are substantially decreased in the substantia nigra (Sofic *et al.*, 1992), while Fe levels tend to increase and accumulate (Dexter *et al.*, 1989; Hirsch & Faucheux, 1998). Defects in the mitochondrial electron transport chain have been reported, such as reduction in the activity of complex I in the substantia nigra, which may lead to energy failure, increased oxidative stress and apoptosis (Parker *et al.*, 1989; Schapira *et al.*, 1989, 1993 for review; Berman & Hastings, 1999; Schapira, 2001). There is also a growing body of evidence to suggest that PD may be caused by an inherited sensitivity to environmental factors or endogenously produced toxic agents (Jenner *et al.*, 1992; Di Monte *et al.*, 2002; Gao *et al.*, 2003), which may accelerate the oxidative damage to DA neurons.

The hallmark cytoskeletal pathology for PD is the presence of Lewy bodies, which are intracytoplasmic neuronal inclusions found throughout the CNS in Parkinson's patients. α-synuclein is a protein found within Lewy bodies, and it has recently been reported that the accumulation or mutation of α-synuclein may elicit neurotoxic effects to

DA neurons specifically (Baba *et al.*, 1998; Giasson *et al.*, 2000). This finding is of utmost importance, since it demonstrates that accumulation of α-synuclein may induce endogenous DA levels to become toxic, providing a novel mechanism for the selective loss and vulnerability of DA neurons in PD patients (Xu *et al.*, 2002). This recent study went on to examine the toxic potential of DA and demonstrated that DA may be a potent promoter of apoptosis in DAergic neurons by acting as a source of endogenous ROS. This entire mechanism is believed to be provoked by α-synuclein accumulation or alterations, a process which may be initiated by Zn^{2+} (Xu *et al.*, 2002).

Zinc and Metallothionein: Implications for Neurodegenerative Diseases

Over the past decade or more, it has become increasingly important to understand the mechanisms surrounding the ability of Zn^{2+} to elicit neuroprotective effects in the brain. Recent studies are beginning to indicate that the potential antioxidant properties of this essential transition metal are not due solely to this element's properties alone, instead the focus is now on the induction of proteins which may act as cofactors to aid Zn^{2+} in the scavenging of free radicals and other oxidative damage (Mendez-Alvarez *et al.*, 2002). Regarding the protection of DAergic neurons, this key protein is MT, to which Zn^{2+} firmly binds. There is a close relationship between tissue MT and Zn^{2+} content, and both MT-1 and MT-2 have been localized within DAergic neurons of the basal ganglia (Miles *et al.*, 2000).

Currently, the roles of MT in homeostatic control and regulation of transport and compartmentalization of Zn^{2+} still remain to be elucidated. However, considerable evidence has suggested that metals such as Zn^{2+} and Cu^{2+} can strongly induce both MT-1

and MT-2, and that Zn^{2+} may reduce oxidative stress by binding to MT, decreasing the oxidation of free hydroxyl (·OH) and superoxide radicals which are produced by the xanthine/xanthine oxidase reaction (Thornalley & Vašák, 1985; Sato & Bremner, 1993; Maret 1994, 2000; Maret & Vallee, 1998; Coyle *et al.*, 2002). It is the large number of cysteine residues in each protein that appear to be responsible for the ability of MT to provide redox control of Zn^{2+} (Coyle *et al.*, 2002). Complementing these theories, it has also been determined that MT protects and sequesters Zn^{2+}, ensuring that this transition metal ion does not accumulate and reach excessive levels, which may lead to damage within the CNS and interference with other metal-dependent processes such as Ca^{2+} homeostasis. Conversely, if the available Zn^{2+} levels are low, MT will release more of it, providing evidence that MT appears to modulate the concentrations of intracellular Zn^{2+} (Maret, 2000). MT has multiple binding sites for Zn^{2+} and plays an important role in buffering Zn^{2+} levels within cells (Aschner *et al.*, 1997).

Recent studies utilizing MT-knockout mice demonstrated that a lack of MT increased susceptibility to apoptosis (Kondo *et al.*, 1997), and that over-expression of this protein was found to protect against apoptosis in mouse cardiomyocytes (Wang *et al.*, 2001a, b). Carrasco and colleagues (2000) have determined that MT-1 and MT-2 knockout mice had increased seizures and hippocampal neurodegeneration following kainic acid administration, along with increased levels of chelatable Zn^{2+}, which may contribute to the neurodegenerative process. Studies by Penkowa *et al.* (1999) demonstrated that Zn^{2+}-MT-2 reduced apoptosis in both neurons and oligodendrocytes. Over-expression of MT appears to reduce the sensitivity of cells and tissues to free radical damage (Ebadi *et al.*, 1996b; Lazo *et al.*, 1998). Rojas-Castaneda *et al.* (1994)

illustrated that the administration of 6-OHDA can induce synthesis of MT in the brain, while Shiraga *et al.* (1993) demonstrated that 6-OHDA reduces both Zn^{2+} and MT levels in rat striatum, but not in any other tested brain regions. This work led Shiraga and colleagues to theorize that MT may reduce the effects of ROS and other free radical production, thereby releasing Zn^{2+} to neuronal membranes and/or receptors, lending credence to an antioxidant role for Zn^{2+} and MT (Bray & Bettger, 1990; Sato & Bremner, 1993).

Current research has also demonstrated that MTs appear to protect against neurodegeneration resulting from epilepsy (Erickson *et al.*, 1997) or ischemia (van Lookeren Campagne *et al.*, 1999). Subsequently, the alteration of Zn^{2+} homeostasis and the derangement of Zn^{2+} metabolism may play significant roles in neurodegenerative diseases such as PD, AD and ALS (Cuajungco & Lees, 1997). MT may play an important role in regulating Zn^{2+} levels and its distribution inside neurons which degenerate in the above disease states, along with protecting the CNS from oxidative damage. Experiments are presently underway to investigate the possibility of MT as a therapeutic agent after a recent study demonstrated that MT-2 injections were able to elicit a significant clinical improvement in an animal model of multiple sclerosis (Penkowa & Hidalgo, 2001).

Rationale

The goal of this thesis is to investigate the effects of Zn^{2+} treatment on cultured PC12 cells and cultured DA neurons isolated from rodent embryonic midbrains, and to illustrate the up-regulation of MT protein when neurons are cultured in the presence of Zn^{2+}. The ability of DA and DA oxidation products (such as 6-OHDA) to react with MT and form covalent adducts was explored, as well as whether MT can be arylated by DA within cultured DAergic neurons.

Hypotheses

This thesis will test the following hypotheses:

1. **Zn^{2+} is protective to PCI2 cells.**

   To evaluate the neuroprotective role of Zn^{2+} within PC12 cells, we will culture PC12 cells with or without Zn^{2+}, and after 24 h. administer increasing concentrations of a potent neurotoxin. A colourimetric acid phosphatase assay will be utilised at the end of the experiment to determine whether the Zn^{2+} treatment had a protective effect on the PC12 cells compared to control.

2. **Zn^{2+} is neuroprotective to cultured DAergic neurons.**

   To evaluate the neuroprotective effect of Zn^{2+} on DAergic neurons, we will use dissociated ED15 rodent midbrain cultures to determine whether Zn^{2+}, BDNF or a combination of Zn^{2+} and BDNF are protective for these cells. After 14 or 21 days of treatment, we will perform immunohistochemistry and count TH-positive neurons to evaluate survival of these cells after varying treatments compared to control.

3. **Administration of Zn^{2+} to cultured neurons will cause MT protein levels to become up-regulated.**

   To determine whether MT protein levels are inducible upon Zn^{2+} treatment, we will use dissociated ED15 rodent midbrain cultures, treat them with or without 100μM Zn^{2+} for 14-21 days, and perform immunofluorescence double labelling on the cultures upon completion of experiments.

4. **DA and 6-OHDA will spontaneoulsy arlyate with MT, forming covalent adducts.**

   Both SDS PAGE and Western blotting will allow us to test whether or not MT can form covalent adducts *in vitro* with DA, or DA oxidation products. We will determine that DA or 6-OHDA is able to arylate MT if we can demonstrate a decreased migration or band shift via SDS PAGE or Western blotting. We will also use a well-known reducing agent (GSH) to block arylation and incorporation of DA into MT. Lastly, via MT immunoprecipitation, we will investigate the ability of this protein to act as a substrate for DA arylation within DAergic neurons in culture.

Materials and Methods

PC12 Cell Acid Phosphatase Assay

Rat pheochromocytoma (PC12) cells were cultured on polystyrene flasks containing RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Wisent) in a humidified 5% CO_2 atmosphere at 37 °C. PC12 cells were dissociated from flasks by treatment with calcium- and magnesium-free Gey's balanced salt solution (CMF BSS), centrifuged in a refrigerated centrifuge at 300 × *g* for 10 min, and the pellet was collected. The pellet was resuspended in 3-5mL of RPMI 1640 and 10% FCS, and the cell suspension was counted with a hemocytometer and Trypan blue (which differentiates between viable and non-viable cells). 100µL of the cell suspension was seeded onto pH 8.5 0.1mg/mL poly-D-lysine (PDL) (Sigma) coated 96-well culture plates (Corning) at a density of 10,000 cells/well. Once cells were added to the wells, $ZnCl_2$ (BDH Laboratory Supplies) was added to half the wells to a final concentration of 100µM, and the remaining PC12 cells were left untreated. The cells were then incubated for 24 h. at 5% CO_2 and 37 °C, and the following day cells received 6-OHDA (Sigma) in increasing concentrations (1, 10, 25 and 50µM) where a lane of control cells would be compared with Zn^{2+}-treated cells for each different 6-OHDA concentration. 24 h. after 6-OHDA administration, the neurotoxin was removed and cells were washed twice with 0.1M phosphate buffered saline (PBS). The 0.1M PBS was washed away and 100µL of buffer containing 0.1M sodium acetate pH 5.5, 0.1% Triton X-100 and 10mM p-nitro phenyl phosphate (Sigma acid phosphatase substrate) was added to each well, and cells were then incubated for two hours at 37°C and 5% CO_2. Lastly, the reaction was stopped by adding 10µL 1M NaOH to each well, and a yellow colour was allowed to develop

within the wells. Colour development within the wells was then measured with a microplate reader set at 405nm. The first column on each plate was used as a (blank) control and contained no PC12 cells. Cell viability was determined by assaying acid phosphatase activity which compared the percent of viable cells to the control column. All test compounds were prepared immediately before adding to cultures, and methods of the assay closely resembled those outlined by Connolly *et al.* (1986).

