AN INVESTIGATION OF THE NEUROPROTECTIVE EFFECTS OF ESTROGEN IN A MODEL OF QUINOLINIC ACID-INDUCED NEURODEGENERATION

THESIS

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" Men ought to know that from nothing else but the brain comes joys, delights, laughter and sports, and sorrows, griefs, dependency, and lamentations. And by this, in an especial manner, we aquire wisdom and knowledge, and see and hear and know what are foul and what are fair, what are bad and what are good, what are sweet and what are unsavory…. And by the same organ we become mad and delirious, and fears and terrors assail us….. All these things we endure from the brain when it is not healthy….In these ways I am of the opinion that the brain exercises the greatest power in the man "

- Hippocrates, On the Sacred Disease
(Fouth Century B.C.)

ABSTRACT

The hippocampus, located in the medial temporal lobe, is an important region of the brain responsible for the formation of memory. Thus, any agent that induces stress in this area has detrimental effects and could lead to various types of dementia. Such agents include the neurotoxin, Quinolinic acid.

Quinolinic acid (QUIN) is a neurotoxic metabolite of the tryptophan-kynurenine pathway and is an endogenous glutamate agonist that selectively injures and kills vulnerable neurons via the activation of the NMDA class of excitatory amino acid receptors.

Estrogen is a female hormone that is responsible for reproduction. However, in the last decade estrogen has been shown to exhibit a wide range of actions on the brain, including neuroprotection. Estrogen has been shown to exhibit intrinsic antioxidant activity and protects cultured neurons against oxidative cell death. This is achieved by estrogen's ability to scavenge free radicals, which is dependent on the presence of the hydroxyl group at the C3 position on the A ring of the steroid molecule. Numerous studies have shown that estrogen protects neurons against various toxic substances and may play a role in delaying the onset of neurodegenerative diseases, such as Alzheimer's disease.

Neuronal damage due to oxidative stress has been implicated in several neurodegenerative disorders. The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and in human disease.

The study aims to elucidate and further characterise the mechanism behind estrogen's neuroprotection, using QUIN as a model of neurotoxicity. Initial studies confirm estrogen's ability to scavenge potent free radicals. In addition, the results show that estrogen forms an interaction with iron (II) and also acts at the NMDA receptor as an agonist. Both mechanisms reduce the ability of QUIN to cause damage to neurons,

since QUIN-induced toxicity is dependent on the activation of the NMDA receptor and the formation of a complex with iron (II) to induce lipid peroxidation.

Heat shock proteins, especially Hsp 70 play a role in cytoprotection by capturing denatured proteins and facilitating the refolding of these proteins once the stress has been relieved. Estrogen has been shown to increase the level of expression of Hsp70, both inducible and cognate forms of the protein. This suggests that estrogen helps to protect against cellular protein damage induced by any form of stress the cell may encounter.

The discovery of neuroprotective agents, such as estrogen, is becoming important as accumulating evidence indicates a protective role *in vivo*. Thus further research may favour the use of these agents in the treatment of several neurodegenerative disorders. Considering how devastating diseases, such as Alzheimer's disease, are to a patient and the patient's families, the discovery of new protective agents are a matter of urgency.

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ABBREVIATIONS

AD -- Alzheimer's Disease
AdCSV -- Adsorptive cathodic stripping voltammetry
AMP -- Adenosine monophosphate
AMPA -- α amino-3-hydroxy-5-methyl-4-isoxasole propionic acid
ANOVA -- Analysis of variance
APP -- Amyloid peptide precursor
ATP -- Adenosine tri-phosphate
BDNF -- Brain derived neurotrophic factor
BHT -- Butylated hydroxytoluene
CAMP -- Cyclic adenosine monophosphate
CEE -- Conjugated equine estrogen
ChAT -- Choline aceyltransferase
CNS -- Central nervous system
CPM -- Counts per minute
CREB -- cAMP response element binding protein
CSF -- Cerebrospinal fluid
DAG -- Diacylglycerol
DNA -- Deoxyribonucleic acid
DPM -- Disintegrations per minute
EAA -- Excitatory amino acids
EPSP -- Excitatory postsynaptic potential
ERE -- Estrogen response element
ERK -- Extracellular regulated kinase
ERT -- Estrogen replacement therapy
GABA -- γ-aminobutyric acid
GS -- Glutamine synthetase
HDA -- Hydroxyalkenals
HAO -- Hydroxyanthranilic acid oxygenase
HD -- Huntington's Disease
HPLC -- High pressure liquid chromatography
HRT -- Hormone replacement therapy
Hsc -- Heat shock cognate
HSP -- Heat shock protein
LTD -- Long term depression
LTP -- Long term potentiation
MAPK -- Mitogen activated protein kinase
MDA -- Malondialdehyde
MPTP -- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA -- Messenger ribonucleic acid
NAD – Nicotinic acid dinucleotide
NFT -- Neurofibrillary tangles
NGF -- Nerve growth factor
NMDA -- N-methyl-D-aspartate
NMDAR -- N-methyl-D-aspartate receptor
NMR -- Nuclear magnetic resonance
NO -- Nitric oxide
PAGE -- Polyacrylamide gel electrophoresis

PBS -- Phosphate buffered saline
PCP -- Phencyclidine
PKA -- Protein kinase A
PKC -- Protein kinase C
QPRT -- Quinolinate phosphoribosyl transferase
QUIN -- Quinolinic acid
ROS -- Reactive oxygen species
SDS -- Sodium dodecyl sulphate
SOD -- Superoxide dismutase
TBA -- Thiobarbituric acid
TBA-RS -- Thiobarbituric acid reacting substances
TBS -- Tris buffered saline
TCA -- Trichloroacetic acid

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Publications arising from this work –

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Heron, P. and Daya, S. (2001) 17β-estradiol attenuates quinolinic acid insult in the rat hippocampus. *Met. Brain Dis.* In Press

CHAPTER 1

LITERATURE REVIEW

1.1.   BASIC NEUROBIOLOGY

1.1.1.   Introduction

Over the years, neuroscientists have continued to unveil more and more, and have gained tremendous insight into the pathophysiology and etiology of several neurodegenerative disorders. As a result, the field of neuroprotection has become a rapidly advancing area of neuroscience, which has led to the ongoing search of suitable agents for therapeutic intervention. The female hormone, estrogen is one such agent that has been shown to elicit a wide range of actions in the brain, including the improvement of cognitive functions (Henderson, *et al*., 1994), neuroprotection (Culmsee, *et al*., 1999; Wise, *et al*., 2000), enhancement of nerve regeneration (Tanzer, *et al*., 1999) and stimulation of neurite growth (Toran-Allerand, *et al*., 1999). In order to understand the specific mechanisms of action that estrogen has on the brain, it is necessary to give a brief outline of the processes involved in neuronal transmission and the structural features of the brain.

1.1.2.   Neuroanatomy and synaptic transmission

The brain is a highly sophisticated and complex organ responsible for mood, behaviour, memory, learning and thought processes to name just a few. The basic structural units that comprise the brain are cells called neurons and glia. From an historical standpoint, no other cell has attracted as much attention and controversy as the neuron (Siegel, *et al*., 1989). Neurons display extensive structural, topographical and functional variation (Siegel, *et al*., 1989), and have two special properties that distinguish these cells from all other cells in the body. Firstly, these are specialized to conduct electrochemical impulses known as nerve impulses or action potentials, and

secondly, these possess specific intercellular connections with other nerve cells and with innervated tissues such as muscles and glands (Cooper, *et al.*, 1996). Neurons consist of a cell body or soma, and numerous neurites that radiate away from the cell body (Figure 1.1). Neurites are of two types: axons and dendrites (Bear, *et al.*, 2001).
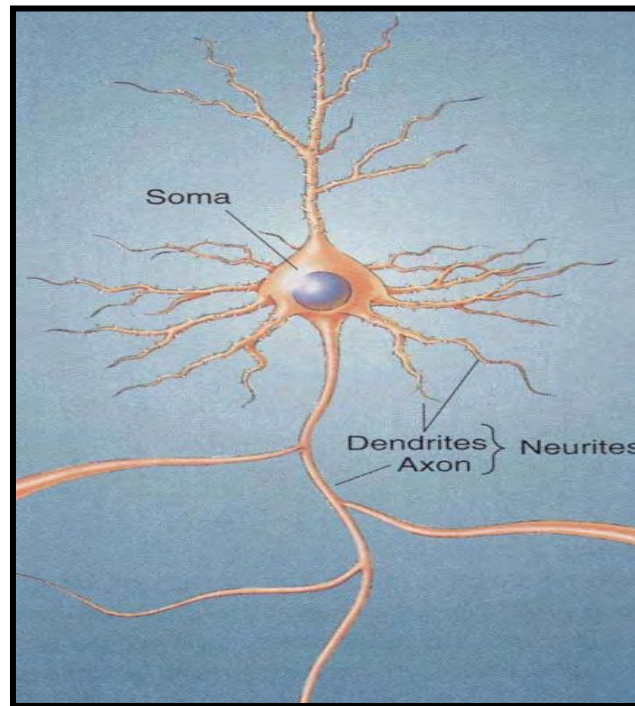
Figure 1.1   A diagram showing the structure of a typical nerve cell with its axon and dendrites radiating away from the cell body (Bear, *et al.*, 2001)

Neurons usually contain a single axon of uniform diameter, which may branch off at right angles to form axon collaterals (Figure 1.2). The axon may extend from less than a millimeter to over a meter long. It is along this projection that the output signal from the cell body travels, the speed of which is determined by the thickness of the axon; the thicker the axon the faster the impulse travels. Dendrites are more numerous and are thinner than axons and rarely extend more than 2mm in length. Many dendrites extend from the cell body and generally taper to a fine point. Dendrites come into contact with many axons of other neurons and thus receive incoming signals from these neurons (Bear, *et al.*, 2001). Thus nerve cells communicate by impulses that travel from one neuron to another.
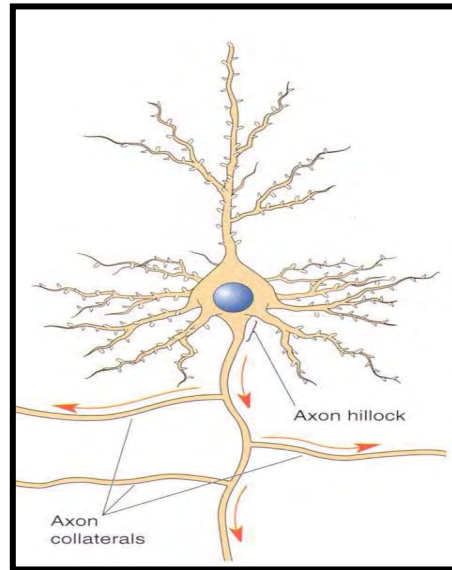
Figure 1.2. A diagram demonstrating the structure of the axon and axon collaterals. The arrows indicate the direction of information flow (Bear, *et al*., 2001)

The connection point between the axon of another cell and the dendrite is known as a synapse. The normal direction of information flow is from the axon terminal to the target neuron. Thus, the axon terminal is termed presynaptic and the target neuron is said to be postsynaptic (Bear, *et al*., 2001). In response to a certain stimulus, the presynaptic neuron releases chemicals known as neurotransmitters from vesicles within the cell body, into the synaptic cleft. These chemicals then act on specific receptors on the postsynaptic membranes, which then stimulate a chain of reactions.

Within the synaptic cleft several uptake mechanisms exist for the removal of neurotransmitters from the synapse. Upon release from nerve terminals, neurotransmitters can either produce excitation or inhibition of the target cell once these act on specific receptor sites at postsynaptic membranes (Siegel, *et al*., 1989). Figure 1.3 illustrates the synaptic transmission between the axon terminal of one neuron with the dendrite of another.
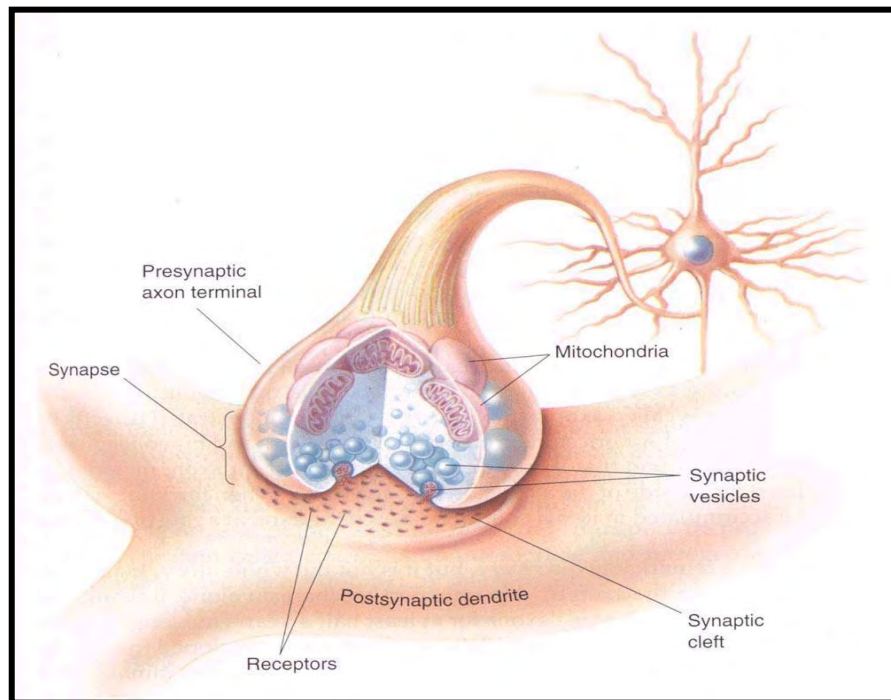
Figure 1.3. Synaptic transmission from the axon terminal (presynaptic neuron) to the dendrite of another neuron (postsynaptic neuron) (Bear, *et al*., 2001)

1.1.3. The structure and function of the hippocampus

The hippocampus is a region of the brain, situated medial to the lateral ventricle in the medial temporal lobe. This region forms part of the cerebral cortex, which is folded onto itself in a peculiar shape (Bear, *et al*., 2001). This is evident in a Nissl-stained coronal section through the caudal telencephalon of a rat brain (Figure 1.4). Figure 1.5 shows the area of the rat brain in which the hippocampus is located, which can easily be dissected from the whole brain. The main pathway involved in hippocampal function is the glutamatergic system, requiring glutamate as the major neurotransmitter, which causes the activation of postsynaptic receptors such as N-methyl –D-aspartate (NMDA) receptors involved in memory.

The rat hippocampus is located approximately 1mm anterior to where the cerebrum starts (from where the cerebellum ends, if one looks at the dorsal view of the brain) and extends about 4mm posterior and approximately 2.5mm lateral of the midline separating the two hemispheres.
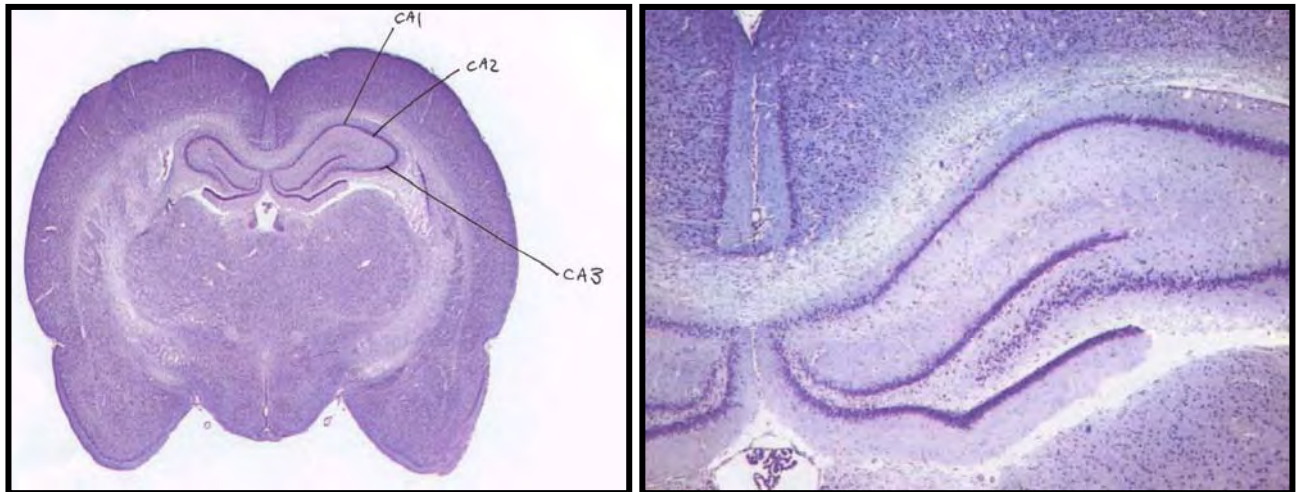
Figure 1.4. (Left) A coronal section through the caudal telencephalon of a rat brain displaying the hippocampal structure and three subdivisions. (Right) A magnified view of the rat hippocampus.
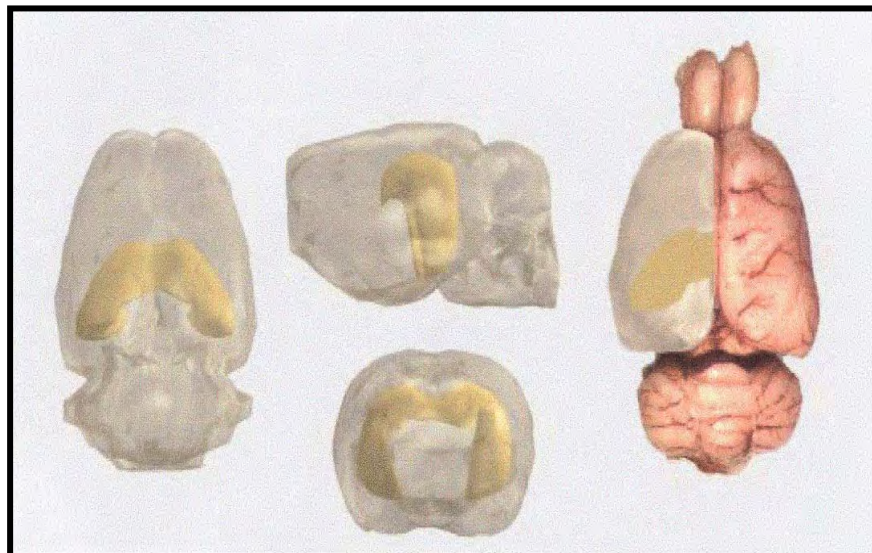
Figure 1.5. Various sections of the rat brain displaying the hippocampus shown in yellow. (Left and Far Right) A dorsal view of the rat brain; (Middle, Top) A midsaggital section through the brain; (Middle, bottom) A coronal section through the brain.

The primary function of the hippocampus is that of declarative memory consolidation. The hippocampus consists of two thin sheets of neurons folded onto each other; one sheet is the dentate gyrus and the other is the Ammon's horn. The Ammon's horn has four divisions, of which the most important are the CA1 and CA3 (Figure 1.4). CA stands for *Cornu Ammonis* meaning Ammon's Horn in Latin. The major input to the hippocampus is the entorhinal cortex, which sends information to the hippocampus via a bundle of axons called the perforant path. These axons synapse on neurons of the dentate gyrus. Dentate gyrus neurons give rise to axons, called mossy fibres, that synapse on cells in the CA3 region. The CA3 cells give rise to axons that branch; one branch leaves the hippocampus via the fornix and the other branch called Schaffer collateral, forms synapses on the neurons of CA1 (Figure 1.6).
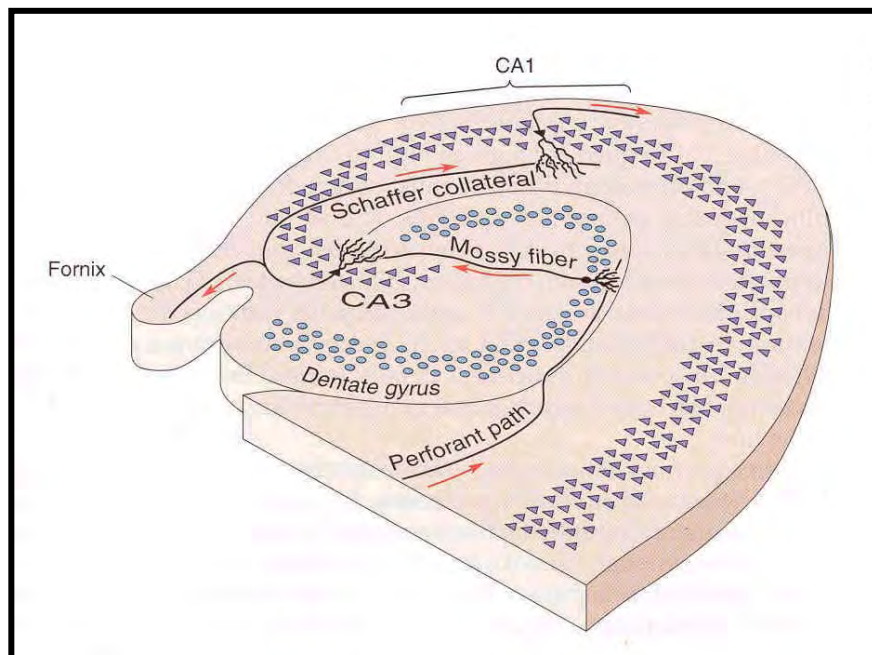
Figure 1.6.　　An illustration of some microcircuits of the hippocampus (Bear, *et al.*, 2001)

1.1.4.　　　　The biochemical basis of learning and memory

Learning is the acquisition of new information or knowledge and memory can be defined as the retention of learned information. Declarative memory can be explained by defining it as the everyday memory of facts and events, however we remember many other things such as skills, habits and behaviours, which is known as

nondeclarative memory and is information that is stored in our brain. Thus declarative memories are available for conscious recollection while nondeclarative memories are not (Bear, *et al*., 2001). Furthermore, the literature refers to two distinct types of learning and memory, which in many cases may be employed in conjunction. Motor learning and memory is the learning of all skilled movements, such as standing and walking; cognitive learning and memory involves all perceptions, ideas and linguistic expressions (Eccles, 1991).

Brief high-frequency electrical stimulation of an excitatory pathway to the hippocampus produces a long lasting enhancement in the strength of the stimulated synapses. This effect is known as LTP (Long term potentiation), and is important in the understanding of how declarative memories are formed. LTP is induced when afferent fibres to the CA1 region are stimulated electrically in close temporal proximity to depolarization of the postsynaptic neurons, a long lasting increase in sensitivity of these cells develops, which may persist for weeks (Siegel, *et al*., 1989). This increase is known as the EPSP (excitatory postsynaptic potential). Recent evidence suggests that LTP is initially a strengthening of the EPSP and that it occurs only when there has been activation of hundreds of synapses on a neuron, which co-operate in making a strong prolonged depolarization of the neuron. Depolarization refers to the change in membrane potential from resting value to a less negative value and is what triggers an action potential or nerve impulse.

LTP is primarily postsynaptic and plays a key role in cognitive memory. The amino acid glutamate has been shown to be the excitatory neurotransmitter in LTP formation and that the postsynaptic receptor activated is the NMDA receptor. However, this receptor can only be stimulated once the postsynaptic membrane has been strongly depolarized. Once depolarized, the magnesium ions that normally block the NMDA receptor channel are driven off. Depolarization occurs as a result of the EPSP's, formed after glutamate has bound to the quisqualate sensitive component of the postsynaptic membrane, causing an influx of sodium ions into the synaptic spine to produce depolarization. The activation of NMDA receptors allows the influx of calcium ions into the synaptic spine. It is not fully understood how this increase in calcium leads to LTP but calcium would by activation of protein kinases, increase the

sensitivity of the quisqualate receptor sites in the spine synapse to glutamate, giving the LTP effect (Eccles, 1991).

The introduction presented in the preceding pages, explain the basic neuroscience principles regarding the communication between nerve cells, the complexity of the neuronal network essential in the formation of thought processes and memory and the biochemical basis behind learning and memory. All this provides adequate background for the following sections that will deal with estrogens mode of action in the brain, and neurotoxicity in general.

1.2.   EXCITOTOXICITY

1.2.1.   Introduction

Excitotoxicity is the excessive stimulation of neurons by excitatory amino acids such as glutamate, aspartate and quisqualate through postsynaptic receptors. Only the excitatory amino acid, glutamate will be discussed in the following sections. Glutamate serves as a neurotransmitter at most CNS synapses. Although glutaminergic neurons contain a high concentration of glutamate, the cytosol of nonglutaminergic neurons also contain very high concentrations of this amino acid neurotransmitter (Bear, *et al*., 1989). Excitoxicity is the process by which overactivation of excitatory neurotransmitter receptors leads to neuronal cell death (Schinder, *et al*., 1996). Several disorders such as cardiac arrest, stroke, brain trauma and seizures, can initiate excess glutamate release. This occurs when the oxygen and glucose supply to the brain is dramatically decreased in cases where blood flow ceases such as during a stroke. As a result, neurons cannot generate enough ATP to drive membrane ion pumps and depolarization of the membrane occurs and consequently calcium leaks into the cell. This calcium triggers the synaptic release of glutamate from vesicles, which further depolarizes neurons and raises intracellular calcium, and still more glutamate is released.