Rodent ED15 Dissection and Cell Culture

As previously described (Pardo *et al.*, 1997), timed pregnant Sprague Dawley rats (Charles River, PQ) were euthanized with halothane and underwent a caesarean section, where the ventral portion of embryonic midbrains from ED15 embryos were dissociated into primary neuronal cultures. On average, 12-18 embryos were isolated and transferred to a Petri dish containing CMF BSS. Embryonic midbrains were dissected under microscope and freed of meninges, and were then transferred to fresh CMF BSS. All experiments were performed in accordance with the Canadian Council of Animal Care (CCAC) and approved by the University Animal Care Committee (UACC). Tissue was then transferred to and gently dissociated in minimal essential media (MEM) (Invitrogen) plus 15% FCS. This cell suspension was then centrifuged in a refrigerated centrifuge at 300 × *g* for 10 min. Finally, the pellet was resuspended in 3mL of MEM, and the number of viable cells was quantified with a hemocytometer and Trypan blue before plating. For immunohistochemistry (IHC) experiments, 10^5 viable cells per cm^2 suspended in MEM and 15% FCS were seeded onto PDL treated sterile glass coverslips (12mm diameter) (Fisher) in 24 well plates (Corning). 100μL of the cell suspension were plated onto each

coverslip, and the cells were incubated for one hour to allow for adhesion. After one hour, 400μL MEM was added to each well and cells were incubated overnight at 37°C and 5% CO_2. The following day cultures were washed once, and then fed with Neurobasal medium (Invitrogen) containing 2% B-27 supplement (Invitrogen), 25μM glutamate, 0.5mM L-glutamine and 0.5μg/mL gentamicin (Sigma). Treatments of 100μM Zn^{2+} and or 50ng/mL BDNF (Peprotech) as required were added at this time and were renewed with each feeding. Cells were subsequently fed with the same medium as above lacking the glutamate, which can be toxic to cells after the first three days of culture. All cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2, and the medium was renewed twice a week for a two or three week period as indicated. Immunofluorescence double labelling (IFDL) and IHC of the midbrain cultures was carried out between the 14^{th} and 21^{st} day after seeding. It was expected that only 0.5-2% of the cells would be TH-positive (Pardo *et al.*, 1997).

Immunohistochemistry and Immunofluorescent Double-Labelling

Upon completion of the 14- or 21-day neuronal culture treatments, cells were fixed onto glass coverslips with 4% paraformaldehyde (Fisher) for twenty minutes at room temperature. The cells were then washed (3 x 5 minutes) with 0.1M Tris-buffered saline (TBS), and then treated with 0.3% H_2O_2 in 0.1M TBS for 30 minutes at room temperature. Coverslips were washed again three times and were then blocked for one hour at room temperature in 10% bovine serum albumin (BSA) and 0.25% Triton X-100 in 0.1M TBS. Cells were then incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-tyrosine hydroxylase antibody [Chemicon] at a dilution of 1:2000) in 3%

BSA, 0.25% Triton X-100 and 0.1M TBS. The following day, cells were washed three times in 0.1M TBS, then incubated with the secondary antibody (biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) at a dilution of 1:500) in 0.1M TBS and 2% normal goat serum (Vector Laboratories) for two hours at room temperature. Cultures were rinsed again three times and then incubated with peroxidase Vectastain Elite ABC reagent (Vector Laboratories) for two hours at room temperature to visualize the antigen-antibody complex. Finally, cells were washed three times and treated with a 0.05% diaminobenzidine (DAB) stain (Vector Laboratories), rinsed one final time with H_2O and mounted onto glass coverslips with an aqueous mounting medium (Geltol, Fisher). Microscopic analysis involved counting TH-positive cells on the entire coverslip.

IFDL followed a similar protocol to the above IHC with minor modifications. The 0.3% H_2O_2 was removed and replaced with a 0.2% Triton X-100 solution in 0.1M TBS on ice for 15-20 minutes to permeabilize cell membranes. Cell cultures were blocked overnight in 4% BSA/0.1M TBS at 4°C and in the morning two primary antibodies (mouse monoclonal anti-MT [Stressgen] at a 1:1000 dilution and rabbit polyclonal anti-TH [Chemicon] at 1:2000) were added for one hour at room temperature. Cells were rinsed three times and incubated with two fluorescent secondary antibodies for one hour at room temperature (Alexa Fluor 488 goat-anti-rabbit 1:500 dilution and Alexa Fluor 568 goat-anti-mouse 1:500 dilution [Molecular Probes]) in the dark. Following immunostaining, cells were washed three times in 0.1M PBS then rinsed with H_2O and later mounted onto glass coverslips with Slowfade. Slides were stored in the dark until further microscopic analysis.

MT Isoforms and MT-1 Adducts

All MT samples (MT-1, MT-2 and MT-1+2), obtained from Sigma and isolated from rabbit liver were initially diluted in H_20 as a 10mg/mL stock solution and stored at –20°C until required for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) or Western blotting. For basic SDS PAGE to identify different MT isoforms (MT-1, MT-2 and MT-1+2), stock solutions were diluted in SDS sample buffer to 1mM and run on 18% tris-glycine gels. To identify the most appropriate isoform for adduct experiments via Western blotting, all three isoforms were initially run on SDS PAGE, followed by a titre of MT-1 in a range of 1.5mM to 100μM. For *in vitro* arylation studies, 500μM MT-1 was mixed with 1mM DA or 1mM 6-OHDA (Sigma). DA and 6-OHDA were first diluted in water as 10mM stock solutions. Samples were mixed on a magnetic stirrer at room temperature for 24 h. as indicated. MT-1 and DA or MT-1 and 6-OHDA was also mixed with 100μM ascorbic acid (AA) (Sigma) or 100μM GSH (BDH Laboratory Supplies) in a similar fashion for a 24 h. period. An arylation time course study for both the MT-1-DA and MT-1-6-OHDA adducts was conducted at 1, 2, 4, 8, 12, 24 and 96 hours. Each sample was added to a standard volume of SDS sample buffer, boiled for 10 minutes to ensure denaturation of all proteins, and stored at –20°C until they were run on an SDS PAGE gel and later transferred to nitrocellulose for Western blotting.

SDS PAGE and Western Blotting

MT samples diluted in SDS sample buffer were loaded into each lane in 10μL aliquots in varying concentrations (1.5 mM - 100μM) next to broad range pre-stained

standards (Bio-Rad), on an 18% tris-glycine gel, and SDS PAGE was run for an hour and a half at 100 volts. Gels were stained with Coomassie blue for analysis or processed further for Western blotting. For Western blotting, upon completion of the SDS PAGE procedure, the proteins were transferred to nitrocellulose at 100 volts for one hour. Following the transfer, the nitrocellulose membranes were soaked in 0.1M TBS then transferred to blocking buffer (0.1M TBS, 5% non-fat dry milk and 0.1% Tween 20) at room temperature for one hour. After blocking, membranes were washed (3 x 5 minutes) in 0.1M TBS + 0.1% Tween 20 (TBST), then incubated with the primary MT mouse monoclonal IgG1 antibody (Stressgen) at a 1:1000 dilution in 1:10 Superblock (Pierce) overnight at 4°C. The next day, membranes were washed once again 3 x 5 minutes in TBST and incubated with secondary antibody (HRP-conjugated sheep anti-mouse at a 1:500 dilution [Amersham]) for 2 hours at room temperature. Membranes were washed one final time (3 x at 5 min. each), added to chemiluminescence solution (Pierce) for 5 minutes and exposed to film.

[^{3}H]-dopamine Immunoprecipitation Studies

Dissociated cells from ED15 rat ventral midbrain were plated to PDL coated wells in 6 well culture plates at 1.0 – 1.5 x 10^5 viable cells per cm^2 and cultured as described above for five or six days. Wells were washed twice in Neurobasal medium containing 0.1mM ascorbic acid and then the medium was replaced with 1.5mL Neurobasal medium containing 2% B-27 supplement, 0.5mM L-glutamine, 0.1mM ascorbic acid and 0.1mM pargyline. Culture plates were placed in a 37°C incubator for 30 minutes before addition of 50nM [7,8-^{3}H] Dopamine (49 Ci/mmol, Amersham). Plates were returned to the

incubator for 24 h. after which wells were thoroughly washed with 5 changes of ice cold 0.1M TBS. Cells were lysed by scraping them into a small volume of lysis buffer (0.1M TBS, 10% glycerol, 1% Triton X-100, 1mM phenyl-methyl-sulphonylfluoride (PMSF), 10μg/mL aprotinin, 1μg/mL leupeptin [Sigma]) and 8-12 wells were combined into 3 ml total volume. The cells were divided equally among 3 microcentrifuge tubes and spun at 4°C for 15 minutes. Lysates were removed to fresh tubes and 5μg antibody for immunoprecipitation (IP) (mouse monoclonal IgG1 anti-metallothionein from Stressgen; or mouse monoclonal IgG1 anti-nerve growth factor receptor from Oncogene Research, a control antibody of the same isotype) was added and left overnight at 4°C. Immunoprecipitates were collected with agarose conjugated anti-mouse IgG1 antibody (Sigma). Agarose beads were thoroughly washed and bound radioactivity was determined by adding the beads to 5mL Universol (ICN).