As mentioned, certain pathological conditions can cause excessive glutamate to be released, however under normal circumstances glutamate from the synaptic cleft is

reaccumulated directly into the nerve terminal or into adjacent glia, which contains glutamine synthetase that converts glutamate into glutamine. Glial cells and neurons possess a similar plasma membrane glutamate uptake carrier, which keeps the extracellular glutamate concentration below levels that damage neurons (Nicholls & Attwell, 1990).

The mechanism by which glutamate raises the intracellular calcium is through the activation of several types of receptors, which allow excessive amounts of Na^+, K^+ and Ca^{2+} to flow across the membrane. The NMDA receptor once activated results in an influx of calcium into the cell. This causes neuronal damage due to the stimulation of intracellular enzymes that degrade lipids, proteins, and nucleic acids. In addition to this, neurons are damaged due to swelling that occurs resulting from water uptake (Bear, *et al.*, 2001)

1.2.2.   N-methyl-D-aspartate receptors

As discussed above the NMDA receptors play a critical role in excitotoxicity and are classified as ionotropic glutamate receptors. These receptors are ligand-gated, voltage-dependent ion channels (Mayer, *et al.*, 1984), which at resting potential is blocked by a magnesium ion that is only displaced following depolarization of the neuron by the binding of glutamate to the receptor along with a coagonist such as glycine to a modulatory site (Carfagno, *et al.*, 2000). The NMDA receptor channel is a complex molecular entity with a number of distinct recognition sites for endogenous and exogenous ligands, each with discrete binding domains (Cooper, *et al.*, 1996).

The NMDA receptor contains six pharmacologically distinct sites through which compounds can alter the activity of this receptor. 1) A transmitter binding site that binds L-glutamate and related agonists, however the action of glutamate in promoting Ca^{2+} and Na^+ entry is dependent on the glycine modulatory site (2) being occupied; 3) a site within the receptor that binds phencyclidine (PCP site) and related noncompetitive antagonists such as (dizocilpine) MK-801, (these agents act most effectively when the receptor is activated ie. open channel block); 4) a voltage dependent magnesium binding site; 5) an inhibitory divalent cation site near the

mouth of the channel that binds Zn^{2+} to produce a voltage-independent block; a polyamine regulatory site whose activation by spermidine and spermine facilitates NMDA receptor-mediated transmission (Cooper, *et al*., 1996). The structure of the NMDA receptor is shown in Figure 1.7.

NMDA channels are rapidly and reversibly blocked by Mg^{2+} ions in a highly voltage-dependent manner. This extreme voltage dependence is the reason that the Mg^{2+} binding site lies deep within the ion channel pore (MacDonald & Nowak, 1990).

The glycine site has been studied as a potential site for the action of new antiepileptic drugs or agents that may be useful in preventing ischemic brain damage. Glycine increases the frequency of the NMDA receptor channel opening, thus conditions that alter the concentration of glycine or compete with its binding site can dramatically alter the NMDA receptor-mediated responses. Furthermore, spermine and spermidine are allosteric modulators and potentiate NMDA currents in the presence of saturating concentrations glutamate and glycine, but in contrast to glycine their presence is not a requirement for NMDA receptor activation. In cases of brain ischemia or trauma where the concentrations of polyamines are increased dramatically, neuronal damage may result due to excitotoxicity (Cooper, *et al*., 1996).
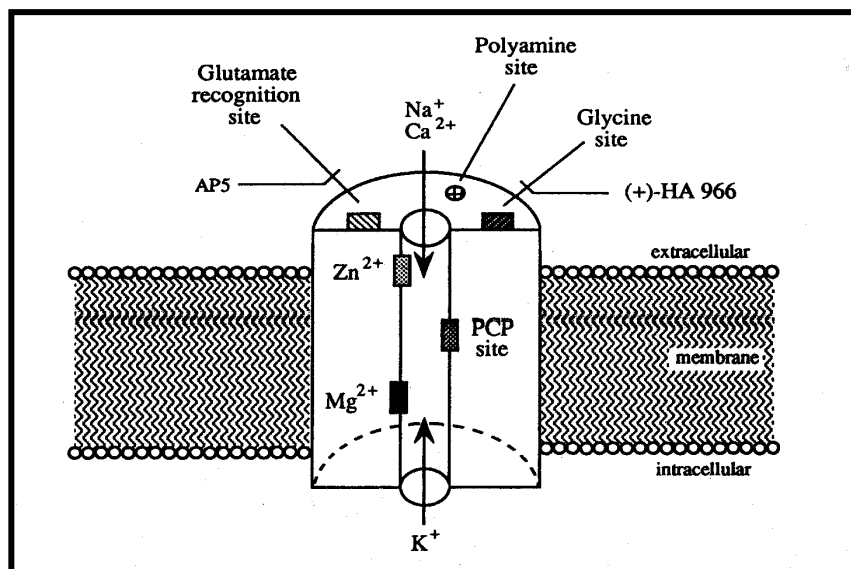
Figure 1.7. Schematic illustration of the NMDA receptor complex and the sites of action of different agents on the receptor (Cooper, *et al*., 1996)

1.2.3.   NMDA receptor activation and cell death

It is believed that excitotoxins can destroy neurons by either an acute fulminating process, which is Na^+ and Cl^- (but not Ca^{2+}) dependent or by a slow process that is calcium dependent. In the latter case, excitotoxic neuronal degeneration occurs even in cases where the duration of exposure to an abnormal concentration of excitotoxin is only one sixth as long as in the former case (Olney, 1989). Several features distinguish the NMDA receptor from other excitatory amino acid (EAA) receptors such as the kainate receptors. NMDA receptors are linked to a Na^+/Ca^{2+} ion channel that has a much higher calcium conductance than ion channels associated with other EAA receptor subtypes. As a result, NMDA receptors are major participants in the neurodegenerative process.

1.2.3.1.   Acute NMDA toxicity

When agonists such as glutamate or NMDA are added to cultures or injected directly into the brain in large concentrations, excessive activation of NMDA receptors occurs rapidly. NMDA receptor channels permit a large Ca^{2+} influx and evidence indicates that it is mainly through these channels that toxic calcium entry occurs. This excessive calcium entry is the major cause of NMDA toxicity. In cerebellar and hippocampal slices, NMDA toxicity towards all vulnerable neurons is abolished when calcium is omitted, which provides evidence that calcium plays a crucial role in cell damage (Meldrum & Garthwaite, 1990).

1.2.3.2.   Delayed NMDA toxicity

When injected into rat hippocampus in amounts not immediately toxic, NMDA induces a gradual neurodegeneration over several hours, which can be inhibited by delayed administration of NMDA antagonists. Several factors contribute to this slow degeneration: 1) There may be a failure of inhibition due to preferential loss of function of inhibitory interneurons, which would amplify ongoing NMDA receptor activation, and 2) neurons may undergo a period of enhanced vulnerability to further NMDA receptor activation. Neurons in cerebellar slices given one non-lethal

exposure to NMDA can be rendered necrotic by a second, non-lethal exposure an hour later. This may be related to the time required to reinstate normal intracellular calcium buffering following the first insult (Meldrum & Garthwaite, 1990).

1.2.4.   The effects of Ca^{2+} influx in excitotoxic neuronal damage

Calcium influx and subsequent calcium overload following NMDA receptor activation, triggers cytotoxic processes responsible for neuronal degradation. Calcium accumulation inside neurons parallels the cytopathological changes in both time-course and the sites involved, starting with the Golgi apparatus (swelling), then the nucleus (chromatin clumping), some mitochondria (swelling) and finally the cytoplasm (Meldrum & Garthwaite, 1990).

Following sublethal calcium entry a number of calcium-dependent enzymes are activated. These include:

1) Calpains I and II, the activation of which induces the breakdown of the cytoskeleton and some membrane proteins are affected (Dodd, *et al*., 1994).

2) Protein kinase C, an enzyme responsible for a prolonged phase of Ca^{2+} influx following EAA receptor activation (Mayer & Miller, 1990).

3) Phospholipise A_2 leads to the production of arachidonic acid, which can be metabolised to give free radicals. This enzyme also potentiates synaptic transmission, increases glutamate release, decreases glutamate uptake into glia and activates Protein kinase C (Meldrum & Garthwaite, 1990).

4) Phospholipase C generates IP_3 (inositol 1,4,5-triphosphate), which raises cytosolic calcium release from internal stores, and DAG (diacylglycerol), which activates protein kinase C and enhances glutamate release (Mayer & Miller, 1990).

5) Ca^{2+}-calmodulin-dependent protein kinase II, which is involved in LTP of synaptic transmission, phosphorylates synapsin I presynaptically and enhances glutamate release (Mayer & Miller, 1990).

6) Endonucleases, which cause fragmentation of DNA and the activation of which, is important in the toxin- or hormone-induced death of some peripheral cell types (Meldrum & Garthwaite, 1990).

7) Nitric oxide synthase, which produces nitric oxide; this contributes to the cytotoxic effects of activated macrophages by inhibiting mitochondrial respiration and DNA synthesis. NO also reacts with $O_2^{\cdot}$ ions to produce toxic hydroxyl radicals (Dodd, *et al*., 1994).

1.2.5. NMDA receptor antagonists

N-methyl-D-aspartate (NMDA) receptor antagonists have therapeutic potential in numerous CNS disorders ranging from acute neurodegeneration (e.g. stroke and trauma), chronic neurodegeneration (e.g. Parkinson's disease, Alzheimer's disease, Huntington's disease) to symptomatic treatment (e.g. epilepsy, Parkinson's disease, drug dependence, depression, anxiety and chronic pain). However, many NMDA receptor antagonists also produce highly undesirable side effects at doses within their putative therapeutic range. This had unfortunately led to the conclusion that NMDA receptor antagonism is not a valid therapeutic approach (Parsons, *et al*., 1999).

However, memantine is an uncompetitive NMDA receptor antagonist effective in the treatment of dementia and is essentially devoid of such side effects at doses within the therapeutic range. This has been attributed to memantine's moderate potency and associated rapid, strongly voltage-dependent blocking kinetics. The action of this antagonist has thus been proposed to have a promising profile in animal models of chronic neurodegenerative diseases. Subsequent evidence has shown that it is indeed possible to develop clinically well tolerated NMDA receptor antagonists, a fact reflected in the recent interest of several pharmaceutical companies in developing compounds with similar properties to memantine (Parsons, *et al*., 1999).

The most potent NMDA antagonist is (+)MK-801 (Dizocilpine, (+)-5-methyl-10,11-dihydro-5H-dibenzocyclo-hepten-5,10-imine maleate) shown in Figure 1.8. MK-801 is a non-competitive antagonist that has been reported to block NMDA receptors and in this way protect against neuronal damage such as that induced by lipid peroxidation

(Santamaria & Rios, 1993). MK-801 is useful in testing whether a specific compound is able to act on the NMDA receptor as an agonist or modulator as explained in chapter 4.
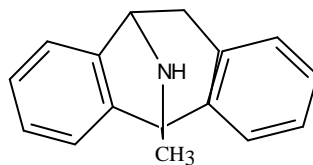
Figure 1.8. The structure of MK-801 (Kemp, *et al*., 1987)

1.2.6. Huntington's disease

Huntington's disease (HD) is a neuropsychiatric disease, characterized by neuronal depletion and neurochemical alterations in the neostriatum. HD is an autosomal dominant neurodegenerative disorder characterized by chorea and dementia with a progressive course leading to severe debilitation and death usually within 15-20 years (DiFiglia, 1990).

A hypothesis has been proposed which links the cell loss found in the striatum with excitotoxic injury. Although most studies are consistent with this hypothesis that an endogenous excitotoxin acting at the NMDA receptor is responsible for nerve cell damage, resembling that of Huntington's disease, some characteristics of the disease are not readily explained by this hypothesis. One such characteristic is the delayed onset of symptoms in middle age and the initial loss of neurons near the lateral ventricles and the dorsal putamen, are not readily explained by the hypothesis. In addition, the early loss in HD of specific subsets of medium spiny projection neurons in the matrix compartment of the neostriatum has not been reported following excitotoxic injury in rodents (DiFiglia, 1990).

This hypothesis has come under scrutiny by Meldrum & Garthwaite, 1990, who refer to it as a 'weak excitotoxic hypothesis'. These authors argue that there is no morphological evidence for specifically excitotoxic neural damage in Huntington's disease; nor is there evidence for elevated levels of Quinolinic acid, a neurotoxic metabolite that produces pathomorphological changes resembling those in

Huntington's disease. However, DiFiglia, 1990, reports that prolonged exposure to low concentrations of quinolinic acid are effective in causing cell death, thus the levels needn't be elevated for excitotoxic insult to occur.

1.3.   QUINOLINIC ACID

1.3.1.   Introduction

Quinolinic acid (2,3, pyridine dicarboxylic acid) was first identified in human brain in 1983, however the role played by this neurotoxin in neuropathology was uncertain. Quinolinic acid (QUIN) is a naturally occuring amino acid and a structural analog of the neurotransmitter candidates L-glutamate and L-aspartate. When introduced into the CNS, QUIN is a potent excitotoxin acting through excitatory amino acid receptors to cause neuronal excitation (Stone & Perkins, 1981), seizure activity and lesions (Schwarcz, *et al*., 1984), and neuronal degeneration (Southgate & Daya, 1999). More recent reports describe QUIN as an endogenous agonist at the NMDA glutamate receptor subtype that is able to cause neuronal damage and cell loss (Santamaria, *et al*., 1996).

This neurotoxin is a metabolite of the tryptophan-kynurenine pathway in the brain, and is normally present in low nanomolar concentrations in human brain and cerebrospinal fluid. The concentration of QUIN varies among different brain regions, with the cerebral cortex containing approximately 1.8 nmol/g wet weight; almost 2 fold that in the hippocampus with 1nmol/g wet weight (Moroni, *et al*., 1984b).

Substantial increases in QUIN have however been found in the brain and CSF in response to a broad spectrum of infectious and other inflammatory neurological diseases (Heyes, *et al*., 1996). Furthermore, Moroni, *et al*., 1984a, showed that the concentration of QUIN in the CNS changes after treatments capable of modifying brain tryptophan content; the QUIN concentration can increase by 150% in response to tryptophan loading. Paradoxically, administration of a tryptophan-free diet to rats for 15 days resulted in a doubling of QUIN concentrations in the cortex. One explanation for this may be that QUIN can also be synthesized by a pathway distinct

from the kynurenine pathway, particularly as some bacteria and plants are able to synthesize QUIN from the condensation of aspartic acid and dihydroxyacetate (Stone, 1993). It has also been reported that the concentrations of QUIN present in the human brain is similar to those in the rat brain (Wolfensberger,*et al*., 1983), and that these concentrations increase in the cortex during the aging process (Moroni, *et al*., 1984b).

QUIN also causes axon-sparing lesions when injected into the brain and the toxicity induced by exogenous QUIN can be prevented or reversed by NMDA receptor antagonists, supporting the notion that the toxicity produced by locally administered QUIN is mediated through this receptor (Cooper, *et al*. 1996). Foster, *et al*., 1984, showed that there is no active uptake of QUIN from the extracellular space, nor is there extracellular metabolism of QUIN. It is therefore unlikely that QUIN is a neurotransmitter in the classical sense. Stone & Perkins, 1981, demonstrated that QUIN is about one-quarter as active as NMDA, and approximately as active as glutamate and aspartate at stimulating NMDA receptors. It must however be remembered that the latter compounds have a rapid, high-affinity uptake system for their removal from the synapse, while QUIN does not. Because QUIN is not removed from the synaptic cleft, QUIN will continue stimulating the NMDA receptor causing extensive damage.

1.3.2.   Synthesis and metabolism of Quinolinic acid

QUIN is synthesized according to the kynurenine pathway shown in Figure 1.9. The kynurenine pathway begins with oxidative cleavage of the amino acid tryptophan by tryptophan 2,3-dioxygenase (EC 1.13.11.11), which is haem dependent. The product of this cleavage is formylkynurenine, which is hydrolyzed by a formamidase enzyme (EC 3.5.1.9) to give kynurenine, the amino acid, which gives this pathway its name. Kynurenine is at a branch point in the pathway and may have a number of different fates. Kynurenine could either be converted to anthranilic acid, utilized in the biosynthesis of aromatic amino acids; or transamination catalysed by kynurenine aminotransferase (KAT, EC 2.6.1.7) yields kynurenate, sometimes employed in the biosynthesis of quinolines. However, the third possibility and the most important route for the synthesis of QUIN, is the oxidation of the benzene ring catalysed by kynurenine 3-hydroxylase (EC 1.14.13.9). At this point kynureninase cleaves the 3-

hydroxy kynurenine to give 3-hydroxyanthranilic acid (Botting, 1995). The synthetic enzyme for QUIN in the kynurenine pathway, 3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6; 3HAO), catalyses the conversion of 3-hydroxyanthranilic acid to an unstable intermediate, α-amino-β-carboxymuconic semialdehyde or otherwise known as 3-acroleyl-3-amino-fumarate. This then spontaneously rearranges to form QUIN (Foster, *et al.*, 1986). 3-HAO is an iron dependent enzyme requiring Fe^{2+} ions and sulfhydryl groups for its activity. It has become an important target for drug action as its inhibition provides the most direct method of reducing QUIN levels.

Finally, QUIN is catabolized to NAD (nicotinic acid dinucleotide) and carbon dioxide by the action of quinolinate phosphoribosyl transferase (QPRTase, EC 2.4.2.19). This enzyme has been identified in rat and human CNS tissue (Foster, *et al.*, 1985). Magnesium ions are required for QPRT activity and there is evidence that an active site cysteine residue is required for catalysis and also essential are lysine and histidine residues (Botting, 1995).

Interestingly, there exists a differential localization of the two enzymes, 3-HAO and QPRT in the brain, with 3-HAO localised almost entirely in the soluble fraction of brain homogenate and QPRT to the particulate component of P2 synaptosomal fractions (Stone, 1993). In view of this, QUIN must be synthesized in one population of glial cells and must exit those cells to be metabolized by QPRT in a separate population of QPRT-containing glial cells and neurons.
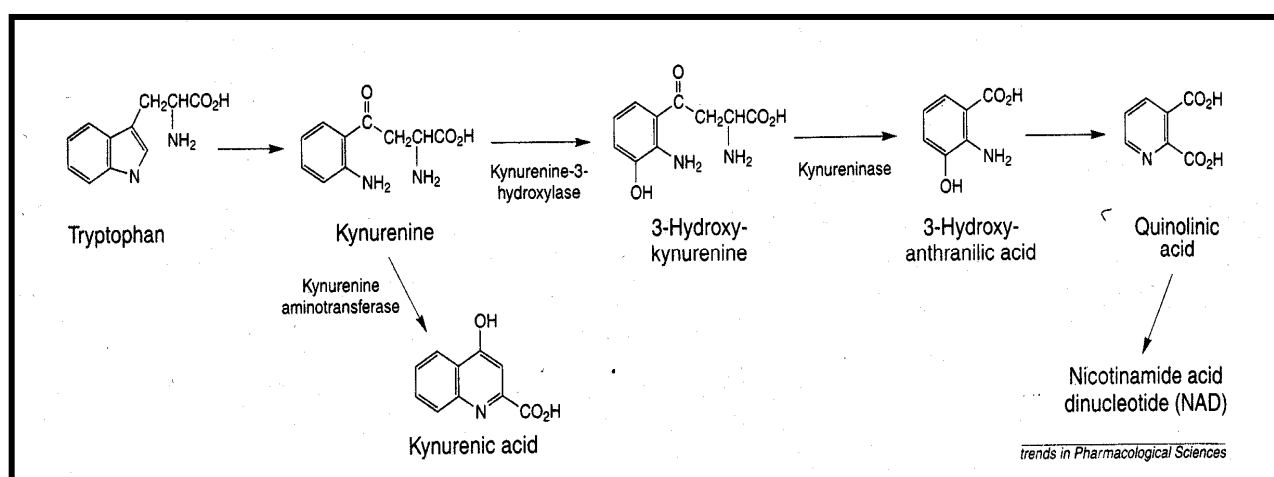
Figure 1.9.   A schematic outline of the kynurenine pathway yielding QUIN (Stone, 2000)

1.3.3.   The implication of QUIN in neurological disease states

QUIN has been recognized as a neurotoxic agent that has been implicated in several neurological disorders. Many factors come into play to render this compound a potent neurotoxin. One such factor is the performance of the two enzymes involved in QUIN synthesis and metabolism respectively. A detailed analysis of the properties of both 3-HAO and QPRT indicate that both have similar Km values but that the reaction velocity of 3-HAO was 80-fold higher than that for QPRT (Foster, *et al*., 1986). Consequently, the production of QUIN occurs at a much faster rate within the brain than the conversion to NAD. This has implications for the accumulation of QUIN in the brain under certain pathological conditions.

Furthermore, it is interesting to note that QUIN is an effective inhibitor of human monoamine oxidase B and a potent inhibitor of major glycolytic enzymes such as PEP (phosphoenolpyruvate) carboxykinase, an important enzyme in the gluconeogenesis pathway that converts 2 phosphoenolpyruvate to fructose 1,6-bisphosphate. This may indicate that QUIN needs to be concentrated in subcellular compartments and as a result the concentrations within these will clearly be substantially higher than the tissue content and could reach the range at which exogenous QUIN is known to be neurotoxic. This in turn raises the possibility that any insult to CNS neurons that results in damage or lysis of QUIN-containing cells in which further excitotoxicity is promoted.

Other factors that promote QUIN's ability to cause neuronal damage are the lack of uptake systems and extracellular metabolism that exists within neurons. An experiment in which radiolabeled QUIN was injected into the hippocampus, the radiolabeled compound was cleared from brain with a half life of 22 minutes, all residual radioactivity recovered after 2 hours still being present as QUIN, assayed by HPLC. Thus this region (as well as the striatum) does not appear to possess mechanisms either for the rapid removal of QUIN or for its metabolic degradation in the extracellular space by QPRT (Foster, *et al*., 1984).

Alzheimer's disease is characterized by a loss of memory and cognitive decline. Moroni *et al*., 1984, analyzed the QUIN content of brain samples taken from patients with senile dementia of the Alzheimer type. Despite clear evidence of neuronal degeneration in these brains, no evidence for a change of QUIN content in the cerebral cortex was obtained in comparison to control subjects. Interestingly though, 3-HAO is localized primarily in areas of the frontal cortex, striatum and hippocampus which possess little detectable QPRT (Stone, 1993). Thus QUIN could reach high enough concentrations to induce excitotoxic damage in these regions, which in the hippocampus, could effect memory processes. The same authors reported that in patients dying in a coma resulting from severe liver damage the concentration of QUIN in the frontal cortex was 3 fold greater than controls, while the QUIN concentration in the CSF was found to be 5- to 6- fold greater.

Pellagra found in patients following tryptophan deficient diets, is accompanied by CNS symptoms including hallucinations and signs of confusion and dementia, signs that could be related to the increased activation of NMDA receptors by elevated QUIN levels (Stone, 1993). Brain slices prepared from excitotoxin-lesioned animals exhibit a substantially higher capacity to produce QUIN from 3-hydroxyanthranilate than controls. It has also been reported that the activity of 3-HAO is approximately 80 times that of QPRT and that 3-HAO activity may normally be restrained by factors such as product inhibition or the availability of Fe^{2+} ions (Stone, 1993). Thus if neuronal damage occurs releasing Fe^{2+} ions, which is normally the case, then the production of QUIN would be elevated, thereby causing more damage and so the vicious circle would continue. An example illustrating this change in QUIN content in the brain reported by Saito *et al*., 1992, indicates that the concentration in the hippocampus of gerbils is normally 65nM. However, the concentration of QUIN 4 days postischaemia increased to 1466nM.

1.3.4. The mechanism of neurotoxicity and the role of QUIN

Neurotoxicity involves presynaptic receptors, transcription factors, apoptosis, cytoskeletal disruption and lipid peroxidation. Neurotoxicity refers to the combination

of neuronal excitation and subsequent neurodegeneration produced by certain compounds such as QUIN glutamate and NMDA.

A) Presynaptic receptors – QUIN is able to produce neurotoxicity only in the presence of afferent fibres to the area of investigation. QUIN is thus ineffective as a neurotoxin in the hippocampus following transection of the perforant path (Keilhoff, *et al*., 1990). This suggests that presynaptic terminals may be an essential feature of the neurotoxic activity of QUIN, ie., either QUIN may promote the release of secondary neurotoxic agents from nerve terminals or the postsynaptic effects of QUIN are dependent on the permissive or enhancing effects of factors released from presynaptic terminals (Stone, 1993).

B) Transcription factors – QUIN can increase the level of *c-fos* mRNA as well as the c-Fos product in the hippocampus and striatum (Aronin, *et al*., 1991). Since this gene encodes nuclear proteins that function as transcription factors to modulate further gene transcription, indicates that NMDA receptor activation could indirectly bring about profound changes in cell function at the genetic level.

C) Apoptosis – The injection of QUIN into the hippocampus does not induce the disruption of DNA molecules characteristic of apoptosis (Ignatowicz, *et al*., 1991). Thus QUIN is not likely to be involved in the initiation of apoptosis.

D) Cytoskeletal disruption – It has been suggested by Stone, 1993, that the initial disruption of the cytoskeleton by QUIN through changes in the protein, fodrin, a major component of the neuronal cytoskeleton in the CNS, leads to the efflux of amino acids leading to cell death.

E) Lipid peroxidation – QUIN is able to increase lipid peroxidation in rat brain homogenate (Rios & Santamaria, 1991). Since lipid peroxidation is related to free radical formation and thus cellular damage, this may suggest that QUIN toxicity could be mediated partly by this route.

1.3.5. The involvement of QUIN in Huntington's disease

Despite shortcomings of the excitotoxic hypothesis described for Huntington's disease in section 1.2.6., the use of excitatory amino acids to produce neuronal depletion confined to the neostriatum has been useful for examining patterns of neuronal reorganization relevant to HD. Schwarcz, *et al*., 1983, showed that QUIN can produce

axon-sparing lesions similar to those observed in HD. These lesions result in a depletion of neurotransmitters contained within striatal spiny neurons, for example, γ-aminobutyric acid (GABA) with selective sparing of these neurons, which closely resemble those of HD. The same authors also showed that in HD striatum, there is an increase in somatostatin and neuropeptide Y, which is due to the preservation of the striatal spiny neurons in which these peptides are co-localized, (Beal, *et al*., 1986). However, Davies & Roberts, 1988, reported that QUIN-induced lesions resulted in a loss of somatostatin and neuropeptide Y and the preservation of a population of cholinergic neurons. The sparing of cholinergic neurons in the striatum has now been confirmed (Norman, *et al*., 1991). It has been proposed that the disputed results of Davies and Roberts, 1988, may have been due to their examining the core of the lesion, where QUIN concentration is the highest and neurons are all killed (Stone, 1993).

 QUIN is also known to produce long-term changes. Excitotoxic injury from a single intrastriatal injection of QUIN results in a more significant reduction in the overall size of the caudate nucleus after long post-lesion intervals (30 weeks) than after short periods (2 weeks). This reduction is due to the slow, progressive depletion of striatal neurons proceeding outward from the lesion site. Thus initial injury triggers chronic, self-sustained neuronal death, which has a striking parallel to that observed in HD (DiFiglia, 1990)

1.3.6.   Seizures and QUIN

QUIN is known to induce seizures through its action on NMDA receptors and has been described as the most powerful endogenous convulsant to date. Schwarcz *et al*., 1984, showed that a dose of 120nmol QUIN injected into the dorsal hippocampus of unanaethetised, freely moving rats, was required to reliably precipitate seizures. These seizure episodes were characterized by repetitive periods of high-voltage spiking typically lasting 20s in encephalographic recordings made. The total number of seizures and the total time in seizures increased in a dose-dependent manner. In addition, seizure episodes were associated with a frozen appearance of the animal with intermittent "wet dog shakes" as well as ataxia. Regarding the level of QUIN in

epileptic states, Heyes, *et al.*, 1990 found no difference in QUIN concentrations in epileptic foci from human patients suffering from intractable complex partial epilepsy. Contrary to these earlier studies, a decrease in QUIN concentration was later found in CSF from an epileptic patient (Stone, 1993). These results are contrary to that expected since QUIN is implicated in the initiation of seizures.

However, it is now thought that an enhanced synthesis of QUIN may be involved in seizures (Stone, 1993), following reports that 3-HAO as well as L-kynurenine is increased (Suzuki & Mori, 1992). Thus 3-hydroxyanthranilic acid may be elevated and together with 3-HAO could elevate QUIN concentrations. This may be further potentiated by the observation by Feldblum, *et al.*, 1988, that QPRT is decreased in epileptogenic zones of the brain.

1.3.7.   **Neuroprotection against QUIN**

Several agents are protective against QUIN-induced toxicity, most of which are linked to NMDA receptor function, for instance, antagonists such as 2AP5, MK-801 and ketamine (Lees, 1987). Zinc and even systemically administered magnesium is effective (Wolf, *et al.*, 1990), however this may be related to its general depressant affect on neuronal excitability, or on the release of necessary presynaptic factors, than to selective suppression of NMDA receptor function (Stone, 1993). QUIN toxicity of striatal neurons can be prevented by systemically applied monosialogangliosides and by the prior implantation of fetal striatal tissue; the mechanism of which is uncertain.

With respect to QUIN toxicity, there have been few reports of agents that can be applied systemically to provide protection. Most antagonists mentioned have little activity in this respect, except MK-801 and more recently, memantine. Memantine when administered orally can be protective against QUIN (Keilhoff & Wolf, 1992).

1.4. OXIDATIVE STRESS

1.4.1. Introduction

Oxygen is essential to life and it plays a vital role in diverse biological phenomena, however, it can also provoke damaging oxidative events within cells. Although there are a variety of free radicals produced by molecules, those that are produced from molecular oxygen have received the most investigative interest (Reiter, *et al*., 1995). The oxygen species that are typically linked to oxidative stress are superoxide ($O_2^{\cdot}$) anion radical, hydroxyl radical (Cheesman & Slater, 1993), hydrogen peroxide (Hoyt, *et al*., 1997), nitric oxide (NO) and peroxynitrite. The collective term for these chemicals is "reactive oxygen species" (ROS), but not all of these species are particularly active in aqueous biological solutions (Dawson & Dawson, 1996). These reactive oxygen species cause an oxidative stress that could lead to cell damage or death (Hoyt, *et al*., 1997).

Oxygen, via the transformation to these more reactive forms, can cause damage to DNA, proteins, essential enzymes, provoke uncontrolled chain reactions such as lipid peroxidation and dissolution of plasma and mitochondrial membranes. Normally there exists a balance in living cells between oxidative events and antioxidative forces. When the normal balance is upset, either by loss of reducing agents or protective enzymes or by the increased production of reactive oxygen species, or by both events simultaneously, the cell is said to be under oxidative stress (Fahn & Cohen, 1992). Thus, oxidative stress refers to cell damage induced by free radicals or reactive oxygen species.

Oxidative stress induces cell injury in a number of neuronal cell culture models and by a variety of proposed mechanisms. Hydrogen peroxide causes apoptosis of immature cultured cortical neurons (Whittemore, *et al*., 1995). ROS increases intracellular free calcium, which leads to the activation of endonucleases that degrade DNA (Floyd & Carney, 1992). Proteins that control calcium homeostasis, such as ATPase is sensitive to oxidative attack, the inhibition of which, leads to dysregulation of calcium levels. Mitochondria are important in buffering excessive calcium in

neurons (Werth & Thayer, 1994) and ROS exposure disrupts mitochondrial function preventing accumulation of mitochondrial calcium as well as decreasing ATP production, both of which have toxic consequences.

1.4.2.   Free Radicals

Free radicals are unstable, highly reactive molecules characterized by the presence of unpaired electrons in their outermost shells (Schipper, 1998). This definition encompasses a wide range of species (Table 1.1.). Usually, electrons associated with atoms or molecules are paired because this makes atoms relatively stable and unreactive. The loss of an electron leaves a molecule much more reactive than its paired counterpart. If two radicals react, both radicals are eliminated, while if a radical reacts with a non-radical, another free radical is produced. This characteristic allows free radicals to participate in chain reactions, which may be thousands of events long (McCord, 1985).

Table 1.1.   Types of free radicals (Halliwell, 1992).

Type of radical	*Example*
Hydrogen centred	Hydrogen atom, $H^{\cdot}$
Carbon centred	Trichloromethyl, $CCl_3^{\cdot}$
Sulphur centred	Glutathione thiyl, $GS^{\cdot}$
Oxygen centred	Superoxide, $O_2^{\cdot}$
	Hydroxyl, $OH^{\cdot}$
	Lipid peroxyl, $Lipid\text{-}O_2^{\cdot}$
Electron delocalized	Phenoxyl, $C_6H_5O^{\cdot}$ (electron delocalized into benzene ring)
	Nitric oxide, $NO^{\cdot}$

The oxygen centred radicals or "oxyradicals" such as $O_2^{\cdot}$, hydroxyl radical and hydrogen peroxide, mediate oxygen toxicity. The radicals are connected via the transfer of a single electron for example, the acceptance of a single electron by an oxygen molecule forms the $O_2^{\cdot}$, while the acceptance of an electron by $O_2^{\cdot}$ along with

two protons forms hydrogen peroxide. Similarly, the acceptance of an electron by hydrogen peroxide forms the highly reactive hydroxyl radical.

1.4.2.1. Superoxide Radical

$O_2^{\cdot}$ is formed *in vivo* in a variety of different ways. A major source is via the cellular electron transport chains, such as those of mitochondria, chloroplasts and the endoplasmic reticulum (Halliwell & Gutteridge, 1990) where some electrons passing through the chain leak directly from the intermediate electron carriers onto O_2. Since oxygen accepts one electron at a time, $O_2^{\cdot}$ is formed (Halliwell, 1992). Some leakage of $O_2^{\cdot}$ can occur, however, mitochondria normally contain high levels of the protective enzymes, $O_2^{\cdot}$ Dismutase (SOD) and glutathione peroxidase to remove toxic species (Jesberger & Richardson, 1991).

The reduction of molecular oxygen to form $O_2^{\cdot}$ also occurs in normal biochemical oxidation-reduction reactions, both enzymatic (xanthine oxidase) and non-enzymatic (autoxidation of catecholamines). In addition, activated phagocytic cells (such as monocytes, neutrophils, eosinophils and macrophages including microglia) produce superoxide, which plays an important part in the mechanism by which bacteria are engulfed and destroyed (Colton & Gilbert, 1987). Thus excessive activation of phagocytic cells (as in chronic inflammation) can lead to free radical damage.

The $O_2^{\cdot}$ can also be generated chemically by auto-oxidative reactions with catecholamines, tetrahydrofolates and reduced flavins. The Ca^{2+}-dependent activation of phospholipase A_2 also yields $O_2^{\cdot}$ through the metabolism of arachidonic acid by the lipoxygenases and cyclooxygenases to form eiconasoids (Chan & Fishman, 1980). Most of the $O_2^{\cdot}$ generated in a cell is converted to H_2O_2 by $O_2^{\cdot}$ dismutase (DiFiglia, 1990; Halliwell, 1992; Barkats, *et al*., 1996

1.4.2.2. Hydrogen Peroxide

A system generating $O_2^{\cdot}$ would produce H_2O_2 by non-enzymatic or SOD-catalyzed dismutation. SOD enzymes in human cells usually work in conjunction with H_2O_2-removing enzymes such as catalases and glutathione peroxidases (Halliwell &

Gutteridge, 1990). Hydrogen peroxide is not itself a free radical and it has limited reactivity, however, it can cross biological membranes, which superoxide can only do very slowly. The toxicity of H_2O_2 to cells is variable, which can be accounted for by the activity of H_2O_2-removing enzymes and by the rate of conversion of H_2O_2 into more highly reactive radicals. One such radical is the hydroxyl radical, which acts close to its site of action and thus the type of damage depends on its site of formation (Halliwell & Gutteridge, 1990).

1.4.2.3.   Hydroxyl radical

The OH^- radical is probably the most reactive of the ROS species (Poeggler; *et al.*, 1993; Dawson & Dawson, 1996) as it will react with almost all molecules in living cells (Fridovich, 1996). The OH^- radical has been implicated in damage to proteins, carbohydrates, DNA and lipids (Bird & Iversen, 1974). Most of the hydroxyl radicals generated in biological systems are formed when transition metals (Oliver, *et al.*, 1990) or biologically active chelators, such as porphyrins and flavins are present simultaneously with an oxidant, such as hydrogen peroxide. A common route of formation of this radical occurs when hydrogen peroxide reacts with a metal such as iron (II). This is known as the Fenton reaction.

1.4.3.   Defence Mechanisms Against ROS Neurotoxicity

The body has many defence mechanisms to deal with oxidative stress (Machlin & Bendich, 1987) see Table1.2. These enzyme systems are normally distributed evenly inside cells (McCord, 1985), and under normal circumstances these defence mechanisms can deal with the production of ROS in the neuron.

Table 1.2. Cellular defence / anti-oxidant mechanisms accessible to neurons to protect against ROS species (Dawson & Dawson, 1996)

Enzymatic	Non-Enzymatic
Cu / Zn – $O_2^{\cdot}$ Dismutase	Ascorbic Acid (Vitamin C)
Mn – $O_2^{\cdot}$ Dismutase	α-tocopherol (Vitamin E)
Glutathione Peroxidase	Glutathione
Glutathione-S-Transferase	
Glutathione Reductase	
Catalase	

These enzymes scavenge reactive chemical species and help to maintain cells in a reduced state. Cellular reducing agents such as glutathione and tocopherol, appear predominantly in the reduced state rather than their oxidized form to enable them to gain electrons (Fahn & Cohen, 1992).

The breakdown of $O_2^{\cdot}$ by SOD yields hydrogen peroxide and oxygen (reaction 1). There are two distinct SOD's in eukaryotes ; the manganese-containing SOD localized in the mitochondrial matrix and the copper- zinc-containing SOD found in the cytoplasm.

$$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$
$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (2)$$
$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad (3)$$
$$2H_2O_2 \rightarrow O_2 + 2H_2O \qquad (4)$$
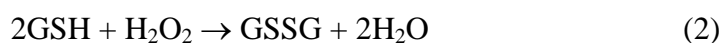
Hydrogen peroxide is decomposed by two reactions. At low concentrations, H_2O_2 is removed by reacting with reduced glutathione to form oxidized glutathione and water, catalyzed by glutathione peroxidase (reaction 2). Reduced glutathione is regenerated by the action of glutathione reductase (reaction 3). At high concentrations, however, H_2O_2 is removed by the enzyme catalase (reaction 4), (Fahn & Cohen, 1992).

1.4.4.   The Involvement of Metal Ions

Ions of transition metals such as copper and iron are involved in many free radical reactions, and often these lead to the generation of very reactive species (Halliwell & Gutteridge, 1989). In the case of iron, only free iron is able to stimulate free radical reactions, whereas iron bound to protein such as transferrin found in human plasma, is not normally available to stimulate such reactions. Iron (II) reacts with hydrogen peroxide in the Fenton reaction to produce the harmful hydroxyl radical and the oxidized form of the metal ie. iron (III) as shown in reaction 5.

$$H_2O_2 + Fe^2 \rightarrow OH^- + OH^\cdot + Fe^{3+} \qquad\qquad (5)$$

$$Fe^{3+} + O_2^\cdot \rightarrow Fe^{2+} + O_2 \qquad\qquad (6)$$

$$O_2^\cdot + H_2O_2 \rightarrow O_2 + OH^- + OH^\cdot \qquad\qquad (7)$$
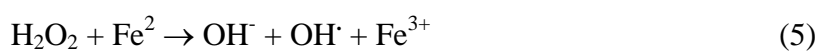
The Fenton reaction can be augmented by the reduction of ferric ion by superoxide thus regenerating ferrous ion (reaction 6). The net result is the production of hydroxyl radicals as in the iron-catalyzed Haber-Weiss type of reaction (reaction 7).

The iron dependent formation of the hydroxyl radical is detrimental to the cell, since, the hydroxyl radical is the most reactive of all oxygen radicals, so much so that no enzyme systems involve it as a substrate. Cells try to prevent its formation, and remove hydrogen peroxide and transition metals to inactive sites (Fahn & Cohen, 1992). The hydroxyl radical does not travel far and has a short half life, however, hydrogen peroxide is able to cross the blood-brain barrier and thus its conversion to hydroxyl radical in the brain causes severe damage to neurons.

One might ask at this point where the hydrogen peroxide originates from in the human body, to be available to form hydroxyl radicals. Hydrogen peroxide in the human body is mainly produced via $O_2^\cdot$. However, certain oxidases, such as uric acid oxidase also produce H_2O_2. It has also been found in micromolar concentrations in blood plasma and in the lens of the human eye. H_2O_2 has even been detected in exhaled human breath, which could arise from pulmonary macrophages, although a contribution from oral bacteria cannot be ruled out. Furthermore, H_2O_2 at

concentrations up to micromolar is present in most natural water supplies (Halliwell & Gutteridge, 1990).

Iron has also been linked to the oxidative stress hypothesis in Parkinson's disease in which the substantia nigra is the main region of the brain where cell damage occurs. The substantia nigra is rich in dopamine and iron (Youdim, 1988a), and patients with Parkinson's disease show an increase in iron in the pars compacta. Furthermore, dopamine is able to undergo enzymatic oxidation via monoamine oxidase to form hydroxyl radicals (Fahn & Cohen, 1992).

There is considerable evidence, which demonstrates that endogenous iron stores are partially available for participating in peroxidative events in tissue (Halliwell & Gutteridge, 1986). Iron accumulates in human and rat brain during aging, and there has been speculation that several degenerative changes in brain functions which occur during aging and in certain pathological states of the CNS may be mediated through or accelerated by iron-induced peroxidative processes (Viani, *et al*., 1991). More recently, Keyer & Imlay, 1996 reported that superoxide accelerates DNA damage by elevating free-iron levels. However, iron also plays essential roles in the brain, especially in learning and memory and these ions are required for the correct binding of certain neurotransmitters to their receptors (Youdim, 1988b).

1.4.5.   Iron Source For Fenton Chemistry

The iron contained in the body is absorbed from the gut, and originates from food and beverages we consume that are fortified with iron. Most body iron is found in haemoglobin, with smaller amounts in myoglobin, various enzymes and the transport protein transferrin. Iron not required for these is stored as ferritin, which can hold up to 45 000 ions of iron (Halliwell & Gutteridge, 1989). Transferrin enters the cytoplasm in a vacuole, which is acidified to release the iron. The transferrin is then ejected leaving a pool of non-protein-bound iron, which will be used in the synthesis of iron proteins. In this way the cells minimize the size of the intracellular iron pool (Halliwell & Gutteridge, 1989).

Oxidant stress can provide the iron necessary for Fenton chemistry by mobilizing iron from ferritin or by degrading haeme proteins to release iron. Reports have stated that the formation of OH^- *in vivo* may be limited by the supply of iron ions, and tissue injury can exacerbate radical reactions if it liberates metal ions from broken cells into the surrounding environment. This is especially true in brain, since CSF has no significant iron-binding capacity, and mechanical disruption of the brain releases iron that can stimulate radical reactions such as lipid peroxidation

1.4.6. Lipid Peroxidation

The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free radical reactions in toxicology and in human disease. Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids, ie. those lipids containing more than two carbon-carbon double covalent bonds (Halliwell, 1992). Cell membranes are rich in polyunsaturated lipids, which together with saturated fatty acids give rise to membrane fluidity. Damage to these polyunsaturated fatty acid side chains reduces membrane fluidity and as a result the biological membrane is not able to function properly.

The fluid nature of membranes, due to weak hydrophobic interactions (Mathews & van Holde, 1995), allows proteins and lipids to move freely with in the lipid bilayer that constitutes all biological membranes. The fluid mosaic model proposed by Singer & Nicholson, 1972, summarises most of the current information concerning membranes. According to this model a membrane is a fluid asymmetric lipid bilayer that carries a host of proteins.

Schematic diagram of the sequence of events in lipid peroxidation:

- Cell damage $\rightarrow OH^{\cdot} + CH_2 \rightarrow C^{\cdot}H \rightarrow$ conjugated diene $+ O_2 \rightarrow CHO_2^{\cdot} + CH_2 \rightarrow C^{\cdot}H$ + lipid peroxide
- Fe^{2+}-complex + lipid peroxide $\rightarrow CHO^{\cdot}$
- Fe^{3+}-complex + lipid peroxide $\rightarrow CHO_2^{\cdot} + H^+ + Fe^{2+}$-complex

Lipid peroxidation is initiated by the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$). A hydroxyl radical can do this, as well as various iron-oxygen complexes. Abstraction from a methylene group leaves a carbon radical, which then stabilizes by molecular rearrangement to form a conjugated diene. This then combines with oxygen (which is hydrophobic and thus concentrates into the interior of membranes) to give a peroxyl or "peroxy" radical ($CHO_2^{\cdot}$). This radical can then abstract a hydrogen from another lipid molecule or it may attack membrane proteins. Once the peroxyl radical is formed it can abstract another hydrogen atom from say a methylene group and combines with this hydrogen to form a carbon radical and a lipid peroxide. This carbon radical may then react with another oxygen to form a peroxyl radical and so the chain of lipid peroxidation can continue.

Iron plays an important role in lipid peroxidation. Not only can it generate hydroxyl radicals via the Fenton reaction, which initiates the chain of events leading to the formation of the alkoxyl radical, but it also plays a second important role in lipid peroxidation. Lipid peroxides are fairly stable at physiological temperatures, but in the presence of iron, their decomposition is greatly accelerated. Thus a reduced iron complex can react with lipid peroxides in a way similar to its reaction with H_2O_2; it causes fission of O-O bonds to form alkoxyl radicals. An iron (III) complex can form peroxy radicals and by further reaction with the iron (II) –complex can form alkoxyl radicals (Halliwell & Gutteridge, 1990).

1.4.7. Free Radicals and Disease

The mammalian brain is especially vulnerable to oxidative stress (Hall & Braughler, 1989) as it consumes 20% of total body oxygen, contains large amounts of polyunsaturated lipids, is relatively deficient in protective mechanisms, and readily accumulates iron (Dawson & Dawson, 1996). Although estimates vary, it is believed that up to 5% of the oxygen (Reiter, *et al*., 1995; Chan, *et al*., 1993) taken up by an organism may eventually end up as damaging oxygen-based radicals. In the human, this means that there could be the equivalent of 2 kg of O_2 produced each year (Reiter, *et al*., 1995).

Free radicals have been reported to be involved in aging as well as many disease states including, iron-overload disease, arthritis, neurological damage, Down's syndrome and diabetic cataract. However, of particular interest to neuroscience and brain related disorders is the role of oxidants in ischemia or reoxygenation injury.

1.4.7.1.   Ischemia and Reoxygenation Injury

Damage to the brain by depriving a portion of the tissue of oxygen (stroke) is a major cause of death,. Early events in ischemia include increased glycogen degradation and anaerobic glycolysis, leading to lactate production and acidosis (Halliwell & Gutteridge, 1990). ATP levels fall and eventually membrane damage becomes visible under the microscope.

If the period of ischemia is not long enough to cause irreversible damage then much tissue can be saved by reperfusing the tissue with blood and reintroducing oxygen and nutrients. However, though beneficial this process may be, it has been shown that this may cause additional insult mediated by oxygen radicals. This damage is known as reoxygenation injury (Patt, *et al*., 1988). This may be reduced in part by including oxidant scavengers in the reoxygenation fluid. The oxygen radicals, particularly, superoxide is generated by a chain of events. Following oxygen deprivation, AMP is degraded to cause an accumulation of hypoxanthine, while xyanthine dehydrogenase becomes converted to the oxidase enzyme by oxidation of essential thiol groups and by limited proteolysis. Upon reoxygenation the hypoxanthine can be oxidised by the xanthine oxidase causing rapid generation of superoxide and hydrogen peroxide, which leads to severe tissue damage (McCord, 1985).

1.4.7.2.   Free Radicals and Aging

Free radicals in brain could represent one of the main causes of cellular dysfunctions occuring during aging (Hall & Braughler, 1989). Apart from the brain being rich in polyunsaturated fatty acid, it also utilises a large amount of oxygen, and is deficient in antioxidant enzymes. Furthermore, it has been demonstrated that a general reduction of the antioxidant protection mechanisms occurs during aging. From among the enzymatic systems, Mizuno and Ohta, 1986, reported an age-dependent decrease in

superoxide dismutase activity. With regard to nonenzymatic antioxidants, it has been reported that during aging there is a decrease in brain ascorbate, glutathione and α-tocopherol (Viani, *et al*., 1991). During aging, the decrease in antioxidant protection, the fall in ATP levels in the brain and the increase in intracellular calcium levels, brings about changes in the composition and structure of plasma membranes. The molecular mechanism of the aging process in the nervous system is still unclear, but evidence points to an involvement of membrane damage. Viani, *et al*, 1991 stated that aging affects membrane properties just as peroxidation does, thus supporting a possible contribution of peroxidative damage to the aging process. This indicates that all pathological states (ischemia, hypertension and diabetes) known to promote a peroxidative injury can have even more dramatic consequences when they occur in old tissue.

1.4.8. Antioxidant Therapy

Antioxidants usually refer to molecules that inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxy radicals (Halliwell, 1992). This would prevent the further attack of adjacent fatty acid side chains or membrane proteins. An important antioxidant in the brain is α-tocopherol. Severe and prolonged deprivation of this antioxidant produces severe neurological derangements (Muller & Goss-Sampson, 1990), however, it takes a long time to increase the α-tocopherol content in the brain tissue in mammals supplemented with vitamin E. Furthermore, oxidative damage can occur without lipid peroxidation (Halliwell, 1992), and so inhibitors of lipid peroxidation may be ineffective.