Calculations and Statistical Analysis

Percent of viable PC12 cells relative to control, from independent experiments were pooled and the results expressed as mean ± standard error of the mean (SEM). For TH immunostained cultured DAergic neurons, statistical analysis was performed using unpaired Student's t-tests for treatment comparisons, and IP [^{3}H]DA data was assessed by ANOVA using the Graph Pad Prism software. Results are expressed as means ± SEM values, and $P < 0.05$ was regarded as significant.

Results

PC12 Cell Acid Phosphatase Assay

Initial experiments (n = 5) investigated the ability of Zn^{2+} to protect PC12 cells. The present data demonstrates that when PC12 cells were exposed to 6-OHDA for 24 h. in the presence of 100μM Zn^{2+}, this metal ion was not neuroprotective to the cells. The colourimentric acid phosphatase assay clearly revealed that upon 6-OHDA administration in 1, 10, 25 and 50 μM concentrations we observed a dose-dependent cell death in PC12 cells over a 24 h. period. There was not a significant difference in cell viability for Zn^{2+} vs. control cells, demonstrating that Zn^{2+} cannot rescue or protect these cells under these conditions (Figure 3).

Immunohistochemical Characterization of Cultured DA Neurons

Cultures were morphologically assessed, and it was determined that ED15 midbrain cultures included different cell types, including glia, astrocytes and several types of neurons. IHC of cell cultures with an anti-TH antibody and DAB staining isolated only TH-positive DAergic neurons within the cultures which were either control, Zn^{2+}, BDNF or Zn^{2+} + BDNF treated (Figure 4). Midbrain cultures were fixed and immunostained with TH antibody and visualized with DAB after 14 or 21 days in culture. Following IHC, morphological examination revealed the appearance of large, densely stained cell bodies with an approximate diameter of 20μM. Cells clearly displayed extensive neurite outgrowth after 14 or 21 days in culture, with projections more than twice the diameter of the cells.

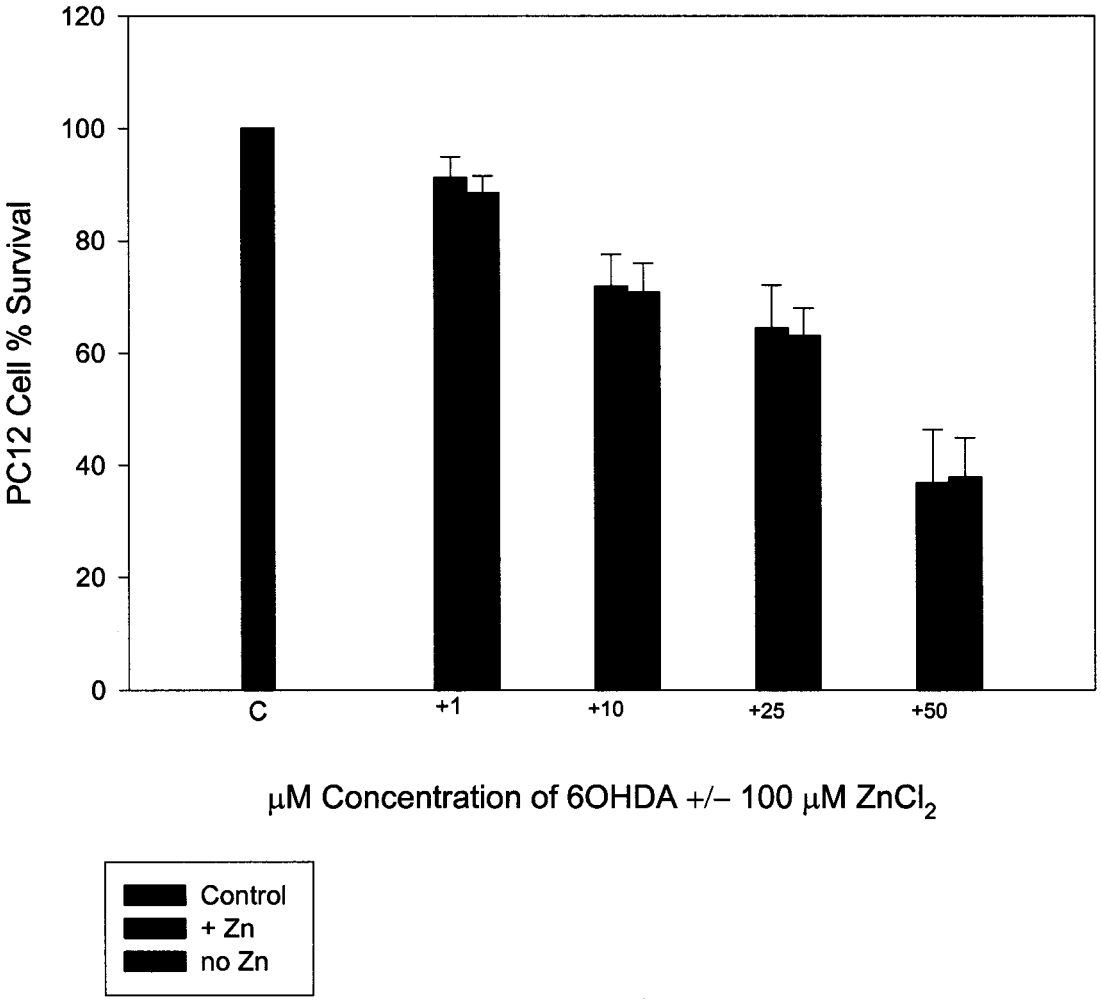


Figure 3. Bar graph depicting the inability of 100μM Zn^{2+} to protect PC12 cells from 6-OHDA treatment in increasing concentrations of 1, 10, 25 and 50μM (n = 5). PC12 cells demonstrate a dose-dependent cell death with increasing 6-OHDA concentrations compared to control over a 24 h. period, as determined by acid phosphatase assay.

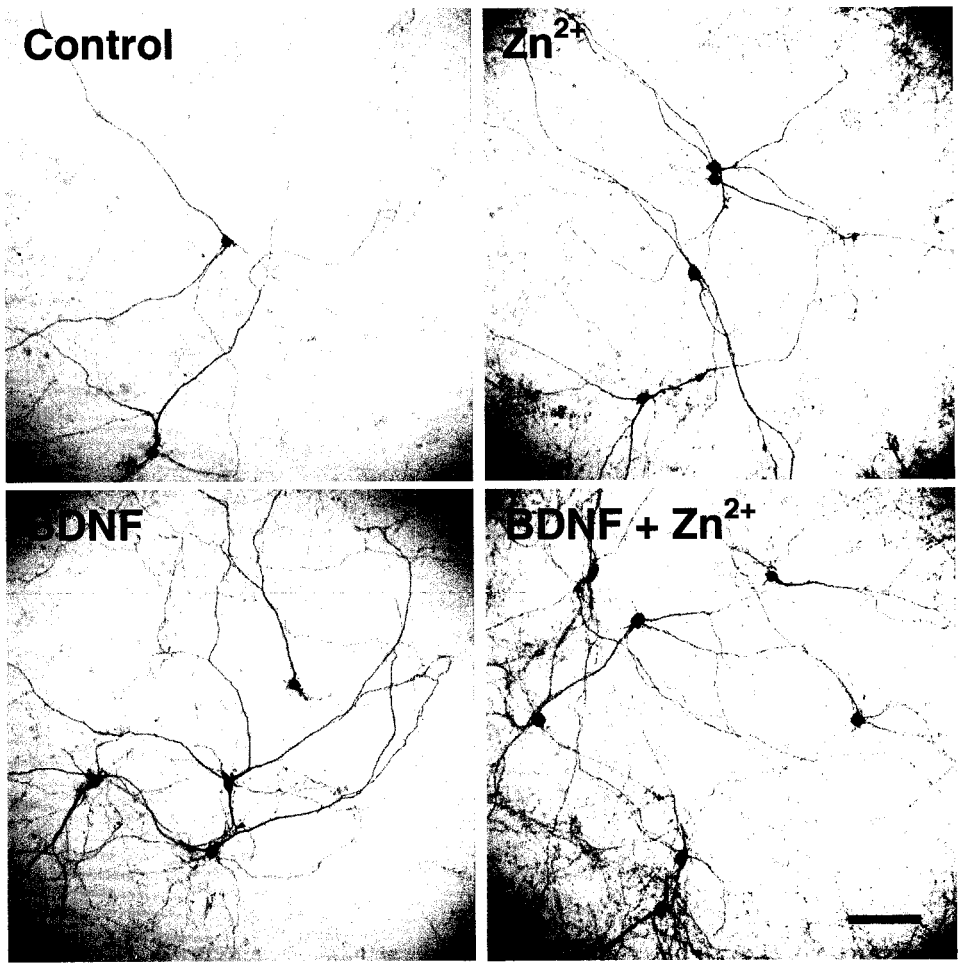


Figure 4. Photographs of DAergic neurons in culture (21 days) stained with DAB IHC to illustrate TH-positive cells. From left to right, top to bottom: Control (medium alone), 100μM Zn^{2+}, 50ng/mL BDNF and Zn^{2+} + BDNF treatments. Scale bar = 100μm

Immunohistochemistry of ED15 Cultured DAergic Neurons

Preliminary studies investigated the effects of 100μM Zn^{2+} added to cultured DAergic neurons from ED15 embryonic rat midbrains. To ascertain cell viability in our midbrain cultures, cells were fixed and then stained with DAB, which stains TH-positive neurons a rich, dark brown colour. TH-positive neurons stained with DAB were quantified under the light microscope, and all neuron counts were performed blindly. All neurons in the microscope field were counted. Results are expressed as percent TH-positive cells relative to control. With Zn^{2+} treatments, we observed a dramatic protective effect on cultured DAergic neurons, compared to control cultures without Zn^{2+} supplementation ($n = 12$, 2 week cultures, Figure 5b; and $n = 5$, 3 week cultures, Figure 5c). The Zn^{2+} treated cultures contained significantly more TH-positive cells than control after both two and three weeks (Student's t-test; $P < 0.05$). As Zn^{2+} is known to alter neurotrophic factor activity (Ross *et al.* 1997), this effect was tested in both the presence and absence of BDNF. In either the absence or presence of BDNF, Zn^{2+} had a significant protective effect on DA neurons in culture, dramatically enhancing the survival of these cells after two weeks in culture ($n = 8$, Figure 5a). All three treatments (Zn^{2+}, BDNF, and the combination of Zn^{2+} + BDNF) resulted in significantly more TH-positive cells than control (Student's t-test; P values for all treatments < 0.03).