1.4.9. Molecular Targets of Oxidative Stress

Oxidative damage does not always lead to the formation of lipid peroxides in injured nervous tissue. Damage to DNA and proteins are of equal or greater importance *in vivo* (Halliwell, 1987). Early events in human cells subjected to oxidative stress, include DNA damage and consequent activation of poly (ADP-ribose) synthetase, decreases in ATP content and increases in calcium, with consequent activation of

calcium-stimulated proteases that can cause such phenomena as ' bleb' formation on the plasma membrane of the cells (Orrenius, *et al*., 1989).

Protein damage is frequently an important consequence of oxidative stress, and Oliver *et al*., 1990 showed that radicals can damage brain proteins, including the enzyme glutamine synthetase (GS), which is responsible for the removal of glutamate. Oxidative inactivation renders GS and other enzymes highly susceptible to proteolysis by proteases such as trypsin and subtilisin as well as by a class of cytosolic proteases that selectively degrade the oxidized protens *in vivo* and *in vitro*. Oliver, *et al*., 1990 proposed that oxidative modification of proteins is a marking step for selective proteolysis. Additional evidence suggests that this process is important in a variety of normal and pathological processes, such as aging, neutrophil function, rheumatoid arthritis and oxygen toxicity (Oliver, *et al*., 1990).

1.4.10.   Heat Shock Response to Oxidative Stress

Heat shock proteins are synthesized in response to any form of stress in the cell that results in a denaturing of proteins. Oxidative stress is one condition in which the inappropriate folding of proteins is favoured. This results in a heat shock response in the cell, which is characterized by a shutdown of protein synthesis and a dramatic increase in the synthesis of heat shock proteins (Hsp's), (Smith, *et al*., 1998). There are several types of Hsp families, each differing with respect to sequence and expression patterns, as well as function and subcellular localization of the respective gene products. Major Hsp families, named to reflect the approximate molecular size (in kilodaltons) of family members, are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small Hsp family (typically 20 to 25 kDa), (Smith, *et al*., 1998).

Hsp70 is the molecular chaperone mainly involved in the refolding of proteins. Chaperones achieve this by controlled binding and release of the substrate protein, facilitating its correct folding, oligometric assembly and transport to a particular subcellular compartment (Hendrick & Hartl, 1993). In addition, this chaperone exhibits a protective effect following tissue injury. Currie, *et al*., 1988, first demonstrated that heat stress promotes recovery of heart tissues from ischemic injury.

This protection correlated with the time course of Hsp70 levels (Yellon & Latchman, 1992), and transgenic mice overexpressing Hsp70 exhibit reduced postischemic injury (Marber, *et al*, 1995). A recent article reported that Hsp70 protects a stressed cell via an anti-apoptotic function (Beere, *et al*., 2000). Apoptosis is programmed cell death. The mechanism underlying this Hsp70-mediated suppression of apoptosis is only just beginning to unfold. However, evidence suggests that Hsp70 targets the APAF-1 (apoptotic protease-activating factor) apoptosome, thereby inhibiting the activation of procaspase-9, which occurs during apoptosis (Beere, *et al*., 2000).

It is believed that one of the major causal factors of senescence is the accumulation of damaged protein induced by oxidative stress (Jawinski, 1996). Thus molecular chaperones may cope with and suppress the accumulation of these damaged proteins, thereby contributing to the delay of senescence and an increased life span. Naturally extreme overexpression of molecular chaperones has some deleterious effects on the growth of an organism (Krebs & Feder, 1997).

1.5.   ESTROGEN

1.5.1.   Introduction

The endocrine physiology in the female and the interplay of the many hormones associated with sex determination, conception, fetal development, birth, growth, puberty, the reproductive years, and finally the menopause beautifully illustrate the complexity and responsivity of this highly differentiated endocrine system. Its integrated operation is dependent upon the interaction of signals – both hormonal and neural – between the central nervous system, the pituitary and the ovary (Norman & Litwack, 1987).

The two most important steroid hormones of the adult female are estrogen and progesterone. Estrogens are formed from androgenic precursors through an enzymatic process known as aromatization. 17β-estradiol, the predominant and most potent estrogen in premenopausal women, is synthesized by developing ovarian follicles. Estradiol is secreted into the bloodstream, bound partially by circulating sex hormone

binding globulin, and then transported to cells throughout the body (Lievertz, 1987). The principle path of estradiol metabolism is reversible oxidation to estrone, a weaker estrogen, and then to estriol. Estrone can also be produced in peripheral tissues through aromatization of androstenedione, an androgen precursor produced by both the ovaries and the adrenal glands (Norman & Litwack, 1987). All of these compounds are metabolized into sulphate and glucuronide forms for excretion (Lievertz, 1987). Several naturally occuring estrogens are typically 18-carbon steroids, which have an aromatic A ring with a phenolic hydroxyl. 17β-estradiol is the most potent natural estrogen; its stereoisomer, 17α-estradiol being much less potent (Emmens, 1969).

1.5.2. Estrogen and the Brain: An Overview

Until about 10 years ago, estrogen was considered to be only a reproductive hormone. We now understand that estrogens modulate far more diverse functions and influence far more tissues than merely those confined to the reproductive axis. Estrogen is now considered to be neurotrophic and has the ability to influence neuronal structure and biochemistry (Dubal, *et al*., 1999)

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Estrogens have numerous effects on the brain throughout the life span, beginning during gestation and continuing into senescence. Estrogens have been reported to have widespread influences on neuronal systems, which make this hormone an important factor to consider in the treatment of depressive illness (McEwen, 1998). A variety of nervous system functions are modulated by estrogen. These include, behavior and cognition (Wise, *et al*., 2000), protection from injury and ischemia (Culmcee, *et al*., 1999), regeneration (Tanzer, *et al*., 1999) and stimulation of neurite growth in culture (Toran-Allerand, 1999). The study of estrogen action on the brain has thus provided novel insights for fundamental neuroscience, including information relevant to coping with stressful life experiences, reproduction, aging, recovery from brain damage and the pathophysiology of many neurologic diseases and mental disorders (McEwen, 1998).

1.5.3.   **Estrogen and Cognition**

Increasing evidence demonstrates that estradiol influences cognition and the incidence and progression of Alzheimer's disease (Sherwin, 1994). Estrogen plays a critical role in preventing the decline in cognitive function associated with normal aging, and hence postmenopausal women who are hypoestrogenic, may suffer increasingly from cognitive dysfunction as they age. Studies in experimental animal models provide a convincing rationale for a role for hormone replacement therapy (HRT) in the treatment and prevention of dementia (Paganini-Hill & Henderson, 1996).

Unfortunately there are very few prospective, adequately controlled, randomized clinical trials that rigorously test whether estrogen replacement therapy 1) improves cognition in young or aging women, 2) decreases the risk of Alzheimer's disease by preventing or delaying its onset, or 3) improves learning and memory in women with AD (Dubal, *et al*., 1999). Numerous authors report that estrogen plays a role in delaying the onset of Alzheimer's disease (Paganini-Hill & Henderson, 1996; Tang, *et al*., 1996), while others maintain that it has no impact on the risk of Alzheimer's disease (Brenner, *et al*., 1994). Nevertheless, substantial evidence suggests a definite role for this hormone in the etiology of the disease and the cholinergic system.

Estrogen elicits many neurotrophic effects on neurons involved in learning and memory. Estrogen helps build and maintain synapses, induces neurite growth and influences LTP and excitability, and enhances gene expression (Dubal, *et al*., 1999). These trophic actions of estradiol may give it the ability to improve such higher mental functions as learning and memory.

1.5.3.1.   **Estrogen and the Cholinergic System**

The basal forebrain and the cholinergic neurons that project to the cerebral cortex and hippocampus play an important role in cognitive function (Blokland, 1995) and affective state (Leong & Brown, 1987). Estrogen has been shown to induce choline acetyltransferase (ChAT) (McEwen, 1998), which is the rate limiting enzyme for acetylcholine formation. Acetylcholine is the principle neurotransmitter of the

cholinergic system and it is via this chemical that neurons are able to communicate with other nerve cells. Thus by increasing the levels of acetylcholine, estrogen is actually revving up activity in the basal forebrain.

1.5.3.2.   Neurite Outgrowth and Synaptogenesis

McEwen and colleagues, in a series of reports showed that estrogen induces morphological changes in the human adult brain. They demonstrated that estrogen induces dendritic spines (Figure 1.10. depicts the structure of dendritic spines) and new synapses in the ventromedial hypothalamus of the female rat but also increases the density of dendritic spines on pyramidal neurons in the hippocampus (McEwen & Woolley, 1994) (Figure 1.11). The effects on dendritic spines were found only in the CA1 regions of the hippocampus, and that spine density changed cyclically during the estrus cycle of the female rat. A later study by Murphy reported that the estrogen-induced increase in dendritic spine density is due to a reduction in GABA neurotransmission in hippocampal neurons (Murphy, *et al*., 1998).

Furthermore, parallel changes in synapse density on dendritic spines revealed by electron microscopy, strongly supported the notion that new synapses are induced by estradiol. Taken together, these morphological studies indicate that synapses are formed and broken down rapidly during the natural reproductive cycle of the rat (McEwen, 1998). In order for estrogen to form new synapses, nerve cell growth has to be induced. Toran-Allerand, 1996 suggested that estrogen may cooperate with neurotrophins, potent stimulators of nerve cell growth, such as nerve growth factor (NGF).

A deficit in axonal sprouting is seen in ovariectomized rats, which is returned to normal with estrogen replacement. A role for apolipoprotein E is implicated in regeneration of synaptic circuitry after neuronal injury. Teter, *et al*., 1999 reported that estrogen increases neuronal sprouting in the same region where sprouting is dependent on apolipoprotein E. Thus, sprouting may be stimulated by estrogen through its up-regulation of apolipoprotein E expression leading to increased recycling of membrane lipids for use by sprouting neurons.

Figure 1.10. A computer reconstruction of a segment of dendrite, showing the variable shapes and sizes of spines (Bear, *et al.*, 2001)
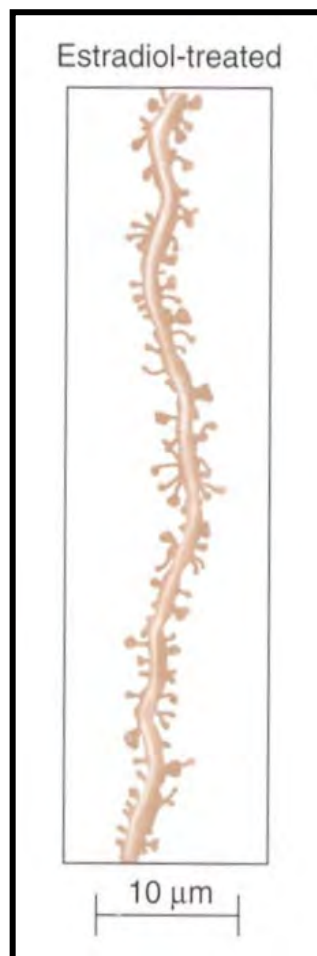
Figure 1.11. The increase in dendritic spine density following estradiol treatment to ovariectomized rats (Bear, *et al.*, 2001)

1.5.3.3.   The Role of NMDA receptors

The regulation of dendritic spine density by estrogen has been shown to be dependent on the activation of the NMDA receptor (Woolley & McEwen, 1994). Two fundamental reports then followed, suggesting that estrogen-induced dendritic changes actually affect neuron function, by linking them to the NMDA receptor. The first report appeared in 1996, in which Morrison & Gazzaley, in collaboration with McEwen and his team, found that estradiol increases the concentration of the NMDAR1 subunit protein. Interestingly, the increase was concentrated in the same hippocampal region where an increase in spine density occurs. Moreover, these receptors were active in transmitting neuronal signals (Woolley, *et al*., 1997).

In short, NMDA receptors are implicated as mediators of effects of estradiol on morphological plasticity and related physiological and cognitive processes in the brain. Estradiol increases dendritic spine density (Gould, *et al*., 1990) and synapses (Woolley & McEwen, 1992) on pyrimidal cells via a mechanism dependent on NMDA receptor activation (Woolley & McEwen, 1994). Less direct evidence of NMDA receptor involvement in estrogen-induced neural modifications includes estrogen's role in facilitating seizure induction (Terasawa & Timiras, 1968) and memory and learning enhancement (Philips & Sherwin, 1992) in human and experimental animals. This connection is based on the NMDA receptor's important role in seizure-triggering mechanisms (Sato, *et al*., 1989) and learning and memory (Morris, *et al*., 1986).

Furthermore, autoradiographic analysis has revealed that NMDA receptor agonist binding sites are increased in the dendritic layer of CA1 in the hippocampus in response to estradiol treatment in ovariectomized rats (Weiland, 1992). This is of particular interest when considering that spine synapses are excitatory and NMDA receptors are likely to occur on them. It is relevant to ask whether activation of NMDA receptors themselves could lead to induction of new synapses, in which case estrogen induction of NMDA receptors would then become a primary event leading to synapse formation. The influx of calcium following NMDA receptor activation, may be an important factor in the extension and retraction of dendritic spines. For example, NMDA receptor activation has been found to promote dephosphorylation of MAP2

and to alter the interaction of this cytoskeletal protein with actin and tubulin (Nasrallah, *et al*., 1989). Subsequent studies revealed that receptors for estrogen and the neurotrophins are found on the same neurons in the rat basal forebrain, and hippocampus. Moreover, estrogen increases the expression of NGF receptors and NGF enhances the binding of estrogen to its receptor. Thus estrogen and NGF amplify each other's growth responses.

Most (not all) of estrogen's actions are mediated through the estrogen receptor. Estrogen exerts many effects via classical genomic mechanisms on a variety of genes. In order to do this, estrogen binds to its receptor and the estrogen estrogen-receptor complex acts as a transcription factor, binding to DNA and increasing the expression of a variety of proteins. These include, brain-derived neurotrophic factor (BDNF), neurotrophin 3 and neurotrophin 4/5 (Dubal, *et al*., 1999). However, many of the effects of acute application of estrogen are too rapid to be accounted for by a genomic pathway.

1.5.3.4.   Long Term Potentiation

Estrogen enhances LTP through the activation of NMDA receptors. Foy, *et al*., 1999 reported that 17β-estradiol enhances NMDA receptor-mediated excitatory postsynaptic potentials and LTP. Other electrophysiological studies have indicated that 17β-estradiol modulates synaptic transmission and enhances the magnitude of LTP recorded from hippocampal slices of adult rats (Foy, *et al*., 2000). Moreover, 17β-estradiol ameliorates cognitive and memory function in postmenopausal women and aging is associated with an alteration of synaptic plasticity eg. higher susceptibility to long term depression (LTD). Vouimba, *et al*., 2000 reported that the induction of LTD recorded from CA1 hippocampal neurons of aged rats is suppressed by 17β-estradiol treatment, which produced only a minimal effect in suppressing LTD in adult rats. The induction of LTP by estrogen has been further supported by Gupta, *et al*., 2001. However, chronic treatment of estradiol to ovariectomised rats has been shown to have no effect on either the excitability or induction of LTP Schaffer collateral-commissural-CA1 pathway *in vitro* (Barraclough, *et al*., 1999).

1.5.4.   The Neuroprotective Effects of Estrogen

1.5.4.1.   Introduction

Accumulating evidence from clinical and basic science studies support a role for estrogen in neuroprotection. Recent basic science studies show that not only does exogenous estradiol decrease the response to various forms of insult, but the brain itself upregulates both estrogen synthesis and estrogen receptor expression at sites of injury. The term neuroprotection encompasses a spectrum of independent processes; estrogen directly promotes cell survival and synaptic plasticity, and prevents axonal and dendritic pruning. Estrogen can also prevent malfunction of neurons by altering levels of neurotransmitters, neurotransmitter receptors and second messengers. This regulation of neurotransmitter function can then, secondarily, promote cell survival and neurite branching. Thus, the protective effects of estradiol are multifaceted (Garcia-Segura, *et al*., 2001).

1.5.4.2.   *In Vitro* Evidence of Estrogen Neuroprotection

Addition of 17β-estradiol to culture media increases the viability, survival and differentiation of primary cultures of different neuronal populations including hippocampal neurons (Sudo, *et al*., 1997). Estrogen has been shown to protect neuronal cells against a variety of stresses, and that much of this protection is independent of the estrogen receptor (Gridley, *et al*., 1998; Moosman & Behl, 1999). Estrogen has been reported to protect neuronal cell cultures against oxidative stress induced by iron (Vedder, *et al*., 1999), glutamate (Singer, *et al*., 1996), α-amino-3-hydroxy-5-methyl-4-isoxasole propionic acid (AMPA) (Zaulyanov, *et al*., 1999), NMDA (Weaver, *et al*., 1997) and kainate (Regan & Guo, 1997). Estrogen also reduces lipid peroxidation induced by iron sulphate (Goodman, *et al*., 1996) and β-amyloid protein (Green, *et al*., 1996) and moreover has a protective effect in response to superoxide anions and hydrogen peroxide (Sawada, *et al*., 1998). The antioxidant activity of estradiol has been attributed to the presence of the hydroxyl group in the C3 position on the A ring of the steroid molecule (Behl, *et al*., 1997).

Other *in vitro* studies show that estrogen prevents neuronal death induced by stimuli such as anoxia, serum deprivation, pro-oxidant hemoglobin and sodium azide (Garcia-Segura, *et al*., 2001). This long list of studies reveals that the neuroprotective effects of estrogen have recently received considerable attention and have provided important insights into the possible mechanisms involved in the *in vivo* neuroprotective effects.

1.5.4.3.   *In vivo* Evidence of Estrogen Neuroprotection

Estrogen has been shown to protect neurons *in vivo* from different lesion paradigms. Experimental forebrain ischemia has been one of the models used to test the neuroprotective effect of estradiol *in vivo*. It has been well documented that pretreatment with 17β-estradiol reduces animal mortality and ischemic area in ovariectomized rats after middle cerebral artery occlusion (Dubal, *et al*., 1998; Culmsee, *et al*., 1999; Simpkins, *et al*., 1997) and common carotid artery occlusion (Wang, *et al*., 1999). Another experimental model that has been of great interest is the nigrostriatal system. Estrogen has neuroprotective properties against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity in the nigrostriatal dopaminergic system (Dluzen, 2000), which is involved in Parkinson's disease.

Systemic administration of kainic acid is used as an experimental model of epilepsy and neurodegeneration. This toxin causes recurrent seizures and loss of somatotatinergic interneurons in the hilus of the dentate gyrus of the hippocampus (Garcia-Segura, *et al*., 2001). Administration of estradiol to ovariectomised rats is able to prevent this partial hilar neuronal loss (Azcoitia, *et al*., 1998). In addition, it was found that the effect of kainic acid on these neurons differed depending on the day of the estrus cycle on which the neurotoxin was injected.

1.5.5.   Mechanisms of Action of Estrogen Neuroprotection

Although estrogen is a well described neuroprotective agent, the mechanism by which estrogens exert their neuroprotective actions is unknown. Estrogens have a plethora of cellular effects including activation of nuclear estrogen receptors (ER), increased expression of anti-apoptotic proteins, interactions with second messenger cascades,

alterations in glutaminergic activation, maintenance of intracellular calcium homeostasis and antioxidant activity (Figure 1.12.) (Green & Simpkins, 2000). Several of these effects have been shown to enhance neuronal survival, however, the exact role of each of these pathways in estrogen-enhancement of neuronal survival remains to be elucidated.
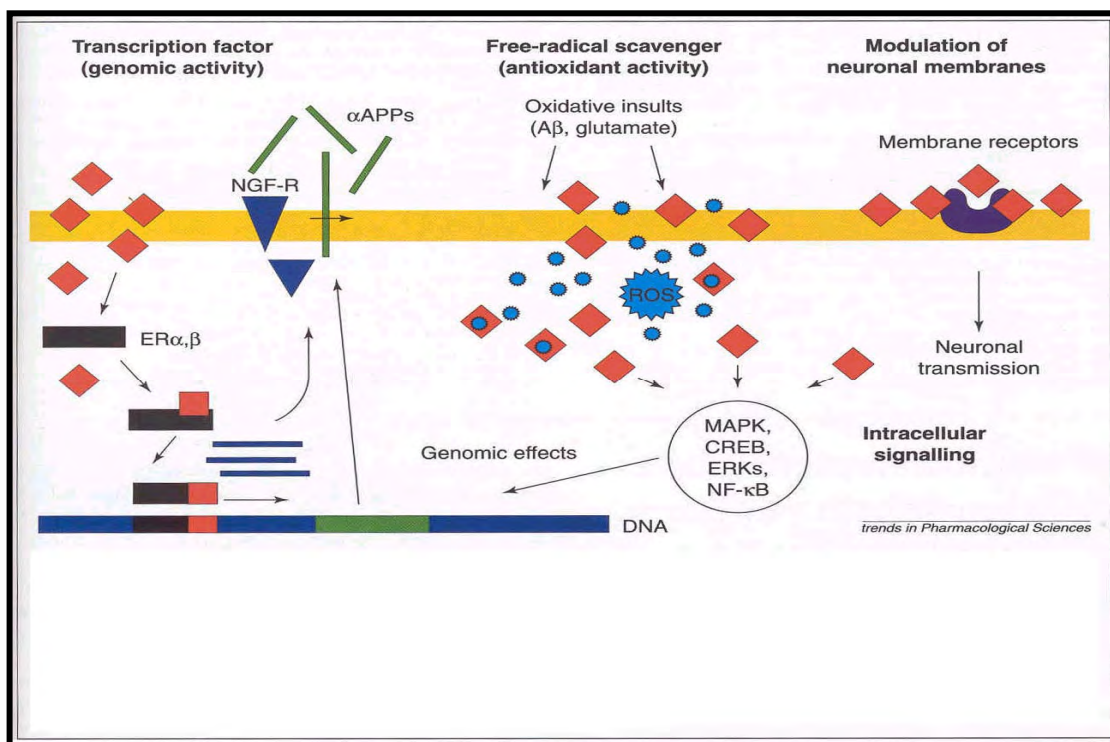
Figure 1.12. Estrogens (shown in red diamonds and squares) exert multiple neuroprotective functions. 1) Estrogen activates its receptor resulting in a genomic method of neuroprotection, 2) estrogen may act as an antioxidant to scavenge ROS, 3) estrogen modulates the function of membrane receptors that might modulate neuronal transmission, and 4) estrogen may interact with various intracellular signaling pathways (Behl & Holsboer, 1999)

1.5.5.1.   Classical Estrogen Receptor Activity

Estrogens classically exert their effects by a nuclear ER mechanism of action, in which the steroid enters the cell and binds to the nuclear ER. The estrogen-ER complex associates with the estrogen response element (ERE) and functions as an enhancer for ERE-containing genes. This may contribute to the neuroprotective effects of estradiol. Exposure to estradiol increases expression of many ERE-containing genes for example BDNF (Sohrabji, *et al*., 1995), c*fos* (Rachman, *et al*.,

1998), the anti-apoptotic bcl-2 gene (Dong, *et al*., 1999) and the *hsp*25 gene (*Gaestel*, *et al*., 1993). Hsp25 is involved in axonal regeneration and may be a critical factor involved in posttraumatic recovery.

1.5.5.2. Activation of the MAPK Signal Transduction pathway

The colocalization of estrogen receptors and neurotrophin receptors in the same neurons leads to convergence or cross-coupling of their signaling pathways. Estradiol has been shown to activate the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 in cerebral cortical explants and neuroblastoma cells (Toran-Allerand, *et al*., 1999). In addition, estrogen-induced neuroprotection in primary cortical cultures exposed to glutamate is associated with a rapid activation of tyrosine kinase and MAPK activity (Singer, *et al*., 1999). MAPK interferes with c-Jun N-terminal protein kinase activation, protecting cells from apoptosis.

1.5.5.3. Activation of the cAMP-PKA-CREB Pathway

The cAMP-PKA pathway is a well described signal transduction pathway in which increased adenylate cyclase activity results in increased cAMP concentrations and downstream activation of protein kinase A (PKA). Estradiol increases cAMP accumulation in hypothalmic neurons and human neuroblastoma cells and has cellular effects in neurons consistent with increased cAMP levels, including increased phosphorylation of cAMP response element binding protein (CREB) (Green & Simpkins, 2000). The activation of the cAMP pathway is associated with decreased susceptibility of neuronal cell to apoptotic signals. Furthermore, elevation of cAMP is sufficient to enhance the survival of spinal motor neurons in culture (Hanson, *et al*., 1998). Increased CREB phosphorylation is also associated with increased resistance to ischemic injury and activation of the cAMP-PKA-CREB pathway may enhance neuronal survival by increased expression of bcl-2, activation of the map kinase ERK or inhibition of the pro-apoptotic raf-1 in non-neuronal cells (Green & Simpkins, 2000).