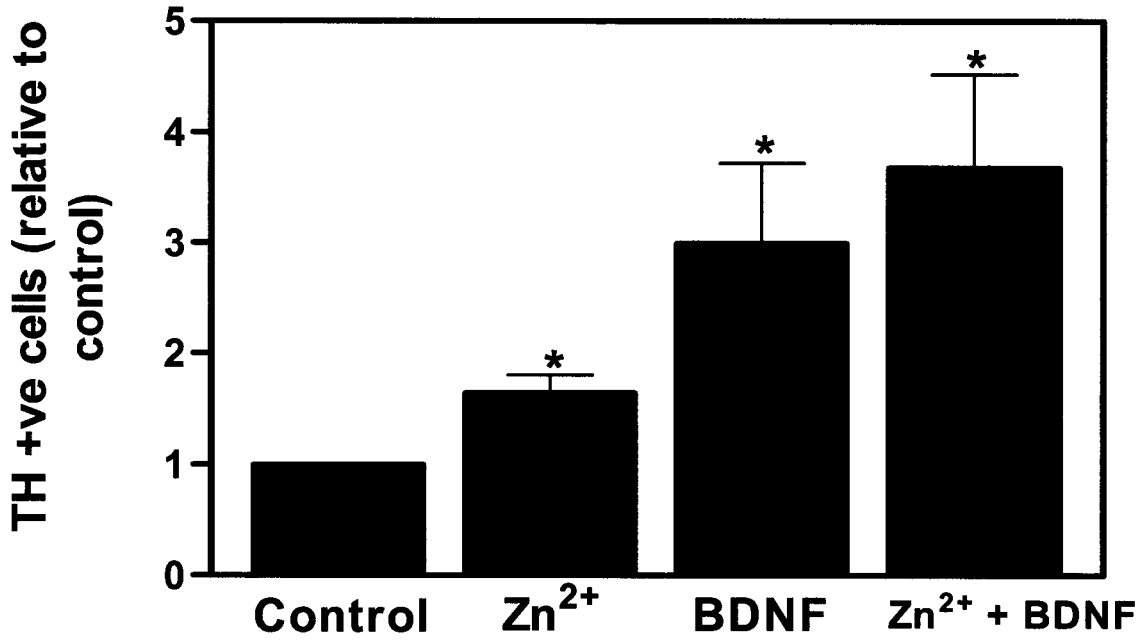
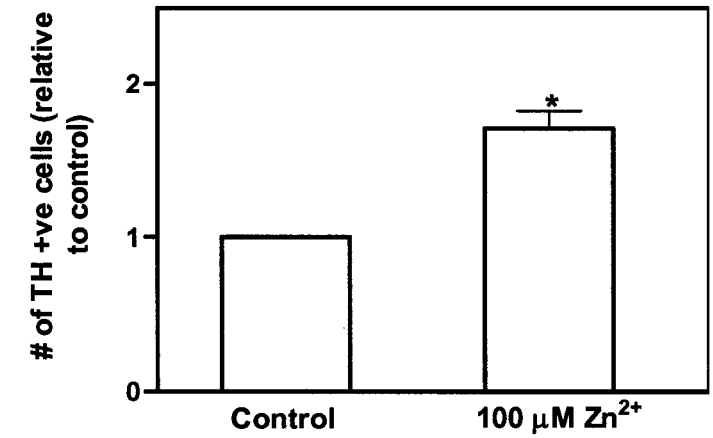


Figure 5(a). Rat ED15 midbrain cells cultured for 2 weeks (n = 8). Cells were either untreated (control), or treated with 100μM Zn^{2+}, 50ng/mL BDNF or both Zn^{2+} + BDNF. The bar graph illustrates the percent TH-positive cells counted relative to control. All three treatments (Zn^{2+}, BDNF, and the combination of Zn^{2+} + BDNF) resulted in significantly more TH-positive cells than control (Student's t-test; P values for all treatments < 0.03 at 95% confidence).

(b)



(c)

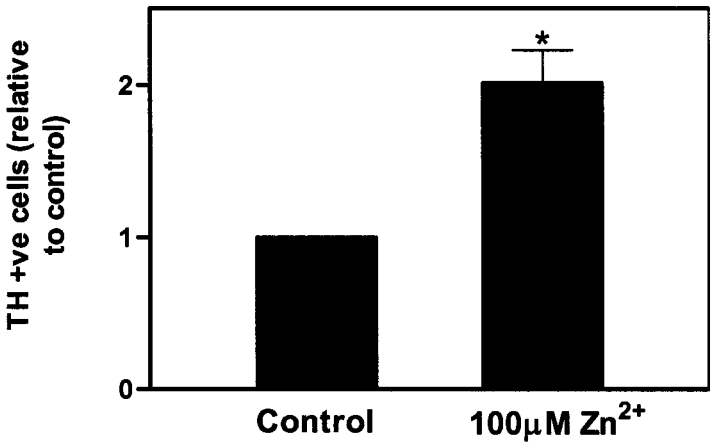


Figure 5(b,c). Rat ED15 midbrain cells cultured for 2 weeks (n = 12) [b, *top*] and 3 weeks (n = 5) [c, *bottom*]. Cells were either untreated (control) or treated with 100μM Zn^{2+}. The bar graphs illustrate the percent TH- positive cells counted relative to control. The Zn^{2+} treated cultures contained significantly more TH-positive cells than control after both 2 and 3 weeks (Student's t-test; $P < 0.05$ for both experiments).

Immunofluorescent Double Labelling of ED15 Midbrain Cultures

The preliminary studies led us to hypothesize that endogenous DA and/or its metabolites were primarily responsible for the basal cell death when Zn^{2+} was absent from the neuronal cultures, and that MT induction may play a role in Zn^{2+}-mediated survival. To test this, we examined MT protein expression in cultures after Zn^{2+} treatment. DAergic neurons were cultured under control or Zn^{2+} treatment conditions for 14 or 21 days and MT protein levels were observed in a qualitative manner by IFDL IHC. The up-regulation of MT via Zn^{2+} treatment was readily observed using fluorescent labelling with both MT and TH of cultured neurons, visualized with a fluorescent microscope with different filters. Immunofluorescence photomicrographs depict both TH (green) and MT (red) staining for control and 100μM Zn^{2+} treated two week cell cultures. Qualitative analysis indicates that MT fluorescent staining is more intense with the Zn^{2+} treatment in all cell populations within the treated cultures. In cultures that were double labelled for both MT and TH, we were not able to visualize co-localization, but did see a more intense MT fluorescence label overall when cells were treated with Zn^{2+} compared to controls (Figure 6).

SDS PAGE and Western Blotting – MT and MT Adducts

Preliminary experiments identified the three different MT preparations available from Sigma: MT-1, MT-2 and MT-1+2 on 18% tris-glycine SDS PAGE gels (Figure 7a,b). Each MT sample was 1mM and 10μL samples were loaded to each well in the SDS PAGE gel. These experiments determined that MT-1 was the most appropriate isoform to use because of the clean single band at approximately 13 kDa. MT-2 showed two bands, and MT-1+2 had several bands with many impurities. For Western blot