1.5.5.4.   Modulation of Intracellular Calcium Concentrations

Increased intracellular calcium concentrations modulate numerous neuronal functions including cell survival, synaptic formation and strength, and calcium-mediated neuronal death (Ghosh & Greenberg, 1995). Estrogens can modulate intracellular calcium levels through interactions with AMPA/kainate and NMDA receptors, and estradiol treatment of ovariectomised rats decreases L-type calcium currents in neostriatal neurons, although voltage gated currents are increased in CA1 hippocampal neurons (Joels & Karst, 1995). Furthermore, estradiol is known to increase the activity of protein kinase C and calmodulin kinase, both of which are calcium-triggered enzymes.

Maintenance of intracellular calcium homeostasis has been shown to be a component of estrogen-mediated neuroprotection. Estrogen treatment has been shown to attenuate the increase in intracellular calcium associated with amyloid β protein and gp-120-mediated toxicity (Brooke, *et al*., 1997).

1.5.6.   Hormone Replacement Therapy

The menopause is associated with a sharp decline in estrogen production, which gives rise to symptoms such as hot flushes, cessation of menstruation, weight change, mood swings, forgetfulness and headaches. Estrogen replacement therapy (ERT) is widely used among postmenopausal and surgically menopausal women to prevent osteoporosis and to reduce the risk of cardiovascular disease. However, numerous authors have established an additional role for estrogen therapy in the treatment or prevention of Alzheimer's disease (Wang, *et al*., 2000; Dubal, *et al*., 1999). Preparations used in estrogen replacement therapy contain a variety of estrogenic compounds. The most common and most frequently prescribed form of ERT is Premarin®, which is extracted from the urine of pregnant mares and is commonly referred to as conjugated equine estrogen (CEE).

Premarin® contains at least ten different estrogens in various quantities, but the two estrogens present in the greatest quantities are sodium estrone sulphate and sodium

equilin sulphate (Bhavnani, 1998). All ten estrogens are biologically active and are the sulphate esters of the ring β saturated estrogens: estrone, 17β-estradiol, 17α-estradiol, and the ring β unsaturated estrogens: equilin, 17β-dihydroequilin, 17α-dihydroequilin, equilenin, 17β-dihydroequilenin, 17α-dihydroequilenin and delta-8-estrone (Bhavnani, 1998). Estrogen sulphates are absorbed directly from the gastrointestinal tract, however hydrolysis of the sulphates also occurs here and the unconjugated estrogens formed are readily absorbed. Finally, Premarin® is marketed in two forms, a tablet form and an injectible form. The injectible form, unlike the tablets can be used for *in vitro* studies because no hydrolytic enzymes are required to break it down into active components.

1.5.7.   Protective Role for Estrogen in Males

Estrogen is sexually dimorphic; it is not restricted to the female as the male sex hormone testosterone (as well as other steroids that contain 19-carbon atom structure) can be converted locally to estrogen in various tissues, including the brain, by an aromatase cytochrome P450 enzyme (Behl & Holsboer, 1999). The possible neuroprotective effect of estrogen has been tested in male rats as well. Estradiol treatment results in an increase in the rate of regeneration of axotomized facial motoneurons of male hamsters, an effect also induced by testosterone and by the non aromatizable androgen dihydrotestosterone (Tanzer & Jones, 1997). In addition it has been shown that chronic administration of 17β-estradiol protects the male rat brain after experimental stroke using 2 hours of reversible middle cerebral artery occlusion (Toung, *et al.*, 1998). Although testosterone is converted to estrogen by an aromatase enzyme, Garcia-Segura, *et al.*, 2001, reported that the circulating levels of testosterone are not sufficient to supply prohormone for the neuroprotective levels of estrogen *in vivo*. In support of this, clinical data has revealed that women recover better than men after traumatic brain injury and in several experimental neural lesion models, male animals are more vulnerable than female animals.

1.6. ESTROGEN PROTECTION AND NEURODEGENERATIVE DISORDERS

1.6.1. Alzheimer's Disease

Alzheimer's disease (AD) is one of the most common and severe progressive neurodegenerative diseases of aging, accounting for a significant number of cases of senile dementia. It is characterized clinically by progressive loss of memory and other cognitive functions, resulting in severe dementia (Heston & White, 1991). The diagnosis of AD is one of exclusion as there is no non-invasive diagnostic test. Hence, diagnosis of AD during life is based on a careful neurological examination, neuropsychological testing, blood studies, and radiological evaluation to exclude chronic infection, tumor, vascular disease, vitamin or other nutritional deficiencies, or alterations in intracranial pressure such as hydrocephalus (Heston & White, 1991).

The etiology of AD is poorly understood but it is likely that both genetic and environmental factors are at the root of the disorder. AD has been reported to be associated with age, gender, family history, low education and socioeconomic status, and head injury.

1.6.1.1. The Pathophysiology of AD

The pathological changes are distinctive and correlate well with the clinical symptoms. On gross examination, there is atrophy of the brain, most marked in hippocampus and in association areas of cerebral cortex. The hallmarks of the disease, however, are observed on microscopic examination. These include loss of neurons and the presence of neurofibrillary tangles (NFT) and neuritic plaques (Siegel, *et al*., 1989). NFT are abnormal neuronal soma in which the cytoplasm is filled with unique submicroscopic filamentous structures. These structures are formed from the microtubule-associated protein tau in a highly phosphorylated state. Accumulation of tau proteins is associated with disturbances of intracellular transport of molecules and organelles in affected neurons, leading to cell dysfunction and death (Siegel, et *al*., 1989).

Neuritic plaques (also referred to as senile plaques) consist of clusters of degenerating nerve endings, both axonal and dendritic, with a central core that usually contains extracellular linear filaments that have optical and ultrastructural characteristics of an amyloid protein. The major component of senile plaques is the A4/beta-amyloid peptide, generated by proteolysis of the amyloid peptide precursor (APP), a transmembrane protein. An abnormal metabolism of the APP is often considered as a central physiopathological mechanism of the disease (Brion, 1996). Other pathological changes often found in the Alzheimer brain include amyloid in arterioles in meninges and cerebral cortex and inclusions, termed granulovacuolar bodies, in hippocampus (Siegel, *et al*., 1989).

The neural systems affected in AD include the cholinergic, noradrenergic and serotonergic projection systems. However, the cholinergic neurotransmitter system is most affected in AD. In AD the levels of choline acetyltransferase are markedly reduced in the cerebral cortex and hippocampus (Davies & Verth, 1976). This reduction is significant since acetylcholine is a neurotransmitter involved in memory. Genetic mutations have also been identified in the pathogenesis of AD. Apolipoprotein E (apo E) genotype has been found to affect the risk of developing the disease, i.e. those homozygous for the apo E4 allele are more prone to develop AD. Apo E accelerates precipitation of beta-amyloid (Weisgraber & Mahley, 1996).

1.6.1.2. Estrogen Protection

The aged-matched prevalence of female AD is twice that of male AD in the age range 55-85 years. This increase prevalence in elderly women suggests that estrogen deficiency may play a role in the development of the disease. ERT has been shown to have favourable effects on cognition and delays the onset of AD. The mechanism of protection on AD remains to be clarified but it may act via several mechanisms. These may include an antidepressant effect, an improvement in cerebral blood flow, direct stimulation of neurons and suppression of apo E (Honjo, *et al*., 1995). Present evidence confirms that estrogen helps maintain cholinergic function, promotes the breakdown of the APP to fragments less likely to aggregate as beta-amyloid, and exhibits antioxidant activity that diminishes the cytotoxic effects of beta-amyloid (Henderson, 1997).

1.6.2. Stroke

A cessation of oxygen supply for 4-5 min is sufficient to do irreparable damage to the human brain. This is related to the specific constellation of ion channels that are found on neurons. A decrease of oxygen supply is generally linked to a decrease in blood flow due either to blockage of an artery or to coronary arrest; the former is referred to as a stroke (Juurlink & Sweeney, 1997). Stroke is a leading cause of mortality and morbidity in the population over middle age. The brain is absolutely dependent on oxidative metabolism for cell survival however, neurons in culture have been shown to survive many hours in the absence of oxygen. The reason for this is that neurons can survive long periods of hypoxia as long as glutamate does not activate glutamate receptors. If glutamate activates its receptor, the neurons die (Rosenberg & Aizenman, 1989). Thus, glutamate release has been shown to play a pivotal role in the development of damage due to ischemia.

Estrogen is thought to protect against stroke in humans, however the precise mechanisms are unclear. Men and women do not differ with respect to the primary risk factors for stroke, which are age and high blood pressure, however, they do differ with respect to the incidence of stroke (Garcia-Segura, *et al*., 2001). Premenopausal women have fewer strokes than men of the same age, while, after menopause the incidence of stroke increases in women compared to aged matched premenopausal women (Paganini-Hill, 1995). The difference observed may be due to endogenous estrogens on lowering of cholesterol levels and on vascular endothelium, protective effects that are lost in postmenopausal women. In addition, estrogen has been shown to protect against glutamate-induced toxicity. Earlier studies have reported that the retention of verbal abilities is better in female stroke victims than in male stroke victims (Kertesz & Benke, 1989), and that women experience a better recovery from aphasia than do men (Pizzamiglio, *et al*., 1985). Accumulative basic science studies have supported a role for estrogen in providing protection against stroke and also improving the recovery following ischemic episodes.

1.6.3.   Schizophrenia

Schizophrenia is characterized by a loss of contact with reality and a disruption of thought, perception, mood and movement. The disorder typically becomes apparent during adolescence or early adulthood and usually persists throughout life (Bear, *et al*., 2001). Symptoms of schizophrenia include, delusions, hallucinations, disordered speech, catatonic behavior, reduced expression of emotion, and poverty of speech. Several factors play a role in the development of the disorder. These include genes and the environment, the dopaminergic system and glutamate. It has been shown that an overdose of amphetamines (which causes dopamine release) can result in a psychosis indistinguishable from that of schizophrenia, and neuroleptic drugs that block dopamine D_2 receptors are effective in the treatment of the disorder. This evidence points to the role for dopamine in the progression of the disorder. However, an indication that there is more to schizophrenia that dopamine came from an observation that PCP, an NMDA antagonist, intoxication is accompanied by many symptoms of schizophrenia (Bear, *et al*., 2001).

The overall lifetime risk for schizophrenia is not different between men and women, however, the age of onset, the severity of the symptoms, and the type of symptoms are different in men and women and point to a protective role for estrogen in schizophrenia (Garcia-Segura, *et al*., 2001). Schizophrenia generally occurs slightly later in women and, until menopause, has a less severe course in women than in men. Moreover, although the peak age of onset for schizophrenia is late adolescence, a second cohort of women develops the disorder at the onset of menopause, as estrogen levels begin to fluctuate and decline (Lindamer, *et al*., 1999). In addition, numerous studies have shown that the severity of the psychiatric disturbance decreases with increasing estradiol levels during the menstrual cycle and during pregnancy. Results from clinical trials with ERT provides more evidence that estrogen is protective, and decreases a specific subset of schizoid symptoms (Garcia-Segura, *et al*., 2001).

1.7.　　　OBJECTIVES

The study was undertaken to observe the neuroprotective properties of estrogen against damage induced by quinolinic acid in the hippocampus. This aim was of interest since QUIN is known to be neurotoxic and the concentration of QUIN in the brain increases with age, and is found in high concentrations in AIDS patients, possibly contributing to AIDS-related dementia, and is involved in the pathogenesis of Huntington's disease. Furthermore, QUIN is known to be an endogenous agonist at the NMDA receptor, causing neuronal damage due to massive calcium influx into the cell. Accumulating evidence has suggested an action of estrogen on excitatory amino acid receptors. In addition, the hippocampus was chosen as the area of the brain to study, since this is the primary region involved in memory formation, and estrogen has been reported to improve cognition and memory.

The objectives of the study was to investigate the protective effects of estrogen against QUIN-induced oxidative stress and resultant neuronal damage and also to elucidate the cellular mechanisms whereby estrogen may exert any protective effects in hippocampal neurons.

CHAPTER 2

LIPID PEROXIDATION STUDIES

2.1.   INTRODUCTION

The membranes of living cells are remarkable in molecular architecture, displaying a variety of different functions. The plasma membrane surrounds all animal cells, including neurons, and separates the cell interior from the extracellular environment, and also compartmentalizes the internal structures of the cell. Membranes are composed of proteins, carbohydrates and lipids, of which the most abundant is amphipathic phospholipids (Campbell, 1996). Membranes not only function as permaeable barriers for the selective transport of molecules into and out of the cell, but are also responsible for the production of ATP and the binding of regulatory molecules such as hormones and neurotransmitters that mediate neurotransmission (Bohinski, 1987).

Lipid peroxidation, via the action of radical species, results in the attack of membrane lipids resulting in the destruction of the cell membrane and ultimately destroying the integrity of the cell. If this occurs in neuronal membranes, neuronal characteristics are altered as well as the activities of transport proteins. Neuronal membranes are particularly vulnerable to oxidative attack by free radicals since the brain consumes a large portion of the total body oxygen, is deficient in protective mechanisms such as glutathione, contains large amounts of polyunsaturated lipids, and some areas of the brain are rich in iron (Halliwell & Gutteridge, 1990).

Any event that leads to tissue injury will result in the formation of highly reactive radical species such as the hydroxyl radical, and the release of transition metal ions such as iron. These two events can independently lead to the initiation and propagation of lipid peroxidation.

2.2 THE COMPARATIVE EFFECTS OF 17β-ESTRADIOL AND CONJUGATED ESTROGENS ON QUIN-INDUCED LIPID PEROXIDATION *IN VITRO*

2.2.1 INTRODUCTION

Since the brain is particularly susceptible to oxidative attack by free radicals, lipid peroxidation is able to cause extensive damage and is known to play a major role in the deterioration of the brain and spinal cord that occurs after traumatic or ischemic injury. Such injuries to the brain result in more extensive tissue damage than do equivalent insults in other tissues. Free radical reactions have been implicated in such damage (Halliwell & Gutteridge, 1990).

The aim of these studies was to determine whether 17β-estradiol is able to protect against lipid peroxidation induced by the neurotoxin, quinolinic acid (QUIN). This neurotoxin is known to increase lipid peroxidation levels in brain homogenate (Southgate, 1999 ; Stipek, 1997). In addition, a comparative study was performed to test whether estrogen in the conjugated form offers protection against QUIN-induced lipid peroxidation. Conjugated estrogens in the form of the drug Premarin®, is the most widely prescribed form of estrgen replacement given to post-menopausal or surgically menopausal women.

All animals used for these studies were adult females and each animal was ovariectomised before conducting experiments to ensure that estrus cyclce did not lead to fluctuations in hormone levels and to reduce endogenous estrogen levels. The assay used to measure lipid peroxidation was the Thiobarbituric acid (TBA) test. This test is the most common technique used to measure lipid peroxidation, and involves the reaction of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) with TBA to yield a coloured complex that can be measured spectrophotometrically. MDA and 4-HDA are degraded lipid products from cell membranes, and are taken as reliable indicators of oxidative stress (Halliwell & Gutteridge, 1990 ; Reiter, 1995).

2.2.2   MATERIALS AND METHODS

2.2.2.1   Chemical Reagents

Quinolinic acid (2,3-pyridinedicarboxylic acid), 17β-estradiol, 2-thiobarbituric acid (98%) (TBA), 1, 1,13,3-tetramethoxypropane (98%) and butylated hydroxytoluene (BHT) were purchased from Sigma St. Louis, MO, U.S.A. Premarin® (conjugated estrogens) was a kind gift from Wyeth-Ayerst, South Africa. Trichloroacetic acid (TCA) and butanol were obtained from Saarchem, Johannesburg, South Africa. All reagents were of the highest quality available.

2.2.2.2   Animals

Adult female rats of the Wistar strain, weighing between 200-250g were used. The rats were housed in separate cages, following ovariectomy, in a controlled environment with a 12h light :dark cycle (lights on at 6am). The animals were given standard laboratory chow and tap water *ad libitum*. Protocols for the experiments were approved by the Rhodes University Animal Ethics Committee.

2.2.2.3   Surgical Procedures

2.2.2.3.1   Anaesthesia

Ether anaesthesia was employed for all surgical procedures carried out. Animals were placed, one at a time, in a dessicator containing cotton wool soaked in ether. Once the animals were sedated, they were removed and placed on the operating surface. A small conical flask containing cotton wool soaked in ether was placed approximately 3cm from the rats nose. This flask remained in this position throughout surgery, except in cases where the respiration became too weak. A good indication of the depth of anaesthesia was monitored by the colour of the limbs and tail, which displayed a faint, almost pale pinkness. This was indicative to the optimum level of anaesthesia, meaning a satisfactory rate and depth of respiration with good narcosis. A purple colour of the limbs was an indication of cyanosis. In instances where animal's

breathing ceased, they were held and gently squeezed in the area of the lungs. This method usually succeeded in reviving the animal. Following surgery, the animal recovered from the anaesthetic in approximately 10-15 min.

Diethylether is a desirable anaesthetic to use because the mortality rate of the animals is lower than if halothane or phenobarbitone was used. Ether is also easy to administer and it is easy to monitor the depth of anaesthesia.

2.2.2.3.2 Bilateral Ovariectomy

Once the animals were anaethetised they were placed on their backs on the operating suface. The lower middle area of the abdomen was moistened with iodine solution with cotton, and the hair was cut to expose the skin. A 2cm vertical incision was made with a scalpel through the skin to expose the abdominal muscles. A further incision was made through the muscle and abdominal wall. The fat surrounding the ovary was just beneath the incision and therefore easily visible. With the aid of forceps, the fat with the ovary and part of the uterus for both the left and right sides, were pulled carefully through the incision. The ovarian artery, vein and part of the uterus just below the fallopian tube were ligatured and the ovary was excised. The incisions were stitched with iodine soaked cotton. All ovariectomies were performed at least 2 weeks prior to the experiments to allow the animals to recover.

2.2.2.4. Brain Removal

Rats were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal suture, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain which was easily removed for use in experiments.

2.2.2.5. Homogenate Preparation

Rats were sacrificed by cervical dislocation and then decapitated. The brains were rapidly removed and placed on ice. Each brain was homogenised in a glass teflon

homogeniser with 50mM Tris-HCl buffer (pH 7.4) so as to give a final concentration of 10% w/v. The homogenate was frozen in liquid nitrogen and stored at -70°C until use. All samples were used within 7 days of homogenate preparation.

2.2.2.6.   Lipid Peroxidation Assay

Lipid peroxidation was determined using a modified method of Ottino & Duncan, 1997. 17β-estradiol was dissolved in absolute ethanol, while conjugated estrogens and QUIN were dissolved in Milli-Q. Homogenate (1ml) containing varying concentrations of QUIN (0, 0.25, 0.5, 0.75, or 1mM) alone or in combination with either 17β-estradiol or conjugated estrogens (0, 0.25, 0.5, 0.75 or 1mM). This was incubated in a shaking water bath for 1 hour at 37°C. At the end of incubation, 0.5ml BHT (0.5g/l in absolute ethanol) and 1ml 25% TCA were added to the mixture. The samples were centrifuged at 2000 g for 20min at 4°C to remove insoluble proteins.

Following centrifugation, 2ml pf protein free supernatant was removed from each tube and a 0.5ml aliquot of 0.33% TBA was added to this fraction. All tubes were heated for 1 hour at 95°C in a water bath. After rapidly cooling the tubes on ice 2ml of butanol was added and the tubes vortexed and centrifuged at 2000 g for 10 min. 2ml of the top layer of the mixture was carefully removed with an autopipette and placed into separate tubes. This fraction contained the extracted TBA-MDA complexes, which were then read at 532nm using a GBC Uv/Vis 916 spectrophotometer. MDA levels were determined from a standard curve generated from 1, 1, 13, 3-tetramethoxypropane. Final results were expressed as nmoles MDA/mg protein.

2.2.2.7.   Preparation of the Standard Curve

1, 1, 13, 3-tetramethoxypropane was used as a standard. A series of reaction tubes each containing appropriate aliquots of water and standard solution were prepared with Milli-Q water to a final volume of 1ml. A calibration curve was generated by measuring the absorbance at 5nmoles/ml intervals. No incubation at 37°C followed and the rest of the reagents and procedures were performed as described in 2.2.5. The

absorbance was read at 532nm and plotted against the molar equivalent weight of MDA in the complex assayed. (Appendix 1).

2.2.2.8.   Protein Determination

All protein determinations were performed using the method described by Lowry *et al*, 1952. A standard curve was generated using BSA as a standard at concentration intervals of 60μg/ml (Appendix 2).

2.2.2.9.   Statistical Analysis

The results were analysed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student Newman-Keuls test was used to compare the treated and control groups. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

2.2.3.   RESULTS

The *in vitro* exposure of brain homogenate to increasing concentrations of QUIN caused a significant increase in lipid peroxidation in a concentration-dependant manner. Beyond concentrations of QUIN higher than 0.75mM, no further increase in MDA formation was evident. (Figure 2.1)

Co-treatment of this homogenate with Quin and increasing concentrations of 17β-estradiol (Figure 2.2), as well as with conjugated estrogens (Figure 2.3), resulted in a significant reduction in lipid peroxidation. Furthermore, 17β-estradiol, at all concentrations tested reduced the amount of MDA formed to a lower level as the concentration of QUIN increased. This pattern was also observed for the form of conjugated estrogens used.

2.2.4.   DISCUSSION

The results demonstrate that both forms of estrogen tested, 17β-estradiol and conjugated estrogens, reduced QUIN-induced lipid peroxidation *in vitro*. Furthermore, both types of estrogen were able to decrease the levels of MDA produced to levels lower than in the absence of QUIN, demonstrating complete protection against this neurotoxin with regard to lipid peroxidation.

Tissue disruption by homogenisation tends to undergo lipid peroxidation more easily than healthy, intact tissue (Halliwell & Gutteridge, 1989). This explains the formation of MDA obtained in the absence of QUIN. Reasons for this increased peroxidizability of damaged tissue include inactivation or dilution of some antioxidants as well as the release of metal ions (especially iron) from intracellular storage sites (Halliwell & Gutteridge, 1989). QUIN has been reported to increase lipid peroxidation in brain homogentae and that this is dependent on the presence of iron and in particular, Fe(II) (Stipek, et al., 1997). These authors showed that QUIN complexes with Fe(II) but not Fe(III).