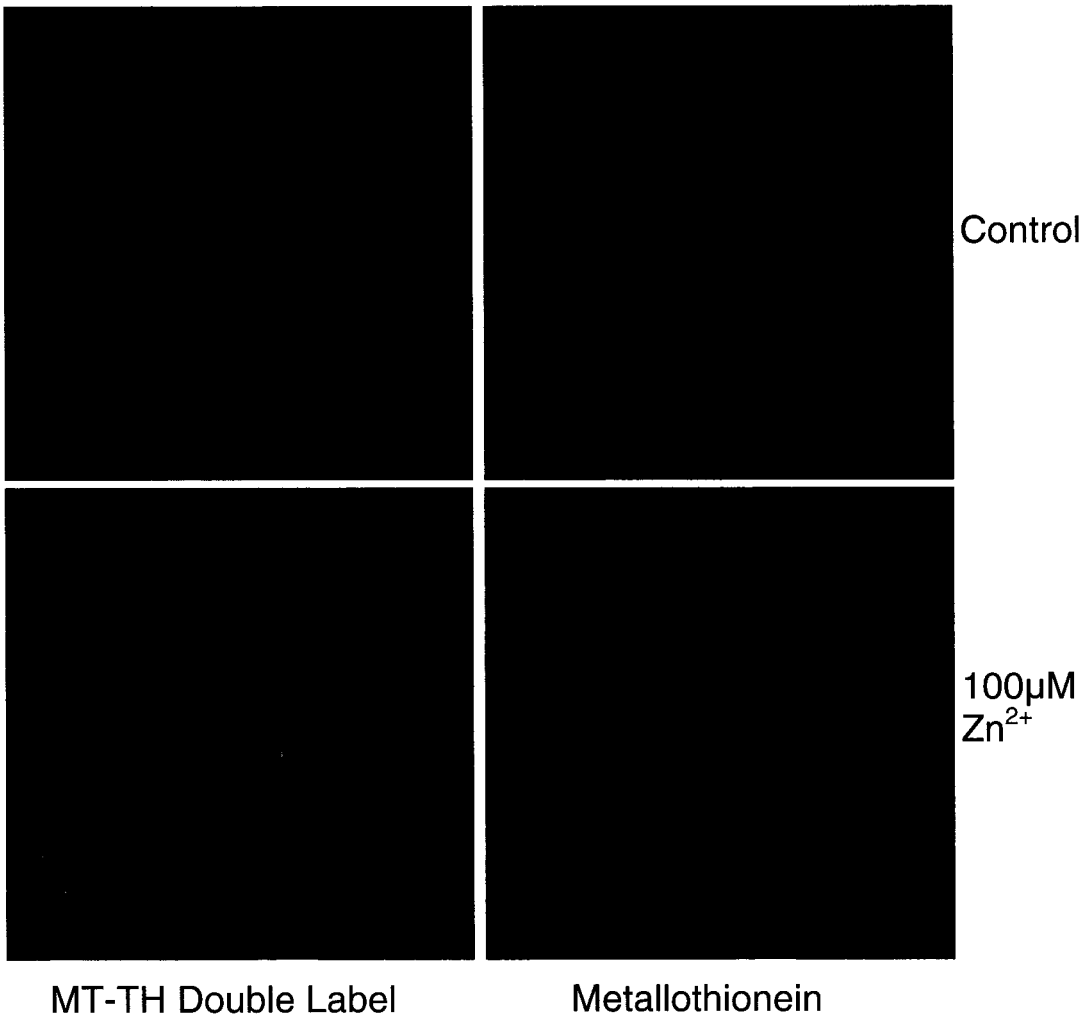


Figure 6. Immunofluorescence photographs depicting labelling of TH (green) and MT (red) staining for control and 100μM Zn^{2+} treated 2 week ED15 midbrain cell cultures. Qualitative analysis indicates that MT fluorescent staining is much brighter with the Zn^{2+} treatment compared to control.

(a)

(b)

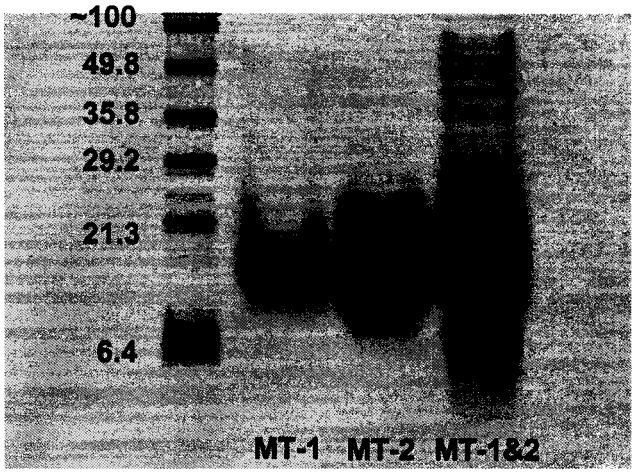


Figure 7(a) [*top*]. 18% SDS PAGE gel stained with Coomassie blue revealing 1mM MT-1+2 and 1mM MT-1 isoforms along with broad range standards to illustrate the molecular weight of MT protein for the two samples.

Figure 7(b) [*bottom*]. 18% SDS PAGE gel stained with Coomassie blue revealing MT-1, MT-2 and MT-1+2 isoforms (all 1mM) along with broad range standards to illustrate the molecular weight of MT protein for the three samples.

experiments, a MT-1 titre with concentrations ranging from 1.5mM to 100μM was conducted to determine the optimal concentration of MT-1 for future adduct experiments (Figure 8). Due to heightened sensitivity of Western blotting compared to Coomassie blue stained SDS PAGE gels, it was determined that the optimal MT-1 concentration was 500μM to compensate for protein degradation during adduct experiments.

Since we were able to illustrate via qualitative IFDL that Zn^{2+} up-regulates MT protein expression in cultured midbrain cells, we hypothesized that MT may be able to scavenge DA oxidation products and metabolites, eliciting neuronal protection. To obtain evidence that MT may have antioxidant or free radical scavenging abilities, we determined whether DA could arylate MT *in vitro*. After 24 h., we observed DA spontaneously arylate both MT-1+2 and MT-1 as determined by a change in migration and a shift in molecular weight of the MT-1+2 protein on SDS-PAGE (visualized with Coomassie blue staining; Figure 9a) and MT-1 protein via Western blotting (Figure 9b, *left*). A time course study allowing the reagents to mix between 1 and 96 hours allowed us to determine the optimal time for maximal arylation of MT-1 by DA to be between 12 and 24 hours (Figure 10). These experiments demonstrate that DA and 6-OHDA can form covalent adducts *in vitro* with MT-1 and MT-1+2, as determined by decreased migration (or upward band shifting) of the MT protein.

As an additional step to explore the nature of DA's ability to form covalent adducts with MT, and also for the reaction to occur through oxidative mechanisms, the reducing agents AA or GSH were added to the reaction. Western blotting of control vs. GSH-containing samples demonstrated an inhibition of arylation and

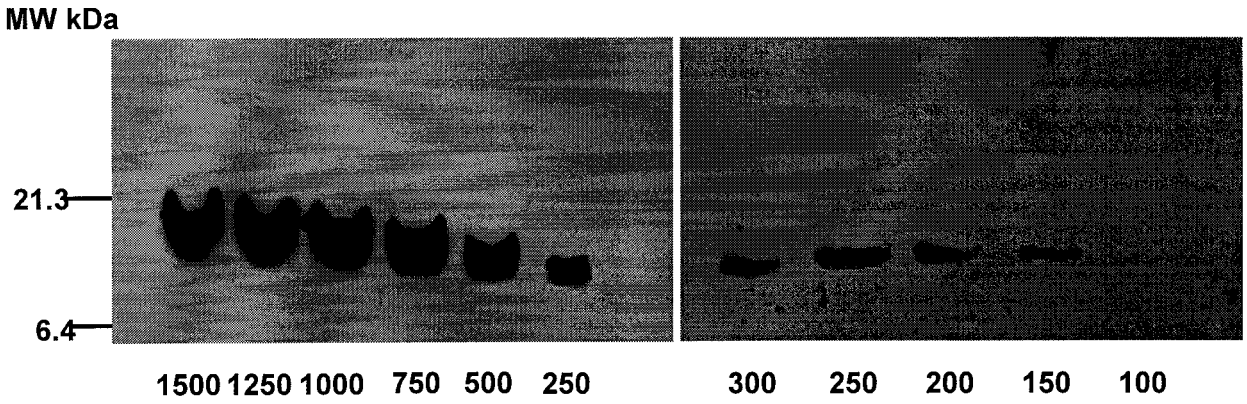


Figure 8. Western blot titre of MT-1 probed with MT-antibody to determine the optimal concentration (in μM) of MT-1 for further Western blot analysis.

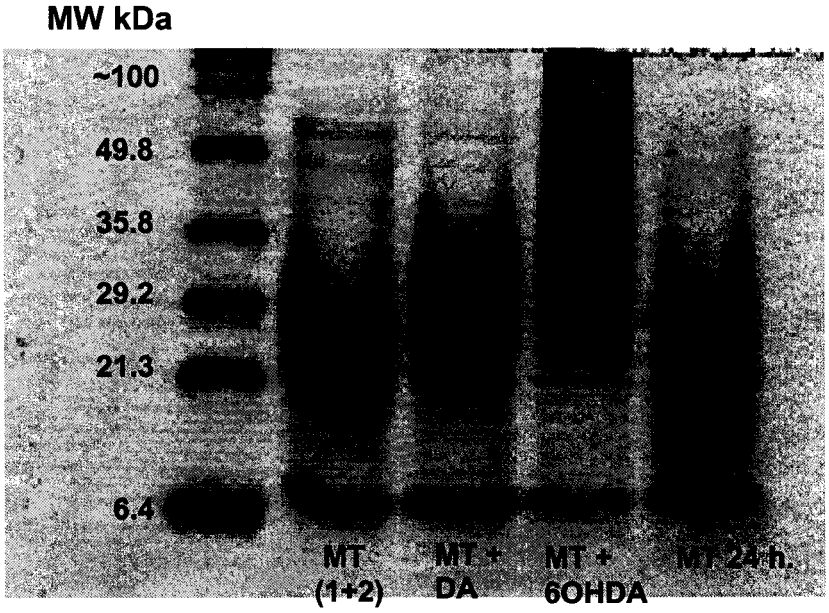


Figure 9(a). 18 % SDS PAGE gel stained with Coomassie blue illustrating MT-1+2, MT-DA and MT-6-OHDA adducts, along with an MT-1+2 24 h. sample for control measures. A clear band shift is noted when MT-1+2 is mixed with DA or 6-OHDA overnight.

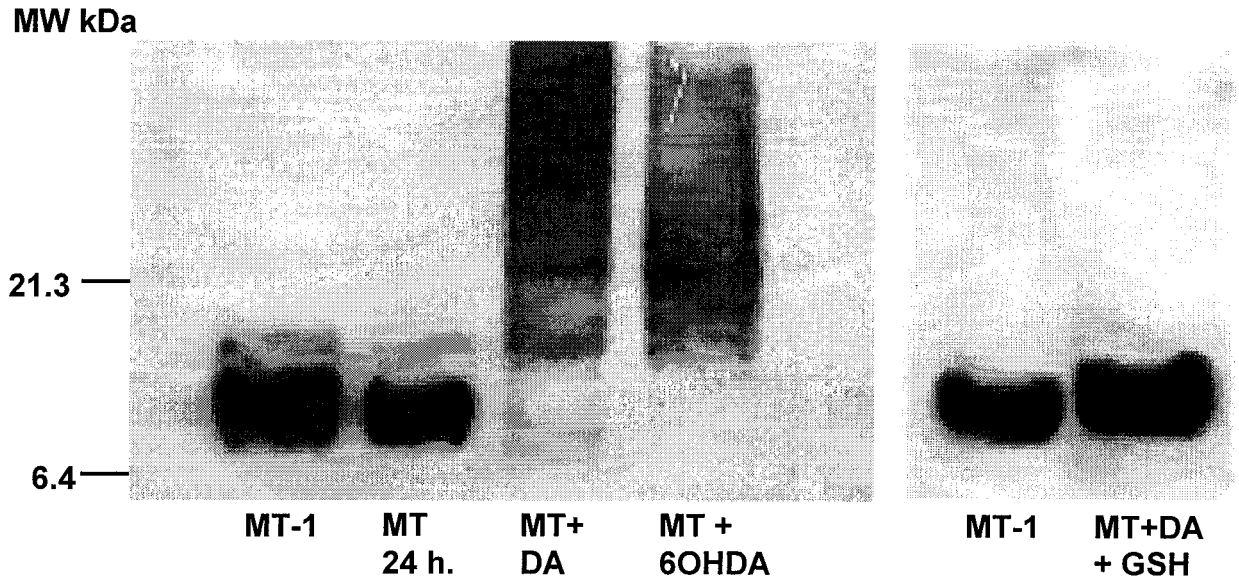


Figure 9(b). Western blot probed with anti-MT antibody illustrating MT-1, MT-DA and MT-6-OHDA adducts, along with an MT-1 24 h. sample for control measures. A clear band shift is noted when MT-1 is mixed with DA or 6-OHDA overnight (*left*). Western blot probed with anti-MT antibody illustrating MT-1 and an MT-DA-GSH sample, both mixed overnight. The presence of 100μM GSH inhibits arylation, and the prominent band shift of the MT-DA adduct (*right*).

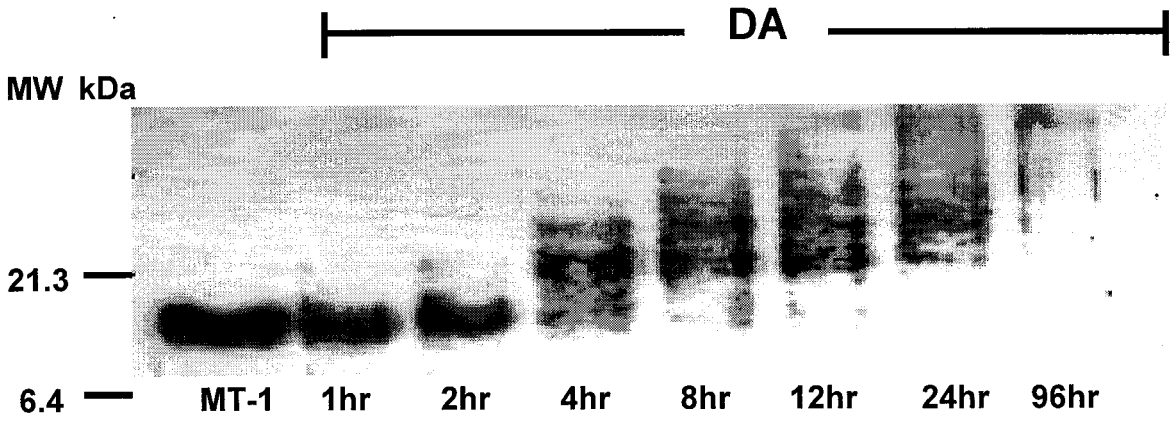


Figure 10. Western blot probed with anti-MT antibody illustrating an arylation time course for MT-DA adducts at 1, 2, 4, 8, 12, 24 and 96 hours. All time course samples were compared to a standard sample of MT, and arylation was determined by a change in migration and a consistent shift in molecular weight of the MT-1 protein.

incorporation of DA into MT-1 (Figure 9b, *right*). However, control vs. AA-containing samples did not inhibit the arylation and incorporation of DA into MT-1 as expected.

[^{3}H]-dopamine Immunoprecipitation Studies

The demonstration that MT could form covalent adducts with DA and 6-OHDA *in vitro* led us to investigate the ability of this protein to act as a substrate for DA arylation within cells in culture. Midbrain cells were treated with [^{3}H]DA, and the ability of MT to covalently incorporate radioactivity was evaluated by MT IP (Figure 11). IP was performed on lysates from ED15 embryonic midbrain cultures which had been allowed to take up [^{3}H]DA for 24 hours. Here, we demonstrate that neurons incorporated significant levels of [^{3}H]DA into MT, suggesting that MT is also arylated by DA within neurons in culture. These results were illustrated by an increased IP of [^{3}H]DA with anti-MT antibody compared to control antibody. Values represent the ratio of disintegrations per minute (DPM) recovered following IP to DPM in the starting cell lysate (mean ± SD, $P < 0.05$ versus control).

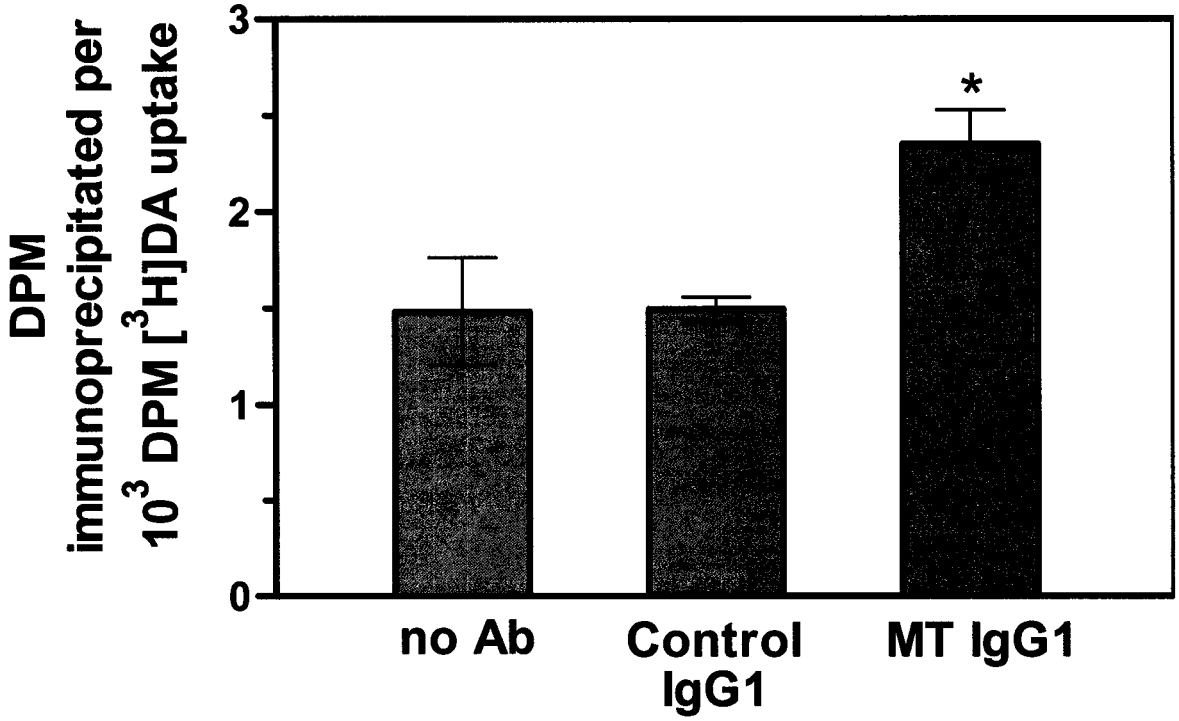


Figure 11. Increased IP of [^{3}H]DA with anti-MT antibody compared to control antibody. IP was performed on lysates from embryonic midbrain cultures which had been allowed to take up [^{3}H]DA for 24 hours. Values represent the ratio of DPM recovered following IP to DPM in the starting cell lysate (mean ± SD, *$P < 0.05$ ANOVA).

Discussion

In the present study, our goal was to investigate the ability of Zn^{2+} to protect both PC12 cells and DAergic neurons in culture. Additionally, we set out to determine whether this metal ion was able to up-regulate MT protein, a mechanism which may have a critical role in aiding the potential antioxidant or free radical scavenging abilities of Zn^{2+}. Furthermore, we investigated whether DA and DA oxidation products can react with MT to form covalent adducts, if MT can be arylated by DA within neurons in culture, and whether the covalent adduct reactions may be blocked by the reducing agent GSH.

Preliminary experiments investigated the ability of Zn^{2+} to protect PC12 cells from the potent neurotoxin 6-OHDA. We revealed that 24 hour Zn^{2+} pre-treatment is not protective to PC12 cells challenged with 6-OHDA. These results closely resemble previous studies in our laboratory which demonstrated that a 24 hour Zn^{2+} pre-treatment did not protect PC12 cells that received DA administration (unpublished observations). Both sets of experiments demonstrate that 6-OHDA or DA (>50μM) poison the cells, and Zn^{2+} is unable to rescue them from the neurotoxic insult. It has been recently reported that 6-OHDA toxicity in PC12 cells is likely associated with both protein degradation and activation of the ubiquitin-proteasome system (Elkon *et al.*, 2001). Preceding this, a key study in 1994 demonstrated that 6-OHDA kills PC12 cells *in vitro* via an apoptotic mechanism (Walkinshaw & Waters, 1994). These authors conclude that because PC12 cells are a neuronal cell line which are often used as a model for DA neurons, and since it

has been reported that the most likely mechanism of cell degeneration in PD is apoptosis, it is probable that DA neurons die apoptotically via specific intracellular mechanisms.