Once a QUIN-Fe(II) complex forms, this reacts with preformed lipid peroxides, generated by tissue disruption, to produce peroxyl radicals (Halliwell & Gutteridge, 1989). These radicals can attack other molecules within the homogenate and so the chain of lipid peroxidation can continue. One way in which the estrogens studied in this experiment, could protect from QUIN-induced lipid peroxidation *in vitro* is by possible binding the free Fe(II) thereby preventing the complex formation between QUIN and Fe(II) to occur.
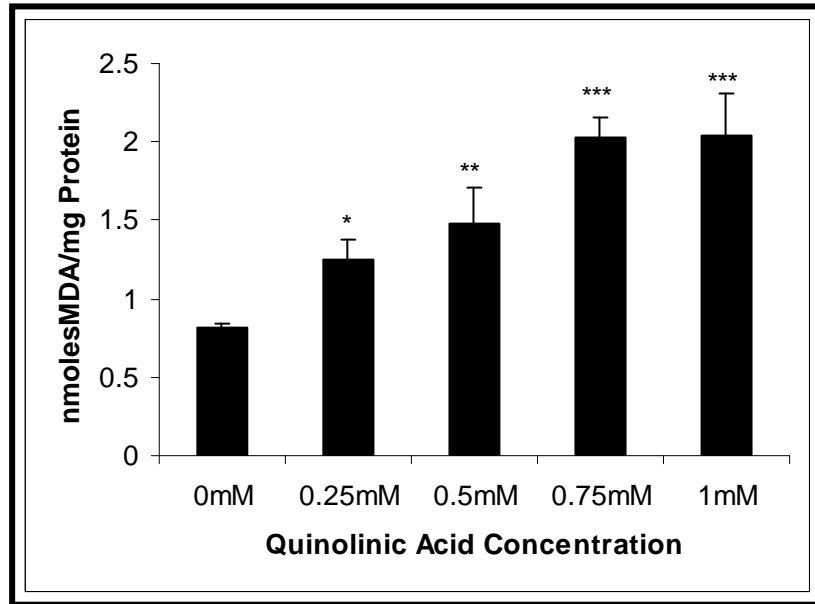
Figure 2.1   Concentration-dependent effect of QUIN on lipid peroxidation in homogenates from whole brain of ovariectomized female rats. Values represent the mean $\pm$ SEM (n=5), (*p<0.05 ; **p<0.01 ; ***p<0.001 in comparison to control)
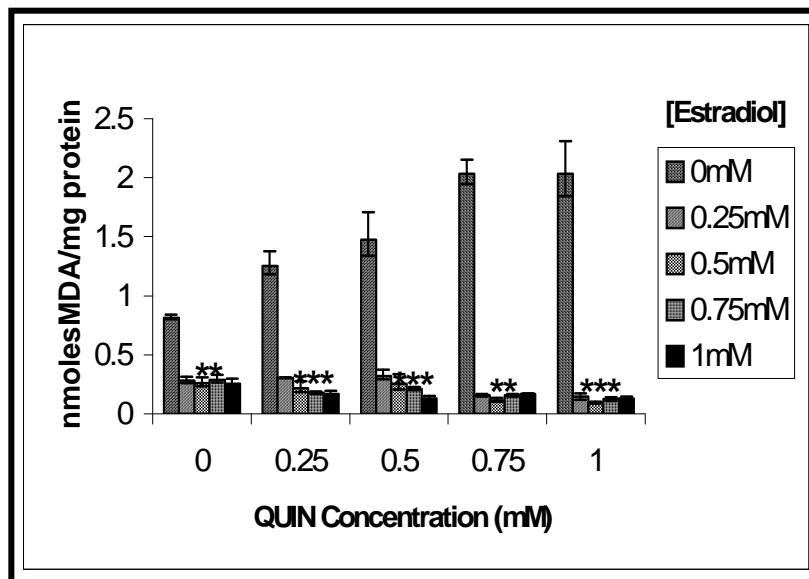
Figure 2.2   The effect of 17β-estradiol on QUIN (0-1mM)-induced increase in MDA production in rat brain homogenate. Values represent the mean $\pm$ SEM (n=5), (*p<0.05 ; **p<0.01 ; ***p<0.001 in comparison to control)
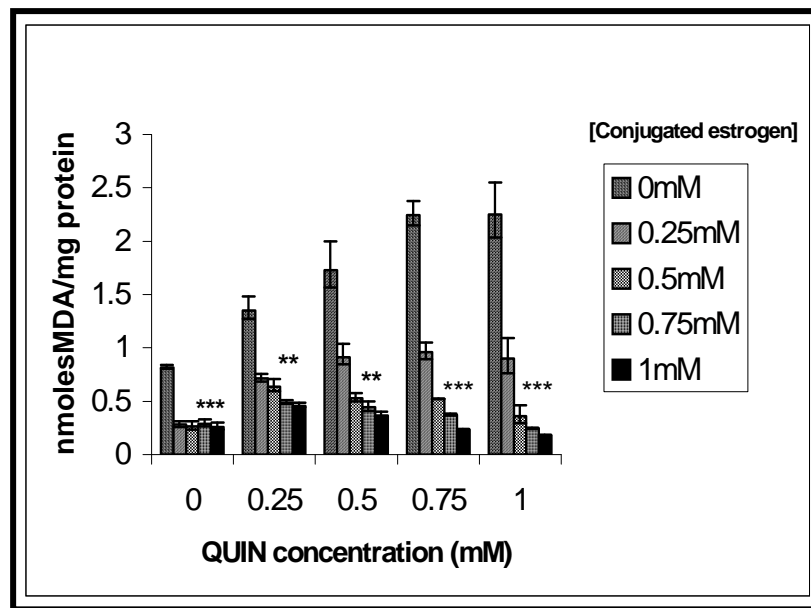
Figure 2.3   The effect of conjugated estrogens on QUIN (0-1mM)-induced increase in MDA production in rat brain homogenate. Values represent the mean $\pm$ SEM (n=5), (*p<0.05 ; **p<0.01 ; ***p<0.001 in comparison to control).

2.3. COMPARISON OF THE EFFECTS OF 17β-ESTRADIOL AND CONJUGATED ESTROGENS ON QUIN-INDUCED LIPID PEROXIDATION *IN VIVO*

2.3.1. INTRODUCTION

The results of the previous experiment demonstrated that estrogen protects against lipid peroxidation in rat brain homogenate following exposure to QUIN. Since QUIN is known to be present in the brain at concentrations in the nM range and that this concentration increases with age (Moroni, et al., 1984a), it was decided to investigate whether estrogen could offer the same protection against lipid peroxidation following the intrahippocampal injection of QUIN *in vivo*.

In addition, the brain is known to be rich in iron, which plays a crucial role in initiating and propagating lipid peroxidation (Halliwell & Gutteridge, 1989) and the concentration of circulating estrogen in post-menopausal women decreases dramatically. However, many women worldwide have been placed on estrogen replacement therapy, which could help protect the brains of these women against oxidative stress implicated in dementia.

2.3.2. MATERIALS AND METHODS

2.3.2.1. Chemical Reagents

As in section 2.2.2.1.

2.3.2.2. Animals

As described in section 2.2.2.2.

2.3.2.3. Ovariectomy

Prior to dosing, the animals were anaethetized with diethyl ether and ovariectomised as decribed in section 2.2.2.3.1. and 2.2.3.2. respectively.

2.3.2.4.   Dosing of the Animals

Following ovariectomy, the animals were given three weeks to recover before dosing commenced. The animals were separated into four groups of five animals each (Table 2.1). The animals in group 3 received single daily doses of 100µg 17β-estradiol in 100µl olive oil, injected subcutaneously, for seven days prior to intrahippocampal QUIN injection. Similarly the animals in group 4 received single daily doses of 100µg Premarin® in Phosphate-buffered saline (PBS), administered in the same way. The animals in groups 1 and 2 received vehicle for 17β-estradiol, viz. olive oil. On the eighth day, the animals were injected with QUIN directly into the hippocampal region. QUIN was dissolved in PBS made up to pH 7.4

Following the intrahippocampal injections of QUIN, the animals in groups 3 and 4 received subsequent daily doses of estradiol and Premarin® respectively, each day for seven days, while as before the animals in groups 1 and 2 received daily doses of olive oil for seven days.

Table 2.1   Treatment regime for each group of animals

Treatment Group	Daily treatment for 7 days prior to stereotaxic surgery (s.c.)	Intrahippocampal injection	Daily treatment for 7 days after stereotaxic surgery (s.c.)
1 (Control)	100µl Olive oil	PBS	100µl Olive oil
2 (EST/ PREM (+))	100µl Olive oil	1 µmol QUIN in PBS	100µl Olive oil
3 (EST (-))	100µl 17β-estradiol in olive oil (100µg)	1 µmol QUIN in PBS	100µl 17β-estradiol in olive oil (100µg)
4 (PREM (-))	100µg Premarin® in PBS (100µl)	1 µmol QUIN in PBS	100µg Premarin® in PBS (100µl)

2.3.2.5.   Bilateral Intrahippocampal QUIN Injection

QUIN was injected intrahippocampally using stereotaxic surgery techniques. Female rats were anaethetised with diethyl ether and placed into a stereotaxic apparatus (Figure 2.4). The skull was orientated according to the König and Klippel stereotaxic atlas (14). After a saggital cut in the skin of the skull, the bregma and lambda suture were located (Figure 2.5) and holes were drilled with a Bosch electrical drill fitted with a drill bit of 0.5mm in diameter at the following coordinates ; 2.58mm anterior, 3.5mm lateral of the sagittal suture. Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3mm, was used to inject 1 µmol of quinolinic acid in 2 µl of PBS, 4mm ventral of the dura.

 The injection was administered at a rate of 1 µl per minute and the cannula was left *in situ* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the canulla tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals recovered from the anaesthesia after approximately 15 min. The rats used as controls were subjected to the same surgical procedures. However, stereotaxic injections into the hippocampus were free of QUIN and comprised solely of PBS.

 Since, QUIN is known to stimulate seizure activity, the animals injected with QUIN displayed several behavioural changes. In particular, seizure episodes were often associated with a frozen appearance of the animal and intermittent "wet dog shakes". Ataxia was also apparent in these animals.

Figure 2.4   A view of the stereotaxic apparatus used for the bilateral intrahippocampal injection of QUIN
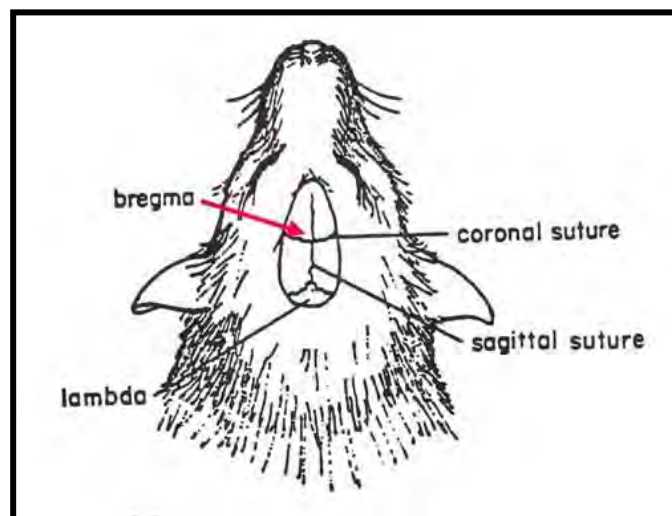
Figure 2.5   A view of the rat skull after the skin has been cut. The sutures shown are used as a reference point for the measurement of the coordinates for the intrahippocampal injection.

2.3.2.6.   Homogenate Preparation

As described in section 2.2.2.4.

2.3.2.7. Lipid Peroxidation Assay

An assay of lipid peroxidation was performed on each treated brain. Since no drugs were added to the homogenate in this assay, no 1 hour incubation step was carried out but instead, homogenate (1ml) containing 0.5ml BHT and 1ml TCA was boiled for 30 min to release protein bound MDA. The rest of the assay was followed according to the protocol outlined in section 2.2.2.5.

MDA, a product of lipid peroxidation, is known to form adducts with protein, nucleic acids and other substances *in vivo*. Since each brain was assayed for lipid peroxidation seven days following the intrahippocampal injection of QUIN, the MDA formed as a result of QUIN-induced lipid peroxidation would have been bound to such molecules (Halliwell & Gutteridge, 1990). As a result, the MDA had to be released from its bound forms. This can be done by hot acid or alkali digestion or hydrolyisis by oxidation of polyunsaturated fatty acids. However, pigments generated during hydrolysis interfere in the colorimetric asessment of MDA. Thus, an alternative method is to boil the homogenate in the presence of BHT and TCA. Thereafter, as before, to avoid adsorption of the TBA-MDA complexes onto insoluble protein, any solid particulate material observed after cooling to room temperature is removed by centrifugation at 2000 g for 10min (Halliwell & Gutteridge, 1989).

2.3.2.8. Protein Determination

Protein was performed as in 2.2.2.7.

2.3.2.9. Statistical Analysis

As described in section 2.2.2.8.

2.3.3. RESULTS

The intrahippocampal injection of QUIN *in vivo* caused a significant increase in lipid peroxidation (Figure 2.6) assessed by the formation of TBA-RS (TBA reacting

substances) during the assay. Administration of either Premarin or 17β-estradiol, by subcutaneous daily injection for seven days prior and seven days following surgery, caused a significant reduction in QUIN-induced TBA-RS formation with a p value less than 0.05. In addition, both forms of estrogen lowered the level of TBA-RS formed to below that of the control.
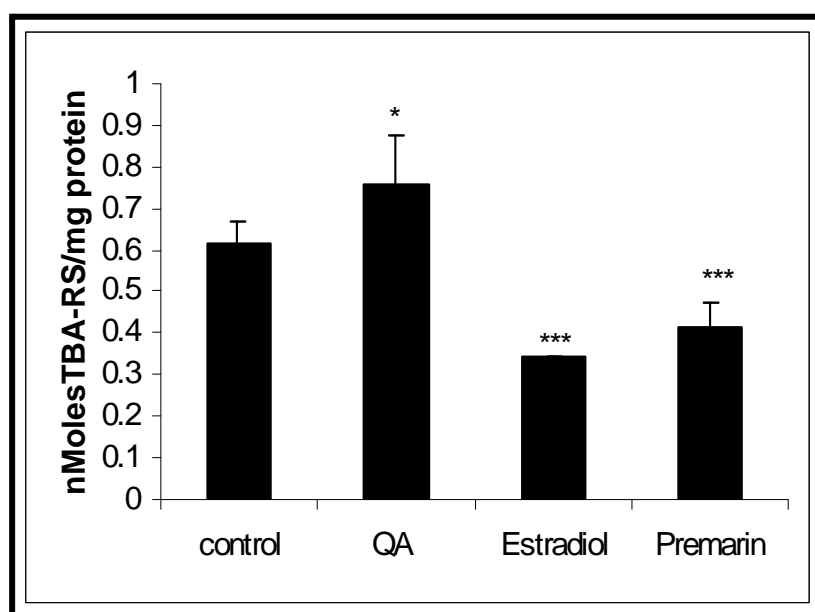
Figure 2.6. The effect of 17β-estradiol and Premarin on QUIN-induced lipid peroxidation *in vivo*. Values represent the mean ±SEM (n=5), (*$p<0.05$ for QUIN in comparison to the control ; ***$p<0.001$ for estradiol / Premarin in comparison to QA)

2.3.4. DISCUSSION

The results obtained show that estrogen in the form of 17β-estradiol as well as conjugated estrogens are able to protect against QUIN-induced lipid peroxidation *in vivo*. Since QUIN is known to be an endogenous agonist of the NMDA receptor that causes neuronal damage due to an influx of calcium into the cell, it is possible that estrogen, in addition to its antioxidant ability, also acts on the NMDA receptor. This assumption is based on the the results of the present study, which shows that both forms of estrogen decrease the level of lipid peroxidation to below that of the control, suggesting total protection against QUIN. Furthermore, if estrogen were to only act as an antioxidant, then cell damage due to QUIN would still occur and estroegn would then only be preventing further damage. However, if estrogen were to act on the

NMDA receptor thereby competing with QUIN to bind, then greater protection would be displayed by estrogen, which is shown in these results.

2.4. CONCLUSION

The results of the studies presented in this chapter demonstrate that QUIN-induced lipid peroxidation is markedly inhibited by 17β-estradiol and Premarin *in vitro* and *in vivo*. QUIN is a potent excitotoxin and it has been shown to induce lipid peroxidation at concentrations as low as 80μM (Rios & Santamaria, 1991). Other workers found that concentrations of QUIN in excess of 3mM inhibited lipid peroxidation (Stipek, 1997). These authors further reported that QUIN-induced lipid peroxidation is dependent on iron and that QUIN forms a complex with Fe^{2+} but not with Fe^{3+}. Iron plays a crucial role in the initiation and propagation of lipid peroxidation. Zaleska *et al*., (1995) found a linear relationship between the susceptibility of various rat brain regions to undergo lipid peroxidation *in vitro* and the content of endogenous iron in these brain regions.

Recently, the possible mechanisms involved in the protective effect of 17β-estradiol on lipid peroxidation was reported by Ayres *et al*., (1998). These authors maintain that 17β-estradiol is able to inhibit the generation of superoxide radicals and thus prevent further chain propagation and that the antioxidant property of estrogens is due to the chemical structure which allows for donation of a proton to a peroxyl radical. This allows estrogens to scavenge free radicals and exert its effect by interfering early or during the propagation phase of lipid peroxidation. This is supported by Moosman and Behl (1999) who reported that the antioxidant neuroprotective effects of estrogens are dependent not on their genomic properties as hormones but rather on their basic chemical properties as hydrophobic phenolic molecules.

In these studies it is evident that estrogen is able to completely protect against QUIN-induced neurotoxicity. Estrogen appears to offer protection against lipid peroxidation *in vitro* due to its antioxidant activity or possibly an interaction with iron. An interaction between estradiol or conjugated estrogens and Fe^{2+} would cause a decrease

in QUIN-induced lipid peroxidation since less Fe^{2+} would be available for QUIN to complex.

Foy *et al*., (1999) reported that 17β-estradiol was able to enhance NMDA receptor-mediated excitatory postsynaptic potentials. From this report estradiol appears to act on NMDA receptors but it is not yet known whether estrogen acts directly on the receptors or indirectly via second messenger processes that in turn influence NMDA receptor/channel processes. It is possible that the protective effect of 17β-estradiol on QUIN-induced lipid peroxidation *in vivo* (Figure 3) is due to the action of estradiol on the NMDA receptor, since Santamaria, *et al.,* 1993, showed that the effect of QUIN on lipid peroxidation is completely abolished by MK-801, a non-competitive antagonist of the NMDA-receptor. Thus estradiol could possibly be acting as an NMDA receptor antagonist *in vivo*.

In conclusion, the present study shows that 17β-estradiol and Premarin offer protection against QUIN-induced lipid peroxidation *in vitro* via a free radical scavenging mechanism. Estrogen has previously been shown to suppress lipid peroxidation induced by amyloid β-peptide and $FeSO_4$ (Goodman et al, 1996) due to its antioxidant properties.

However, there is some room for speculation as to whether estradiol in addition to scavenging free radicals, is also able to interact with iron. The protective nature of estradiol against lipid peroxidation induced by the intrahippocampal injection of QUIN could possibly be due to the action of estradiol on the NMDA receptor of which QUIN is known to be an agonist.

CHAPTER 3

IRON INTERACTION STUDIES

3.1.   INTRODUCTION

The ability of quinolinic acid to induce lipid peroxidation was shown in the previous chapter. It has been reported that this induction of lipid peroxidation in rat brain homogenate is dependent on the presence of iron, in particular, iron (II) (Stipek, *et al*., 1997). These authors state that at concentrations between 0.015 and 1.5 mM, QUIN does not significantly increase lipid peroxidation in rat brain homogenates. However, paradoxically at higher concentrations of 3mM -15 mM, QUIN inhibits lipid peroxidation. This behaviour is attributed to the finding that QUIN forms a complex with iron (II) and as a result chelates this iron, rendering it unavailable to play a role in lipid peroxidation (Stipek, *et al*., 1997). The complex between QUIN and iron (II) has been reported to have a metal:ligand ratio of 1:2 and the overall formation constant to be 10^7, thus indicating a weak complex. Stipek, *et al*., 1997, therefore suggest that the increase in lipid peroxidation observed in brain homogenate is due to the action of iron and not QUIN alone. However, other studies (Southgate & Daya, 1999; Rios & Santamaria, 1991) as well as those of the previous chapter have shown that QUIN at low concentrations does indeed cause a significant increase in lipid peroxidation.

Lipid peroxidation is known to be initiated by any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group of a polyunsaturated fatty acid in a membrane. Reactive species able to accomplish this include, the hydroxyl radical ($OH^.$), the protonated form of the superoxide radical ($HO_2^.$) and various iron-oxygen complexes (Halliwell & Gutteridge, 1989). Iron ions are themselves free radicals, which when added to a peroxide-free unsaturated lipid initiates peroxidation. However, it is extremely difficult to obtain a tissue fraction that is peroxide-free since lipid peroxides are formed enzymatically in tissues by cyclo-oxygenase and lipoxygenase enzymes. Thus, injured cells and membrane fractions isolated from

disrupted tissue contains lipid peroxides. Consequently, brain homogenate, for example, is known to undergo lipid peroxidation much more quickly than isolated intact brain (Halliwell & Gutteridge, 1989). Iron ions therefore are known to accelerate lipid peroxidation rather than initiate it. This occurs in the presence of certain iron complexes, which react with preformed lipid peroxides to produce alkoxyl and peroxyl radicals. These then initiate further damage in the propagation of lipid peroxidation.

An iron (II) complex reacts with lipid peroxide to generate iron (III) and an alkoxyl radical. Iron (II) and its complexes stimulate membrane peroxidation more than iron (III) (Halliwell & Gutteridge, 1989). It is for this reason that it was decided to investigate the possible interaction between estrogen and iron (II) as a mechanism for estrogen's protection against lipid peroxidation induced by QUIN, which is known to complex iron (II). The formation of the complex between QUIN and iron (II) may be the cause of QUIN's stimulatory action in increasing lipid peroxidation in rat brain homogenate. Three techniques were used to investigate the interaction between estrogen and iron (II), namely, UV/VIS spectrophotometry, electrochemistry and NMR. Finally, computational modelling was performed to illustrate the most likely site of interaction of iron (II) to estradiol.

Furthermore, due to the involvement of free radicals and free radical damage during aging and age related diseases, and the role played by iron ions in this damage, a possible interaction between iron (II) and estrogen as an additional protective mechanism of this hormone was investigated.

3.2. THE EFFECT OF CONJUGATED ESTROGEN ON QUIN-IRON (II) COMPLEX FORMATION

3.2.1. INTRODUCTION

Absorption spectra in the near ultra-violet and visible region were studied to observe interaction of QUIN with iron (II) as well as the interaction of estrogen, in the conjugated form, with the preformed QUIN-iron (II) complex. Ultraviolet spectroscopic studies were chosen as a starting point to observe any possible changes in the absorption spectra of the QUIN-iron (II) complex due to the addition of estrogen, since the UV spectra of the QUIN and Iron (II) complex has been reported (Stipek, *et al*, 1997) and also because of the relative simplicity of this method. The ultraviolet absorption spectra of molecules are as a result of the electronic transitions from the ground state, or from low energy levels to high energy levels.

In addition, the spectra of metal-ligand interactions, such as metalloproteins and metalloenzymes, may also exhibit bands arising from 1) the d-d transitions of the metal ion, 2) ligand-to-metal charge transfer transitions (LMCT) involving the metal centre and surrounding ligands or 3) metal-to-ligand charge transfer bands (MLCT) also involving the metal centre and the surrounding ligands (Kendrick, *et al*., 1992). However, transitions that are assigned as d-d transitions are usually weak and hence the band related to QUIN in the visible region border was monitored. In optimum cases this technique may be used to determine differences in the metal-ligand geometry as well as metal oxidation states (Cowan, 1997), although this may not be the case in this study the technique is indeed useful in studying metals in biological systems.

3.2.2.   MATERIALS AND METHODS

3.2.2.1.   Chemicals and Reagents

Ferrous Sulphate was purchased from Merck, Midrand, South Africa, and Premarin®
(conjugated estrogens) was a kind gift from Wyeth-Ayerst, South Africa. Quinolinic
acid (2,3-pyridinedicarboxylic acid) was purchased from Sigma, St. Louis, MO,
U.S.A.

3.2.2.2.   Ultraviolet Spectroscopic Studies

An absorbance spectra of QUIN mixed with iron (II) was prepared by mixing a 10mM
solution of QUIN with an equal volume of a 10mM solution of ferrous sulphate, to
produce an equimolar solution. Both solutions were prepared in Milli Q water just
prior to the experiment to prevent oxidation of iron (II) taking place. This mixture was
used as the control scan. Similarly, a 10mM solution of Premarin® in Milli Q water,
was mixed with an equimolar solution of QUIN and iron (II). Quartz cuvettes were
used and the absorbance spectra were analysed using a Varian Cary 500 Scan UV-VIS
NIR Spectrophotometer (EL 99053199). The wavelength range was set to scan each
sample from 800nm to 200nm.

3.2.3.   RESULTS

Figure 3.1 shows the absorbance spectrum of the QUIN-iron (II) complex alone (blue
spectrum) or in combination (red spectrum) with conjugated estrogen. It is apparent
from this plot that upon the addition if estrogen to the sample of complexed QUIN-
iron (II), there is a shift in the peak from 405nm to around 455nm with a decrease in
peak height being observed.
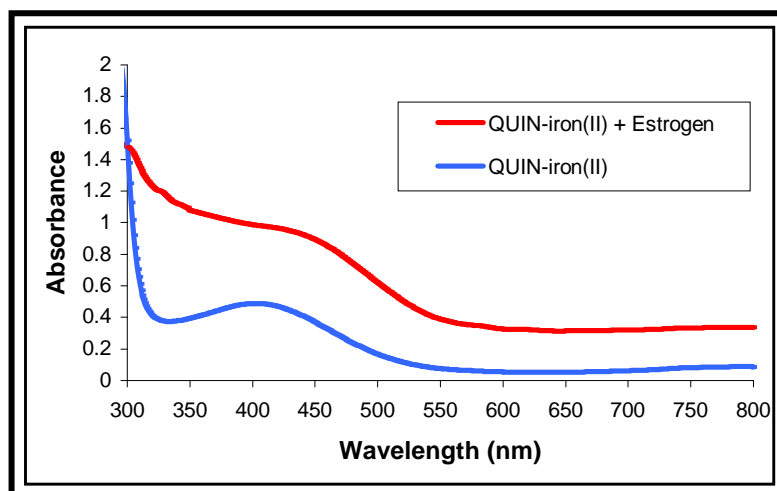
Figure 3.1. Absorbance spectra of QUIN mixed with ferrous sulphate, and QUIN-iron (II) mixed with conjugated estrogen

3.2.4. DISCUSSION

The change in the UV/VIS absorption spectrum would suggest that estrogen, in this form, is able to interfere with the formation of the QUIN-iron (II) complex; known to induce lipid peroxidation. Since the stimulation of lipid peroxidation by QUIN is thought to be dependent on the presence of iron (II), a disruption in the formation of this complex by conjugated estrogen, would protect against the increase in lipid peroxidation in rat brain homogenate. However, this study is not sufficient on its own to elucidate the exact mechanism of action by which estrogen might interfere with the ability of QUIN to induce lipid peroxidation as far as the involvement of iron (II) is concerned.