The preliminary studies on PC12 cells led us to explore the possibility that Zn^{2+} may be protective to cultured DAergic neurons. Embryonic midbrain cultures are a good model for studying mechanism and quantification of cell death, as well as characterizing different cell morphologies. Additionally, cultured embryonic midbrain neurons are valuable models since they retain many of their morphological and biochemical properties that are comparable to *in vivo* neurons. Although these were not pure DAergic neurons (mainly because neurons are very sensitive to manipulation and there are such a small number compared to the overall midbrain cell population, which is rich in glia and astrocytes), we did perform IHC experiments that allowed us to stain the TH-positive cells, which are specific markers for DAergic neurons. We compared control cells to 100μM Zn^{2+}-treated cells after both two and three weeks in culture. From these experiments, we demonstrated that Zn^{2+} is able to protect rodent DAergic neurons from cell death and other forms of neuronal degeneration. This was an exciting finding because much of the literature today focuses on the deleterious effects of Zn^{2+} in the CNS, and its toxic potential which may promote neurodegeneration in diseases like AD, ALS and PD. It is important to note that while Zn^{2+} on its own is not generally toxic within the CNS, an accumulation of Zn^{2+} ions may lead to excitotoxic mechanisms. Here, it was demonstrated that Zn^{2+} alone has a protective role for ED15 rodent DA neurons in culture, which is depicted in Figure 5b and c. In another set of experiments (Figure 5a), cultures were treated with BDNF or Zn^{2+}, or a combination of Zn^{2+} and BDNF. These studies were able to confirm that BDNF alone had a very significant

neuroprotective effect, but furthermore, cultures that were treated with Zn^{2+} plus BDNF demonstrated the most profound level of neuroprotection. This was a key distinction to prove that the protective effects of Zn^{2+} are definitely due to the metal ion alone, but may be enhanced by BDNF. Although BDNF is a potent survival-promoting factor for DA neurons, it is a metal ion-sensitive trophic factor whose protective abilities were initially shown to be inhibited by Zn^{2+}. Ross *et al.* (1997) demonstrated that Zn^{2+} was able to induce a conformational change in BDNF, along with NGF and neurotrophin-3 (NT-3), hindering their ability to bind to receptors (e.g. Trk and p75) and elicit a trophic role. Similarly, previous studies in our laboratory investigated the role of BDNF and Zn^{2+} in cultured embryonic rodent motoneurons and demonstrated that a 100μM Zn^{2+} treatment to motoneurons maintained with BDNF elicited a significant increase in apoptotic death (unpublished observations). The experimental results in this thesis are both surprising and exciting because BDNF and Zn^{2+} appear to protect DA neurons in concert.

The ability of Zn^{2+} to protect rodent DAergic neurons in culture is a novel finding. In these studies, it was decided that cultured cells isolated from embryonic rodent midbrains were a more appropriate model for examining the neuroprotective abilities of Zn^{2+}. Here, we were able to administer a precise amount of Zn^{2+} to the cultures and characterize and quantify specific DAergic neuron populations. These studies demonstrate that, although PC12 cells are a good model for understanding potential mechanisms in PD pathogenesis, primary cell cultures dissected from embryonic midbrains may provide further advantages since they likely mimic a more equivalent environment to actual *in vivo* DAergic neurons. One fundamental difference between PC12 cells and DA neurons is that while neurons synthesize and metabolize DA

endogenously, it has been suggested that PC12 cells perform these functions exogenously. This may have important implications regarding the inability of Zn^{2+} to protect PC12 cells. Regarding DAergic neurons, we propose that MT, up-regulated via Zn^{2+} treatment, binds to DA or DA metabolites endogenously to block ROS and toxic quinone formation. Although PC12 cells do contain MT, this specific mechanism may not apply to these cells because it might be strictly intracellular.

There has been much debate as to whether Zn^{2+} is potentially neuroprotective on its own within the CNS, or whether this key transition metal ion acts in concert with various proteins to elicit neuronal protection. Over the past decade, there has been a wealth of evidence to support the theory that a ubiquitous family of proteins called MTs may be partially responsible for the antioxidant and protective properties of Zn^{2+} (Ebadi *et al.*, 1996a; Hussain *et al.*, 1996; Aschner, 1998; Hidalgo *et al.*, 2001; Coyle *et al.*, 2002; Cai & Cherian, 2003). Previous studies in our laboratory demonstrated that when PC12 cells were treated with Zn^{2+}, there was a significant increase in MT mRNA levels (unpublished observations). One of the major aims of this thesis was to determine whether MT protein levels increased with Zn^{2+} treatment. Initial theories revolved around determining MT protein content by comparing Zn^{2+}-treated and control neurons on a Western blot. However, these experimental ideas were soon abandoned due to the fact that it was virtually impossible to isolate only the DAergic neurons from the ED15 midbrain cultures. When this type of experiment was performed using PC12 cells, the Coomassie blue stained SDS PAGE gels themselves were inconclusive for MT protein content, and the Western blots were difficult to interpret due to a high level of cross-reactivity between the proteins within the PC12 cells and the primary MT antibody.

However, success was met with IFDL experiments. Two and three week embryonic ED15 rodent midbrain cultures, control and Zn^{2+} treated, were immunostained for MT protein and TH enzyme. Although we were not able to visualize a co-localization of TH-positive DA neurons and MT, it was clear that upon Zn^{2+} administration, there was a significantly more intense fluorescent MT label throughout all midbrain cells (Figure 6). It is possible that MT protein may be degraded within the DA neurons faster than in other midbrain cell types, hence the lack of co-localization visualized in the IFDL experiments. To date, it is still extremely difficult to quantify exact amounts of MT protein within cultured cells. Methods are debatable and controversial, and plenty are still under investigation (Dabrio *et al.*, 2002; Butcher *et al.*, 2003). However, it was easy to identify qualitatively that there was at least a two-fold increase in MT protein levels compared to controls when the cultures were treated with Zn^{2+} for 14 or 21 days. These experiments provide direct evidence that MT and its isoforms are up-regulated upon Zn^{2+} administration, and our results are consistent with many other studies that have demonstrated that Zn^{2+} can induce MT-1 and MT-2 (Hidalgo *et al.*, 1994; Ebadi *et al.*, 1996a; Agullo *et al.*, 1998; Coyle *et al.*, 2002).

The ability of MT to form covalent adducts with both DA and one of its toxic metabolites, 6-OHDA, provides a possible mechanism for the observed neuroprotection of DA neurons, which involves scavenging toxic DA oxidation products and metabolites. MT's thiolate clusters, rich in cysteine residues, are likely responsible for the interaction with DA and 6-OHDA, along with possible free radical scavenging of DA's toxic metabolites and auto-oxidation products (Thornalley & Vašák, 1985). Since compounds rich in thiols (e.g. GSH) are known to prevent neuronal apoptotic death following many

different stimuli, it is likely that MT interacts directly with DA to prevent quinone covalent modification of macromolecules along with downstream apoptotic cascades and generation of ROS (Stokes *et al.*, 1999) (Figure 12). This *in vitro* adduct experiment complements additional studies which have demonstrated that many proteins are susceptible to covalent arylation by DA, including catecholamine arylated proteins (CATNAPs; Ross *et al.*, 1993; Ross *et al.*, 1996; Modi *et al.*, 1996), one member of which includes catecholamine-regulated protein 40 (CRP-40) (Sharan *et al.*, 2002; Gabriele *et al.*, 2002; Nair & Mishra, 2001). CATNAPs are thought to play an important role in scavenging the highly reactive DA oxidation products, preventing them from undergoing more damaging reactions. Mechanistically it is possible that MT may act as a CATNAP within DAergic neurons, mediating the neuroprotective properties of Zn^{2+} in this cell population. Supplementary experiments determined that GSH, but not AA, was able to block arylation of the MT-DA adducts. AA is a potent extracellular antioxidant (Halliwell & Gutteridge, 1990; Otero *et al.*, 1997), and GSH is a tripeptide that is considered to be one of the most essential intracellular non-protein thiol/sulfhydryl compounds in mammalian cells (Meister, 1988; Bains & Shaw, 1997). The hallmark role of GSH is as an antioxidant and a free radical scavenger. However, it is also a phase II detoxification substrate for glutathione-S-tranferase, is involved in modulating the redox state of many cells, DNA synthesis and repair, protein synthesis, amino acid transport, enzymatic activation and the enhancement of immune function (Chance *et al.*, 1979; Lomaestro & Malone, 1995). The ability of GSH to block the arylation of covalent MT-DA adducts lends credence to the experiment by demonstrating that the reaction may

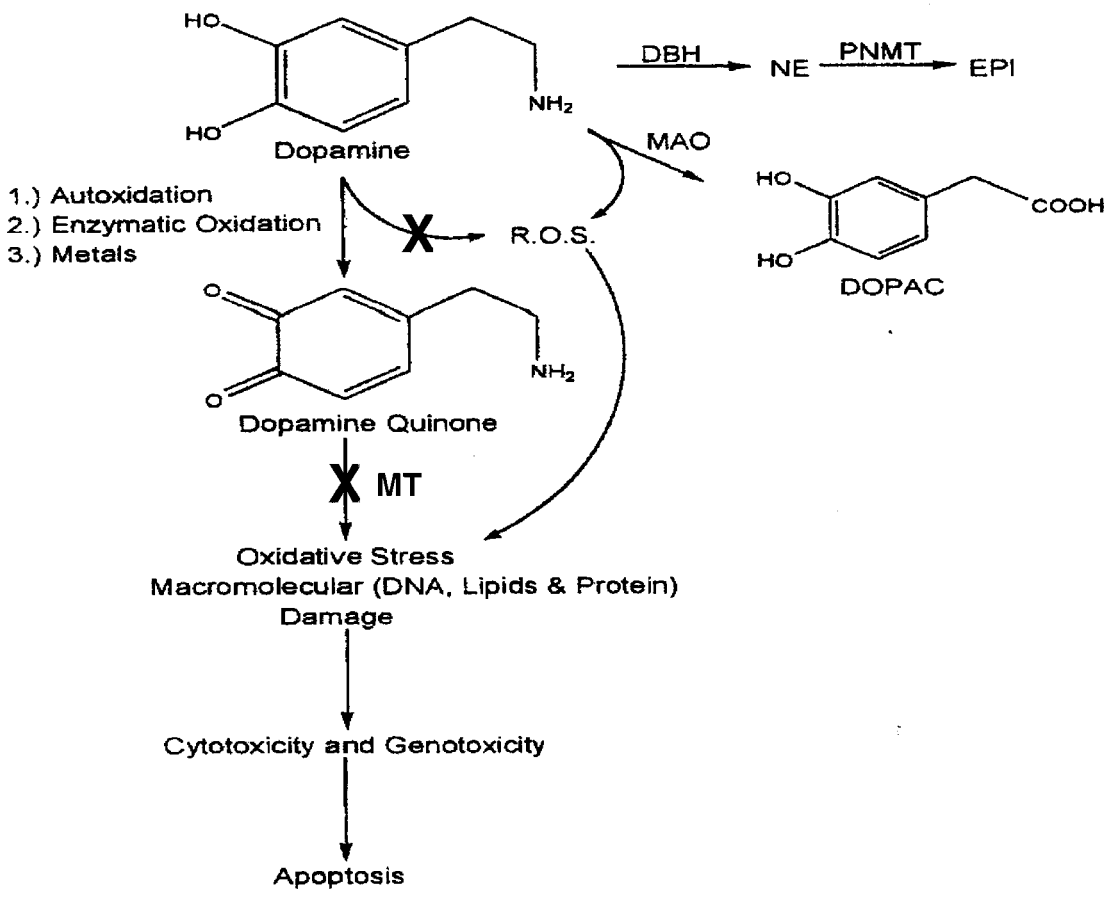


Figure 12. Proposed schematic for MT-mediated Zn^{2+} protection of DA neurons (adapted from Stokes *et al.*, 1999).

occur through oxidative mechanisms. Conversely, the inability of AA to block covalent DA-MT adduct arylation compliments a previous study by Offen *et al.* (1996) which demonstrated that AA was not effective in protecting DAergic neurons from DA-induced cell death, whereas GSH was highly effective. AA and GSH are both antioxidants, yet they exert their protective effects through separate mechanisms. Since MT and GSH possess structural similarities, and have both been implicated as extremely efficient ·OH scavengers (Thornalley & Vašák, 1985; Bains & Shaw, 1997), they may provide protection from oxidative stress and free radical damage in a similar fashion.

To supplement the preceding experiments (primarily because we were unable to detect co-localization in primary cultures via IFDL), an *in vivo* IP study was performed to determine whether DA could be incorporated into MT in neurons, and if MT could act as a substrate for DA arylation within DAergic neurons in culture. Since the experiment determined that neurons incorporated significant levels of [^{3}H]DA into MT (Figure 11), we were able to conclude that MT is also arylated by DA within neurons in culture.

It has become clear that Zn^{2+} may play both protective and pro-apoptotic roles within various populations of neurons. Our research suggests that MT may be a key factor involved in protecting such neurons from Zn^{2+} toxicity or general oxidative damage within the DAergic pathways, perhaps by sequestering Zn^{2+} ions present in elevated concentrations or blocking the formation of ROS and toxic quinone moieties. Our current findings, along with other recent studies, suggest that oxidation products may play a role in the reaction mechanism (Dryhurst *et al.*, 2001). It is evident that both Zn^{2+} and MT may be altered in conditions where oxidative stress has taken place. A recent study demonstrated that intracerebral injection of 6-OHDA (a potent neurotoxin known

to produce free radicals) in rats resulted in a reduction of both Zn^{2+} and MT concentrations in the striatum (Shiraga *et al.*, 1993). These authors postulated that MT may reduce the effects of generating ROS and free radicals by releasing Zn^{2+} to neuronal membranes and/or receptors, suggesting an antioxidant role for both Zn^{2+} and MT. The exact mechanism by which Zn^{2+} is neuroprotective to DAergic neurons which degenerate in PD is currently unknown, but may relate to the up-regulation of MT which can act as a free radical scavenger and/or antioxidant. MT-bound Zn^{2+} may protect these neurons from oxidative damage and further degeneration, possibly due to endogenous DA toxicity mediated by quinone formation or α-synuclein aggregation (Graham, 1978; Halliwell & Gutteridge, 1985; Stokes *et al.*, 1999; Zhou *et al.*, 2000; Kirik *et al.*, 2002). Evidence to support the role of MT as an antioxidant was strengthened when a collection of recent investigations demonstrated that MT-1 and MT-2 can be induced by ROS through mechanisms which are similar to other MT inducers (Dalton *et al.*, 1994; Dalton *et al.*, 1996; Dalton *et al.*, 1997; Andrews, 2000). Therefore, it is possible that the neuroprotective effects of Zn^{2+} may be due to the induction of MT and not simply the effects of the metal ion alone. Zn^{2+} requires a cofactor to implement its neuroprotective abilities, and one of the key cofactors is MT.

Interestingly, several recent papers have been published that support these concepts. Asanuma *et al.* (2002) recently reported, using MT-1 and MT-2 knockout mice, that MT-1 and MT-2 both exhibit neuroprotective and antioxidant effects *in vivo* against the toxic DA metabolite, 6-OHDA. Concomitantly, other studies examining MT-1/2 knockout mice illustrated an increased number of cells that had undergone apoptosis as well as increased oxidative stress (Penkowa *et al.*, 1999). It is important to note that

the cells that were primarily affected were neurons. Another study (Xu *et al.*, 2002) investigated α-synuclein accumulation in cultured human DA neurons, and demonstrated that this protein aggregation (potentially initiated by Zn^{2+}) induced endogenous DA to become toxic, generating ROS and therefore potentially contributing to neurite degeneration in PD (Xu *et al.*, 2002). These studies indicate that endogenous DA induces neurodegeneration, mediated by α-synuclein due to the contribution of DA to the ROS pathway (Xu *et al.*, 2002). Much of the current literature suggests Zn^{2+} is a toxic ion involved in cell death, and that Zn^{2+} may initiate α–synuclein build-up. However, it is possible that although Zn^{2+} may be initiating α–synuclein protein aggregation leading to endogenous DA auto-toxicity, MT induction may override these deleterious mechanisms, suggesting that the protective effects of MT may be greater than the toxic potential of Zn^{2+} ions. MT is a potent antioxidant that can sequester Zn^{2+}'s toxic potential (Hidalgo *et al.*, 2002). It is important to note that α-synuclein alone is not toxic, and is also not deleterious to non-DAergic neurons, but instead elicits neuroprotective effects (Xu *et al.*, 2002). Studies by Nappi & Vass (2000) have postulated that MT may be an Fe^{3+} sequestering molecule, which may provide critical neuroprotective implications since accumulation of Fe^{3+} can lead to cytotoxicity of neuronal DA which may contribute to the neurodegenerative process in PD. Other recent studies have illustrated that although both MT-1 and MT-2 have free radical scavenging abilities, it is MT-1 that seems to be the superior free radical scavenger and therefore perhaps the better antioxidant of the two (Kumari *et al.*, 1998). However, research by Hussain *et al.* (1996) contradicts this theory with data demonstrating that MT-2 has a six-fold higher capacity to scavenge superoxide radical than MT-1. Regardless, the importance of the latter study lies in the ability of *in*

vitro experiments to demonstrate that MT reigns superior in scavenging superoxide radicals in a dose-dependent manner compared to other sulfhydryl molecules such as cysteine, N-acetyl-cysteine and even GSH (Hussain *et al.*, 1996). Subsequently, current studies by Mendez-Alvarez *et al.* (2002) have suggested that the potential basis for the antioxidant ability of Zn^{2+} in neurodegenerative diseases is fundamentally due to the induction of Zn^{2+}-containing proteins and not the metal ion's basic properties as previously proposed. We propose MT is a key Zn^{2+}-containing protein, and that MT-bound Zn^{2+} may allow this essential metal ion to protect the neurons which degenerate in various neurological disease states, such as PD.

Several studies have demonstrated significant alterations and up-regulation of MT-1, MT-2 and MT-3 isoforms in the brain and spinal cord in neurodegenerative disease states (Duguid *et al.*, 1989; Sillevis Smitt *et al.*, 1992; Adlard *et al.*, 1998; Zambenedetti *et al.*, 1998; Chuah & Getchell, 1999). Subsequently, recent studies by Penkowa & Hidalgo (2000, 2001) demonstrated that MT-2 had significant therapeutic benefits for an animal model of multiple sclerosis. Penkowa and colleagues (2002) have also demonstrated recently that endogenous MT-1 over-expression and exogenous MT-2 treatment have significant neuroprotective roles during CNS pathological conditions. It is likely that the protective roles of MT-1 and MT-2 in the CNS are related to their Zn^{2+} binding capacity and antioxidant abilities. Therefore, the roles of Zn^{2+}-bound MT as an antioxidant and potential therapeutic agent may have significant implications for understanding the underlying physiological and pathological mechanisms of neurodegenerative diseases. These protective roles may be important in PD, since DAergic neurons have been known to produce the largest amount of oxygen free radicals

in the mesencephalon, and appear to be poorly protected against deleterious effects of ROS, leaving them selectively vulnerable (Hirsch & Faucheux, 1998). It is likely that both Zn^{2+} and MT perform critical roles in protecting the DAergic neurons which degenerate in this chronic, progressive disorder, possibly by mechanisms responsible for the detoxification of endogenous DA auto-oxidation products.

Conclusion

This thesis has been successful in demonstrating that Zn^{2+} is protective to cultured rodent DAergic neurons, but not to PC12 cells. This study has also demonstrated that although there are many similarities between DAergic neurons and PC12 cells, there are also significant differences which may require further investigation. We have also concluded that the observed protective effects of Zn^{2+} for rodent ED15 midbrain cultures may be mediated by the induction of MT, which reacts directly with DA, and DA oxidation products, to enhance survival of DAergic neurons. By up-regulating MT isoforms, which can then scavenge the DA oxidation products directly, reduction of oxidative stress is achieved. Arylation studies further demonstrate that DA is incorporated into MT in neurons, that MT can form covalent adducts with both DA and 6-OHDA, and that the covalent reaction can be blocked by the reducing agent GSH, providing a possible mechanism for the observed neuroprotection. Understanding the mechanisms of MT activity in the oxidative metabolism of DA (and its modification by Zn^{2+}) may be important in neurological diseases such as PD, where oxidative stress is implicated.

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