3.3. THE INTERACTION BETWEEN 17β-ESTRADIOL AND IRON (II): AN ELECTROCHEMICAL STUDY

3.3.1. INTRODUCTION

To further characterize the role played by estrogen in protecting against QUIN induced lipid peroxidation in rat brain homogenate, an electrochemical study was performed to test whether 17β-estradiol is able to interact with iron (II) ions not complexed to QUIN. Such an interaction would render the iron (II) ions unavailable to complex to QUIN and as a result lipid peroxidation would not be stimulated. The protection against QUIN-induced lipid peroxidation has been shown to occur by the addition of desferoxamine, an iron chelator, to rat brain homogenate (Stipek, *et al.*, 1997).

An electrochemical technique, adsorptive cathodic stripping voltammetry (AdCSV), was employed to show the capability of estradiol to bind iron (II) *in situ*. The first step in AdCSV is the formation of a metal-ligand complex, followed by controlled interfacial accumulation of the complex formed onto the electrode (in the presence of mercury) during the deposition step, and the reduction of the absorbed metal complex by application of a potential in the negative direction during the stripping step (equations 1 to 3).

$$M^{n+} + xLig_{aq} \rightarrow M(Lig)_{xaq} + xnH^+ \qquad (1)$$

$$M(Lig)_{xaq} \rightarrow M(Lig)_{xads} \qquad (2)$$

$$M(Lig)_{xads} + xnH^+ + ne^- \rightarrow M^{n+} + xLig_{aq} \qquad (3)$$

Where Lig = Ligand.

An efficient ligand must satisfy several requirements; it must freely bind the analyte in solution, adsorb onto the electrode, and freely release the analyte during the stripping step. The extent of the increase in current response of the metal on addition of the ligand is an indication of the affinity of the ligand for the metal. The extent of the shift in the reduction potential of the metal after the formation of the metal-ligand

complex is a measure of the stability of the complex (Wang, 1989; Limson, *et al*., 1998; Lack, *et al*., 1999 and Matlaba, *et al*., 2000).

In this study the ability of 17β-estradiol to bind iron (II) using AdCSV and the hanging mercury drop electrode (HMDE) was investigated. Benefits of the mercury drop electrodes include a renewable electrode surface and a high cathodic potential window, whilst the limited anodic range and toxicity are a potential disadvantage of the instrumentation (Wang, 1994).

3.3.2.   MATERIALS AND METHODS

3.3.2.1.   Chemicals and Reagents

17β-estradiol and TBABr (tetrabutylammonium bromide) were purchased from Sigma, St. Louis, U.S.A. Ferrous Sulphate was purchased from Merck, Midrand, South Africa.

3.3.2.2.   ACSV Technique

Fresh solutions of 1mM 17β-estradiol (dissolved in absolute ethanol) and 1g/L iron (II) (0.05M citric acid buffer, pH 4.5) were prepared daily. TBABr (1mM) was the chosen electrolyte since this has a wide potential window. Adsorptive cathodic stripping voltammograms were obtained utilizing the BioAnalytical Systems (BAS, Lafayette, Indiana, U.S.A.) CV-50W voltammetric analyzer. The working electrode was the model MF-9058 controlled growth mercury electrode (CGME). A silver/silver chloride (KCl = 3M) electrode and a platinum wire served as reference and auxiliary electrodes respectively.

Experiments were performed by placing a known concentration of the metal ions in the electrochemical cell, then adding a known amount of 17β-estradiol and diluting the solution in the cell to the required volume with electrolyte. A minimum purge time of 5 min, with nitrogen gas and simultaneous solution stirring was employed before running the electrochemical experiments. The reason for purging the solution is to

minimize any oxygen effects that may occur. All experiments were run under a blanket of nitrogen gas. The potentials quoted are cited with reference to the silver/ silver chloride reference electrode for all data accumulated.

The deposition potential for the iron (II)-estradiol complex was 200mV, whilst a deposition time of 60s was employed to effect the adsorption of the metal ligand complex onto the electrode. Voltammograms were recorded in a positive direction from the deposition potential to -1000mV, relative to the silver/silver chloride electrode at a scan rate of 100mV/s during the stripping step. The deposition potentials employed were obtained by optimization at fixed deposition and deaeration times. The deposition time was then optimized at the optimum deposition potential.

3.3.3. RESULTS

A peak for 17β-estradiol alone was observed at –415mV. The plot in Figure 3.2. illustrates the AdCSV of the iron (II) (1mM) reduction peak in the absence and presence of 17β-estradiol in increasing concentrations from 10μM to 30μM. The addition of 17β-estradiol resulted in the enhancement of the iron (II) stripping peak and a shift in the reduction peak position from –336mV (iron (II) alone) to –398mV; indicative of the *in situ* formation of a iron-estradiol complex. A negative shift of 62mV for the iron (II) reduction peak on the addition of estradiol, shows the resulting iron-estradiol complex is less easily reduced than the iron (II) alone. The shift of 0.062V is relatively small, indicating that the complex formed is not very stable.
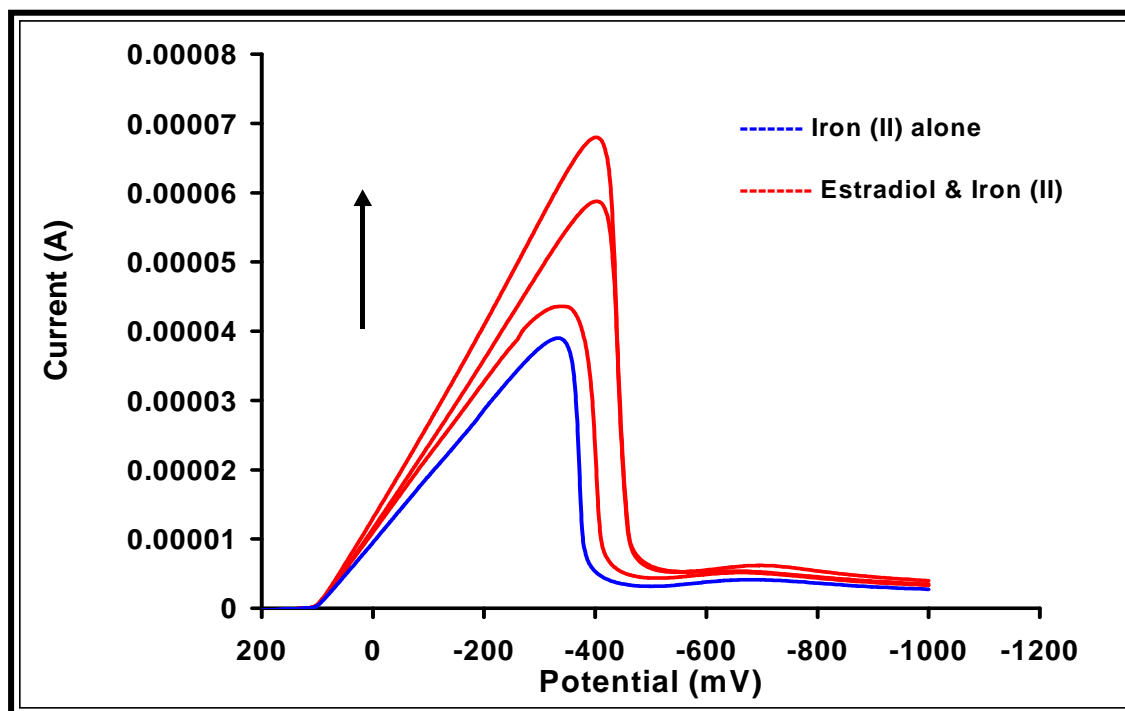
Figure 3.2. AdCSV obtained for Iron (II) alone and in the presence of increasing concentrations of 17β-estradiol

A linear variation of the AdCSV currents of the peak assigned to the iron (II)-estradiol complex with changes in the estradiol concentration was obtained under optimal experimental conditions as illustrated in Figure 3.3. The iron (II) concentration was kept constant at 1mM, while the range applied for the estradiol concentration variation was from 2μM to 34μM. The relative standard deviation of the mercury drop electrode, under optimal experimental conditions, for the detection of iron (II)-estradiol complex was less than 5%. The detection limit for the determination of the above mentioned complex was in the order of 2×10^{-6}M.
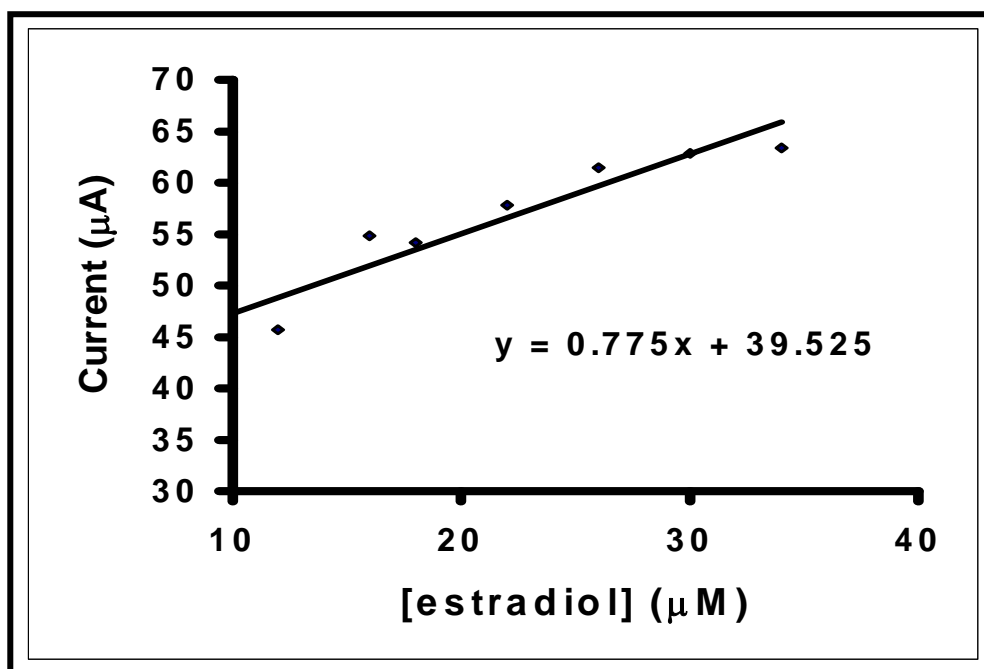
Figure 3.3.   The variation of the concentration of 17β-estradiol with the adsorptive cathodic stripping currents in the presence of 1mM iron (II). Scan rate equals 100mV/s. Deposition potential and time were 200mV and 60s respectively.

3.3.4.   DISCUSSION

The present study shows that 17β-estradiol interacts with iron (II) in a concentration dependent manner. The proposed *in situ* complex is not very stable as indicated by the relatively small shift in peak potential between the iron (II) reduction peak and that of the proposed complex. Nevertheless, although this complex is not stable, the interaction that takes place is significant in that the iron (II) would be unavailable for QUIN to complex, thus reducing lipid peroxidation.

3.4. A COMPARATIVE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY ANALYSIS OF THE INTERACTIONS BETWEEN 17β-ESTRADIOL AND IRON (II) AND IRON (III)

3.4.1. INTRODUCTION

Of all the physical techniques available, nuclear magnetic resonance spectroscopy (NMR) is generally the most useful. The important applications of NMR concerned with binding events include studying the ligand binding to a metal centre or proton uptake or release from an ionizable residue. NMR can be used to study these processes since the response (both chemical shift and the relaxation times T_1 or T_2) is different in the presence or absence of the bound species. Furthermore, certain paramagnetic compounds, such as chromophores, have been studied using NMR spectra. These compounds influence the spectra in two ways 1) by providing additional mechanism for extending the range of chemical shift and 2) by enhancing the nuclear relaxation, which is often accompanied by a significant increase in spectral line width (Cowan, 1997).

The basis of the NMR experiment is to subject the nuclei to radiation, which will result in a transition from the lower energy state to a higher level. The precise difference in energy levels between the two spin orientations is dependent on the particular location of the atom of the molecule, since each nucleus is subject to the differing effects of the magnetic fields of neighbouring nuclei. Only nuclei which are in exactly the same magnetic environment will have exactly the same energy difference between spin orientations, when placed in a magnetic field. In NMR spectroscopy these differences in energy are detected and provide information on the variety of locations of the nuclei in the molecule (Gordon, 1996).

By convention, frequency, and therefore magnetic field strength, increases from left to right in the NMR spectrum. Tracing the spectrum from left to right is referred to as moving upfield, whilst moving from right to left is a downfield shift. Upfield absorptions are said to be more shielded and the downfield absorptions are the result

of deshielding. The position of an absorption peak in the NMR spectrum may be represented either on a frequency scale (Hz) or on a scale of magnetic field (tesla), but by convention the frequency scale is used. However, in order to make rapid comparisons between spectra recorded on instruments operating at different frequencies, the positions of absorptions are normally quoted on the σ scale, which is independent of the instruments operating frequency. The σ value is obtained by dividing the position in Hz by the instrument frequency (in MHz) and is expressed in parts per million (ppm) (Vogel, 1994).

The one-dimensional (1D) experiment involves the excitation of a single type of nucleus, ^1H and ^{13}C being the most common. Two-dimensional (2D) experiments are more efficient for the simultaneous determination of a large number of correlations. In the present study 1D and 2D NMR experiments were used to substantiate and elucidate the proposed iron (II) –estradiol complex structure and compared with a possible iron(III)-estradiol interaction.

3.4.2.   MATERIALS AND METHODS

3.4.2.1.   Chemicals and Reagents

Deuterated methanol (methanol-D4) was purchased from Merck, Germany. Anhydrous ferric and ferrous chloride were purchased from Sigma, St. Louis, U.S.A, along with the 17β-estradiol compound.

3.4.2.2.   NMR Analysis

All NMR experiments were recorded on a 600MHz Bruker AMX pulse Fourier transformed spectrometer. Equimolar concentrations of iron (II) or iron (III) with 17β-estradiol were dissolved in deuterated methanol and the 1D ^1H and ^{13}C spectra obtained were compared to that of the 17β-estradiol alone. 5mm NMR tubes were used throughout, with a total volume not exceeding 1ml in each tube.

3.4.3.   RESULTS

The NMR data for the ^{1}H and ^{13}C spectra of 17β-estradiol is summarized in Table 3.1.; with numbering of the structure relative to Figure 3.4. The chemical shift values compared favourably with those reported by Breitmaier & Voelter, 1986 and Savignac, *et al*., 1986).
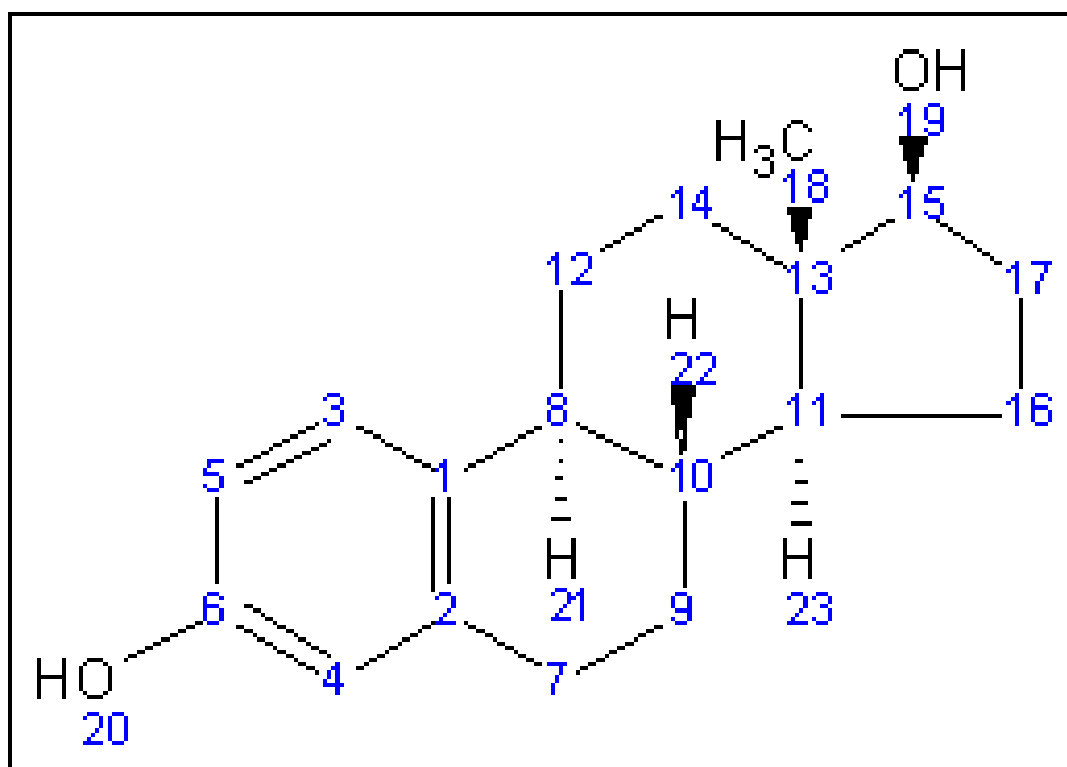
Figure 3.4.   Chemical structure of 17β-estradiol

Table 3.1. Summary of NMR data for 17β-estradiol

¹H NMR		¹³C NMR	
Atom No.	Chemical shift (ppm)	Atom No.	Chemical shift (ppm)
7-α (t)	2.63	1	132.73
7- β (t)	2.68	2	138.88
8 (q)	1.99	3	127.23
9-α (q)	1.08	4	116.12
9-β (q)	1.65	5	113.79
10 (sex.)	1.25	6	155.92
12-α (q)	2.10	7	30.74
12-β (q)	1.38	8	45.40
14-α (q)	1.02	9	28.57
14-β (q)	1.82	10	40.58
15-α (q)	1.32	11	51.39
15-β (q)	1.12	12	27.65
16 (q)	3.40	13	44.42
17-α (q)	1.88	14	38.09
17-β	1.40	15	82.58
19	0.65	16	24.08
21	0.81	17	30.79
		18	11.74

(t)   = triplet signal

(q)   = quartet

(sex)   = sextuplet
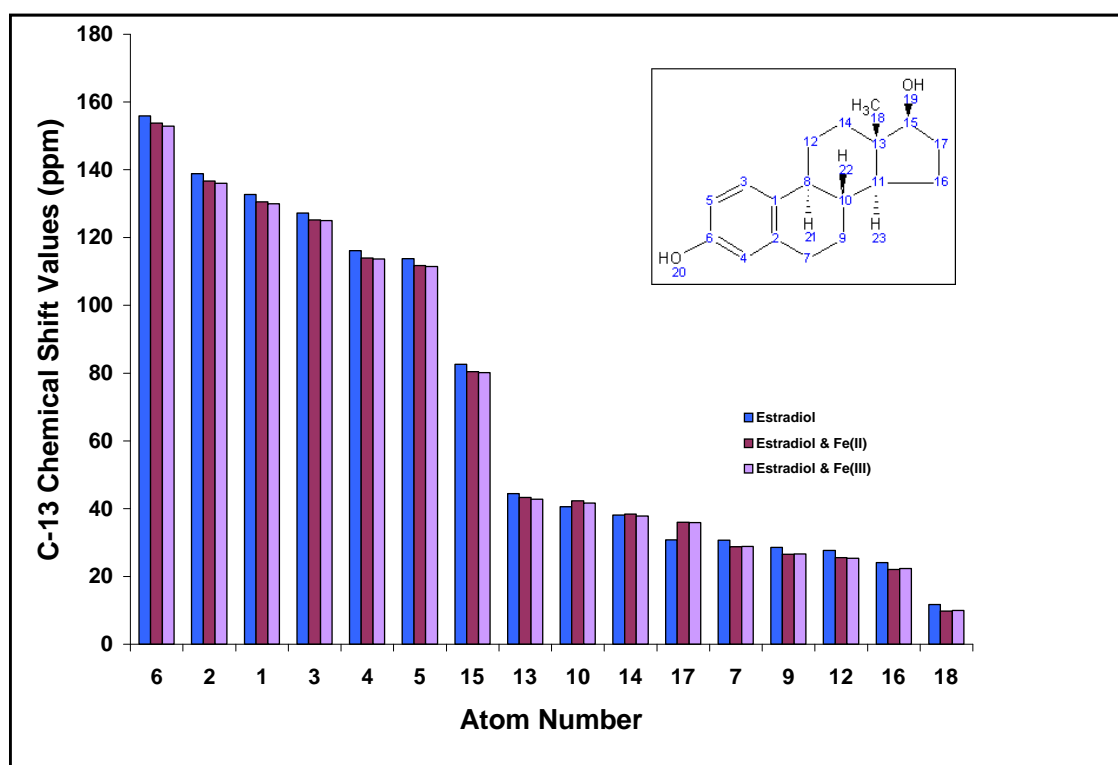
Figure 3.5. A graphic representation of the chemical shift values for ^{13}C NMR.

3.4.4. DISCUSSION

^{13}C NMR chemical shifts have been used to predict and refine chemical structures. The ^{13}C NMR spectrum of a compound contains a pattern of frequencies that correspond directly to the quantum mechanical properties of the carbon nuclei in the molecule and which reflect the proximity and connectivity of nearby atoms (Beger, *et al*., 2000. The quantum mechanical description of a molecule depends largely on its electrostatic features and three-dimensional geometry (Emsley, *et al*., 1965).

The greatest change in chemical shift values upon addition of iron (II) and (III) can be seen at carbon 17 (Figure 3.5.). As a result of this change, the most likely site of interaction is through the oxygen bonded to carbon 15. However, one would expect there to be changes at carbon 15, but there is no net effect because oxygen counteracts

the effect that the iron (II) would have on this carbon. There appears to be a slight difference in Fe^{3+} and Fe^{2+} carbon spectra, but this could be because Fe^{3+} is more paramagnetic.

The NMR data from this experiment suggests that both ferric and ferrous iron electrostatically interact with the oxygen bonded to carbon 15.

3.5. COMPUTER MODELLING OF THE INTERACTIONS BETWEEN QUIN AND ESTRADIOL WITH IRON (II)

3.5.1. INTRODUCTION

Advances in computing, in particular the ready availability of high-resolution graphics, have greatly increased the interest in computer-based molecular modelling. Molecular modelling is widely used as an aid to interpret experimental results and in the design of new materials including the study of the interaction of metal ions with biological substrates (Comba, 1995). Two types of information are obtained from any molecular mechanics experiment: the minimum value of the strain energy and the structure associated with that minimum. However, the modelling of large biomolecules, and their interactions with metals, is fraught with difficulties; the major problems arising from the flexibility of the molecule, resulting in a manifold of adopted conformational geometries (Comba, 1995).

However, despite the difficulties with biological models, when no uneqivocal determination of a structure is available by experimental methods, then structure prediction may be the only means of obtaining a three-dimensional model of the molecule. In metal-macromolecule adducts this is often the case and structures obtained by molecular modelling can be a genuine aid in the visualisation of these interactions (Comba, 1995).

A visual picture of the energy minimized 17β-estradiol-iron (II) complex is obtained, illustrating the proposed site of action.

3.5.2.   MATERIALS AND METHODS

3.5.2.1.   Computer Modelling Analysis

The Cerius^2 version 4.5 software on an O_2 Silicon Graphics machine was utilized for all modelling experiments. The pre-drawn 17β-estradiol underwent a molecular mechanics dynamic simulation by applying the universal force field to find the global minimum of the structure. Atomic charges in the molecule were calculated using the charge equilibration package. From the NMR data, the iron (II) molecule was placed at the 17β hydroxy site and global minimums of the proposed structure were obtained.

3.5.3.   RESULTS

Figure 3.6. (a) and (b) are electrostatically generated models of estradiol alone or in combination with iron (II) respectively. The phenol oxygen was shown to have a charge of –0.6976, whilst the 17β oxygen on the hydroxy group has a charge of –0.7189. Space-filled and ball and stick diagrams of the proposed estradiol-iron (II) complexes are illustrated in Figure 3.7. (a) and (b), graphically indicating the point of interaction.
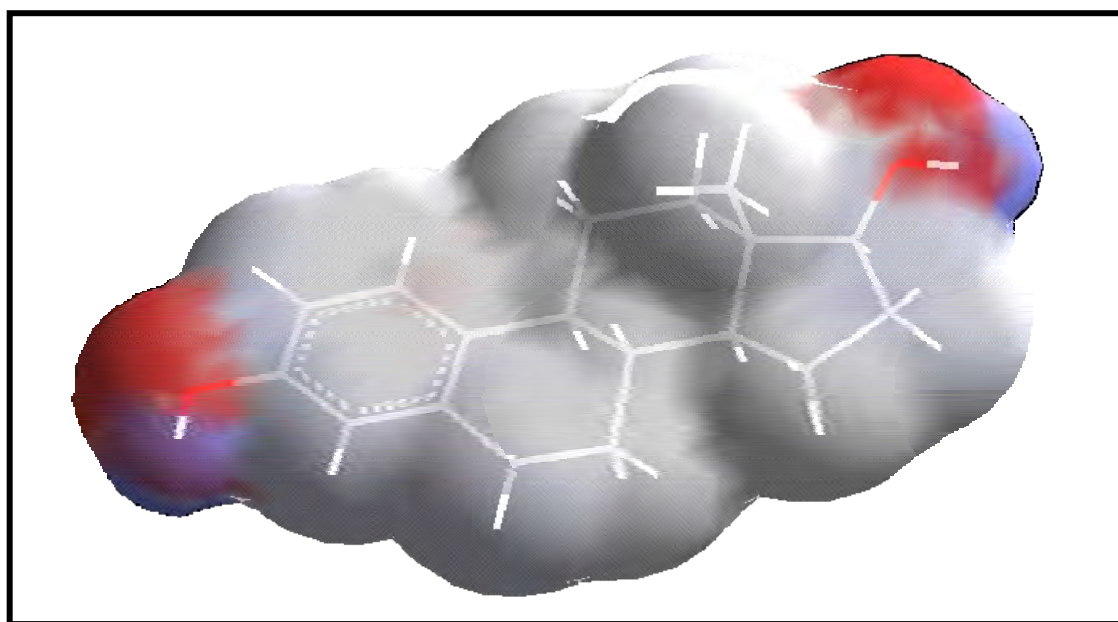
Figure 3.6.(a)   The electrostatic model of 17β-estradiol
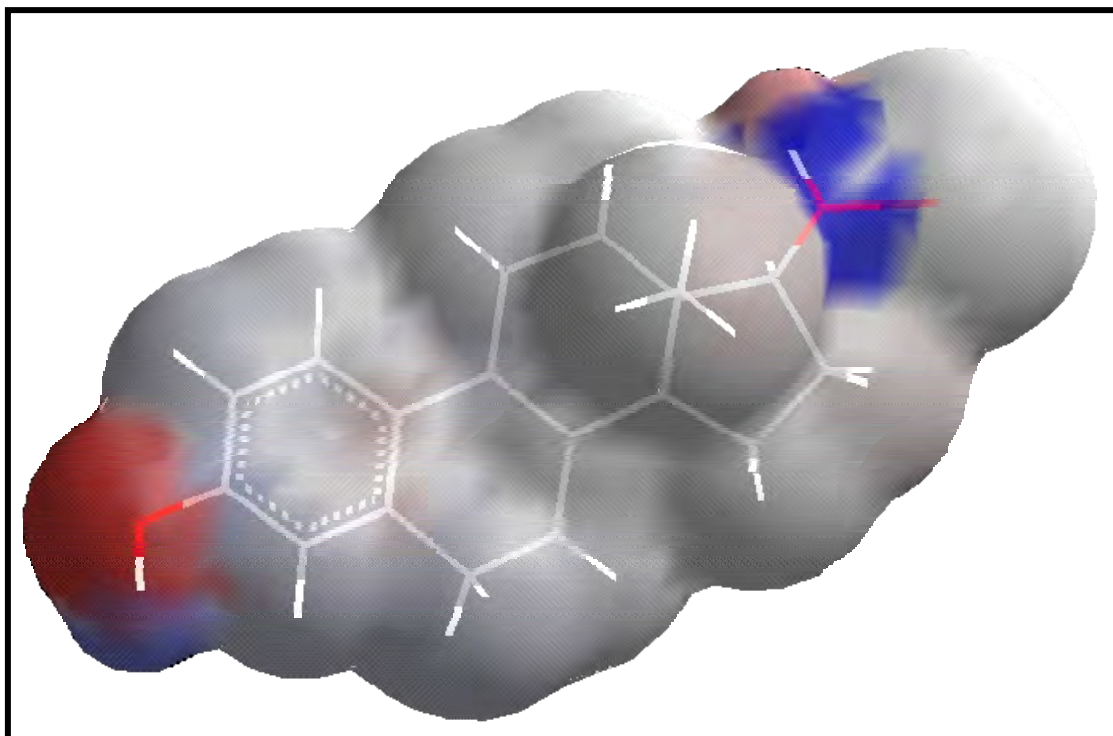
Figure 3.6. (b)   The electrostatic model of the estradiol-iron (II) complex
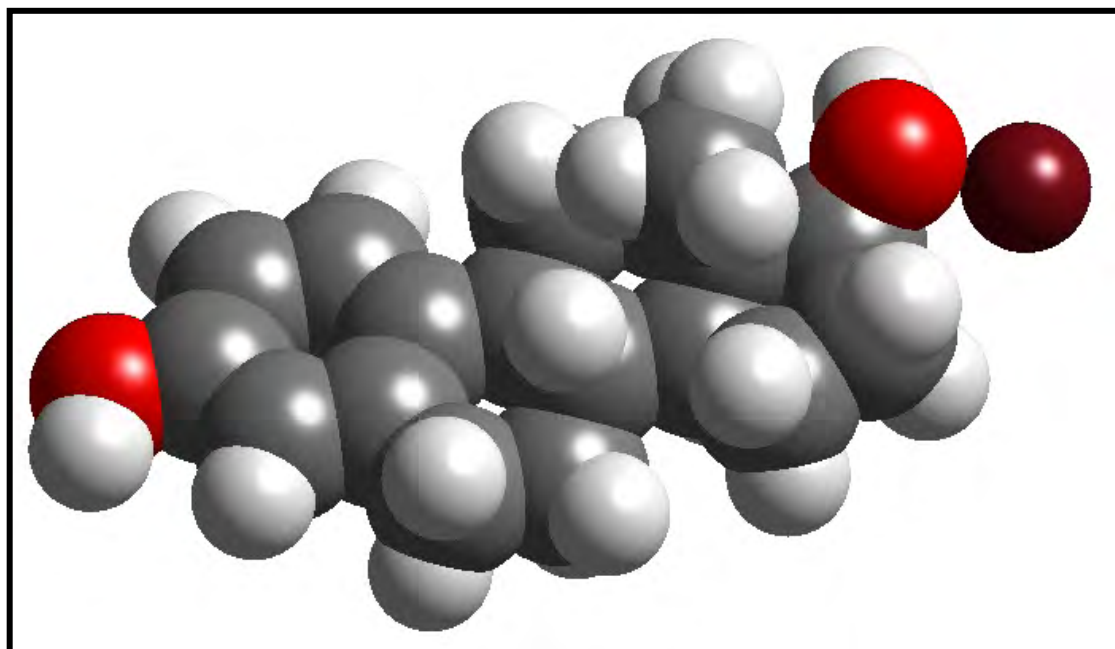
Figure 3.7. (a)   A space-filled model of the global energy minimized estradiol-iron (II) complex
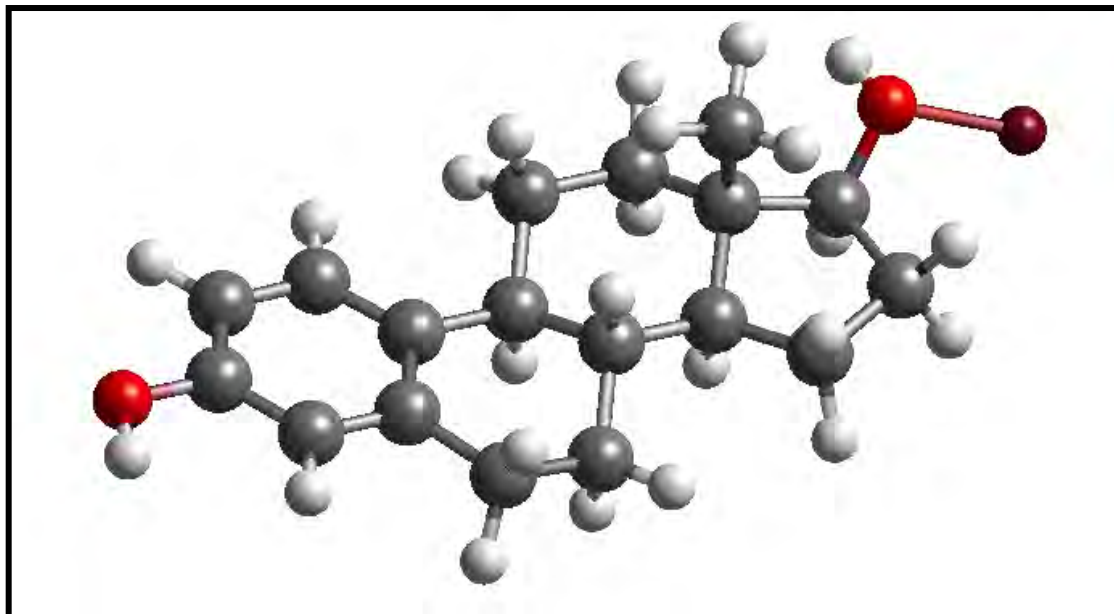
Figure 3.7. (b)   A ball and stick model of the estradiol-iron (II) complex

3.5.4.   DISCUSSION

In comparison, Figure 3.6. (b) displays a decrease in electron density at the 17β hydroxy site upon the addition of iron (II) to the system, with that observed for estradiol alone in Figure 3.6. (a). The model confirms the favoured 17β hydroxy group as a likely site of attack by the iron (II) molecule, based upon electrostatic charge values.

The computer modelling technique employed high quality computer graphics as an aid in visualizing the probable interactions between 17β-estradiol and iron (II) in support of the NMR data obtained in the previous experiment (Figure 3.7.). Since metal-macromolecule interactions are complex, the model generated is but one of the possible representations with the interaction and the model corresponding to one of the most likely energy minima on a complex potential energy surface.

3.6.   CONCLUSION

The purpose of this chapter is to elucidate another possible mechanism of protection shown by estrogen against QUIN-induced lipid peroxidation. The impetus of this study arose as result of a report stating that QUIN-induced lipid peroxidation in rat brain homogenate is iron (II) dependent (Stipek, *et al.*, 1997). As a result, it was reasoned that an interaction between estradiol and iron would render the iron unavailable to cause extensive tissue damage.

The UV studies showed that there existed a definite interaction between estrogen, in the form of premarin®, and the complex that forms between QUIN and iron (II). This interaction itself would be somewhat effective in protecting against QUIN-induced lipid peroxidation, since the actual lipid peroxidation is induced by the complex and not QUIN alone (Stipek, *et al.*, 1997).

Many transition metals, especially iron, have been known to stimulate free radical reactions and to accelerate lipid peroxidation (Halliwell, 1992). Estrogen is a powerful antioxidant, but the extent to which this hormone is able to protect against QUIN-induced lipid peroxidation, could possibly indicate further mechanisms of protection. One of these could be an interaction with iron itself and in particular with iron (II) since this oxidation state of iron is known to react with hydroperoxides at a faster rate than iron (III) (Hallwell & Gutteridge, 1989).

The electrochemistry results show that estradiol does indeed interact with iron (II) but that the resulting *in situ* complex is not stable. The NMR data confirms the presence of this interaction and in addition, suggests the possible site of attack by iron (II) on the estradiol molecule. The proposed site is the hydroxyl group located on carbon 15. An interaction here tends to distort the bond slightly, forcing the bound iron atom closer to carbon 17, which is where the greatest shift is observed. The reason why iron (II) preferentially binds at this hydroxyl site as opposed to interacting with the hydroxyl on the aromatic ring, is that the latter electrons are delocalized through the ring, suggesting a slightly less dense electron cloud at the oxygen on carbon 6. However, the β hydroxy group is less likely to delocalize its electrons to the non-

aromatic ring, thus appearing more electron dense, which favours the interaction of a metal such as iron.

Finally, the computer-generated models of the proposed interaction between estradiol and iron (II) depict a clear image of the site of interaction. The estradiol–iron interaction data presented in this chapter suggest that there is an interaction, but that the proposed complex is not stable. In view of this, it is not likely that this is the predominant mechanism by which estradiol exherts is protection against QUIN-induced lipid peroxidation.

CHAPTER 4

HISTOLOGICAL STUDIES

4.1.   INTRODUCTION

Quinolinic acid is well known to cause considerable neuronal damage *in vivo*; Southgate, *et al*., 1998, showed that QUIN injected directly into the hippocampus, induced cell damage, which was evident by the appearance of swelling and overall degeneration. These authors reported that the CA1 and CA3 regions of the hippocampus were susceptible to QUIN-induced neuronal damage. Furthermore, they were able to show that the pineal hormone, melatonin protects against this damage.

In chapter 2, it was shown that estrogen in the form of 17β-estradiol and conjugated estrogen, protected against QUIN-induced lipid peroxidation both *in vivo* and *in vitro*. Since, these studies were only a measure of the TBA reacting substances, which are degraded lipid products, and even though the decline in the formation of these is indicative of decreased cell damage, it is still important to examine the cells following QUIN administration.

The present study aims to examine the hippocampal neurons following an intrahippocampal injection of QUIN, and pretreatment with subcutaneous injections of 17β-estradiol. Histological techniques were employed and the cells examined under a light microscope attached with a camera.

## 4.2.	THE EFFECT OF 17β-ESTRADIOL AGAINST QUIN-INDUCED DAMAGE TO HIPPOCAMPAL NEURONS

### 4.2.1.	INTRODUCTION

Histology is derived from the Greek word for web or tissue, and involves the examination of preserved, sectioned and stained tissues. Most of our knowledge of internal tissue structure has come from this branch of science (Hodgeson & Bernard, 1992). The Nissl stain, introduced by the German neurologist Franz Nissl in the late nineteenth century, is commonly used to study neurons under the light microscope. This stain is extremely useful since it distinguishes neurons and glia from one another and allows histologists to study the arrangement or cytoarchitecture of neurons in different parts of the brain (Bear, *et al*.,2001).

In this experiment it was decided to investigate whether 17β-estradiol offers neuroprotection against QA-induced intrahippocampal lesions in the rat brain. After treatment, the brains of the rats were sectioned and the hippocampus was examined microscopically for evidence of any morphological changes.

### 4.2.2.	MATERIALS AND METHODS

#### 4.2.2.1.	Chemicals and Reagents

Quinolinic acid and 17β-estradiol were purchased from Sigma St. Louis, MO, U.S.A. Paraffin wax was obtained from Lasec (South Africa). Cresyl violet stain was purchased from BDH Chemicals Ltd (England), while DPX was purchased from Philip Harris Ltd (England). Haupt's adhesive consisted of the following: 1 g gelatine, 100 ml water, 2 g phenol and 15 ml glycerol. All other chemicals were of the highest quality available and were purchased from commercial distributors.

4.2.2.2.   Animals

Adult female rats were cared for as described in section 2.2.2.2.

4.2.2.3.   Surgical Procedures

The anaesthesia employed and the ovariectomy procedure was performed exactly as described in section 2.2.2.3. The bilateral injection of QUIN into the hippocampus was carried out as in section 2.3.2.5.

4.2.2.4.   Treatment Regime

As described in section 2.3.2.4., with the exception that the animals were divided into three groups. The Premarin group was not included in this study.

4.2.2.5.   Histological Techniques

The histological techniques were followed according to the methods described by Southgate, 1999.

4.2.2.5.1.   Fixing the brain

The animals were sacrificed and the brains removed as in section 2.2.2.4. Immediately after death, animal tissues begin to break down as a result of autolysis and bacterial attack. Fixation functions to chemically stabilise proteins, and thus preserve structures (Southgate, 1999). Brains were rapidly fixed in a mixture of formol (30%), glacial acetic acid, and ethanol (2:1:7 v/v) for 2 hours. After fixation, the brains were stored in 70% ethanol.

4.2.2.5.2.   Specimen Preparation and Embedding

In order to be cut, the slices need to be supported. Imbedding involves the infiltration and orientation of tissue in the paraffin wax support medium. The tissue was dehydrated (using increasing concentrations of ethanol), followed by the removal of the ethanol using xylene. Finally the tissue was immersed in molten paraffin wax, which removed the xylene, while infiltrating the tissue without encountering water. The method used is shown in Table 4.1

Table 4.1.   Procedure for embedding brains in paraffin wax.

Step	Processing Agent	Time (Hours)
1	70% Ethanol	1
2	90% Ethanol	1
3	Absolute Ethanol	1
4	Absolute Ethanol	1
5	Xylene	1
6	Xylene	1
7	Melted Paraffin Wax	1
8	Melted Paraffin Wax	1

4.2.2.5.3.   Blocking Out

The brain material was fixed into a block so that it could be cut with a microtome. The mould used was a plastic ice tray. This were coated with ethanol-glycine to prevent the block sticking to the mould. The brain was removed from the final molten wax stage (previous section) and placed into the mould with warmed forceps. The brain was then completely covered in molten wax. Air was gently blown over the surface of the wax until the top solidified. The blocks were left overnight to ensure that the wax had completely solidified.

4.2.2.5.4.   Sectioning

The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly. The sides were cut so as to leave about 2 mm of wax around the tissue. The wax block was attached to a small wooden block with a small amount of molten wax.

Sectioning was done using a rotary microtome. The microtome was set to cut sections of 10µm thickness. As sections were cut these would stick to one another, so as to form long ribbons. When the part of the brain containing the hippocampus was reached, every second section was removed and placed in a water bath (40° C) using forceps..

4.2.2.6.5.   Transferring Sections to Slides

Three sections at a time were removed from the water bath and placed onto microscope slides containing a thin layer of Haupt's adhesive. The slides were left overnight in an oven at 40°C.

4.2.2.6.6.   Staining

The sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple, the nuclei purple, and leaves the background clear (Bauer, *et al*., 1974). Before the section could be stained, it first had to be dewaxed and rehydrated as the stain is water soluble. This was done as per Table 4.2.

Table 4.2.   Procedure for dewaxing and rehydrating brain sections

Step	Processing Agent	Time (minutes)
1	Xylene (dewaxing)	5
2	Xylene	5
3	Xylene / Absolute Ethanol (1:1)	3
4	Absolute Ethanol	5
5	Absolute Ethanol	Overnight at 30°C

Sections were stained by placing in a 0.1% cresyl violet solution for 2 hours. The cresyl violet solution contained 0.25g cresyl voilet, 250 ml milliQ water, 0.75 ml glacial acetic acid and 0.0512g sodium acetate. The pH was adjusted to 3.5 before use. The slides were differentiated rapidly in 95% ethanol by rinsing in a flat dish until the background was clear. Sections were then dehydrated again as shown in Table 4.3.

Table 4.3. Procedure for dehydrating brain sections after staining

Step	Processing Agent	Time (minutes)
1	Absolute Ethanol	5
2	Absolute Ethanol	5
3	Xylene	5
4	Xylene	5

4.2.2.6.7. Mounting of the Slides

While the slides were kept moist with xylene, enough DPX was added to just cover the tissue. A cover slip was placed over the tissue. The slides were allowed to dry on a flat surface for 48 hours.

4.2.2.6.8. Photo-microscopy

The slides were photographed using a combination Olympus camera and light microscope.

4.2.3. RESULTS

The photomicrographs in Figure 4.1., demonstrate that 17β-estradiol protects hippocampal neurons against QUIN-induced neurodegeneration. The hippocampal neurons in the control group from both the CA1 region (Fig. 4.1.(a)) and the CA3 region, (Fig. 4.1.(d)) appear undamaged. The neurons in the CA1 region, (Fig. 4.1. (b)) and those in the CA3 region (Fig. 4.1. (e)) of those animals treated with QUIN show signs of neuronal damage as evident by the swelling of the cells and overall degeneration. However, in the presence of 17β-estradiol the neurons in both the CA1

and the CA3 regions (Fig. 4.1. (c) and (f)), appear to be undamaged and in addition look healthier than the cells of the control group.
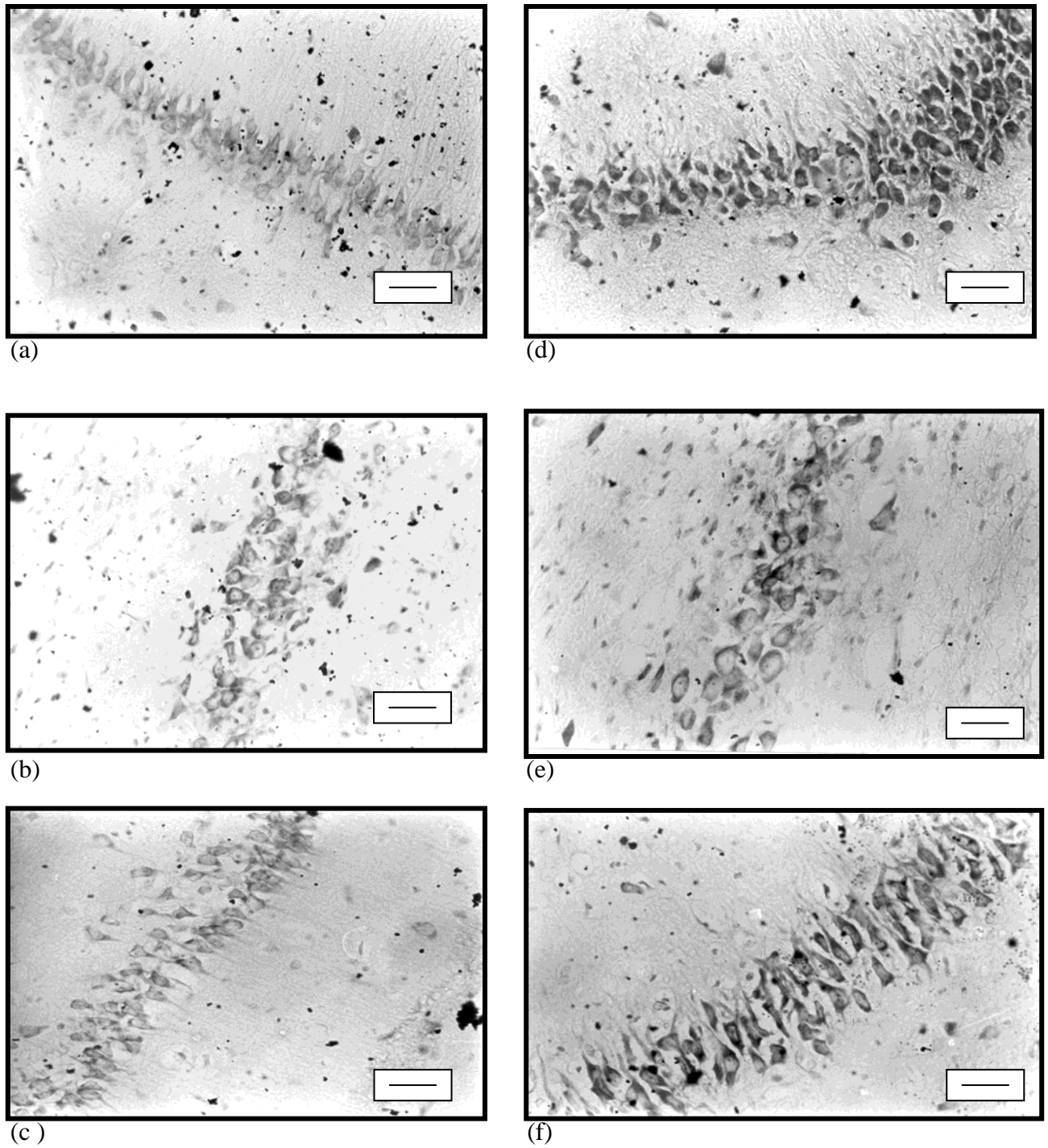
Figure 4.1. QUIN toxicity and the protective effects of 17β-estradiol on rat hippocampal neurons. Micrographs (a-c) indicate cells in the CA1 region of the hippocampus from a control animal (a), an animal treated with QUIN (b) and an animal treated with QUIN and 17β-estradiol (c). Micrographs (d-f) indicate cells in the CA3 region of the hippocampus from a control animal (d), a QUIN treated animal (e), and an animal treated with QUIN and 17β-estradiol. (Bar = 10μm)

4.2.4. DISCUSSION

The cell damage induced by QUIN is dependent on the NMDA receptor, since pre-treatment with an antagonist prevents this damage. However, in order for NMDA receptors to be activated, another non-NMDA glutamate receptor, such as AMPA must be activated. This activation results in an influx of Na^+ ions into the cell causing a depolarization of the membrane. The depolarization results in the removal of the magnesium block, allowing the opening of the channel once QUIN has bound. Once opened, Ca^{2+} ions move through and into the neuron.

In cases where there are high concentrations of glutamate within the synapse, excessive activation of glutamate receptors occurs, resulting in acute toxicity. Acute toxicity occurs because of the rapid influx of Na^+ ions into the neuron, which causes passive Cl^- and water entry via osmotic pressure. This toxic process may be associated with abnormalities in membrane permeability and may be lethal, via osmotic lysis Southgate, 1999). The process that takes place may be direct, by over-stimulation of the neuron leading to prolonged depolarization and depletion of energy reserves, or indirect, by excessive Na^+ ion influx resulting in Ca^{2+} build up via Na^+-Ca^{2+} exchange. In addition, the activation of the NMDA receptor by QUIN results in a further influx of Ca^{2+} ions. This together with the water uptake results in the swelling of the cells evident in the photomicrographs.

From the histology studies (Fig. 4.1.), it appears that 17β-estradiol not only protects neurons from damage induced by QUIN but cells treated with17β-estradiol appear healthier than cells from controls. This follows from the observation that ovariectomy in the female rat results in a dramatic reduction in the hippocampal compensatory process, with a change in hippocampal axon sprouting. This deficit in sprouting could be returned to normal with hormonal replacement (Morse *et al*, 1992). This demonstrates that 17β-estradiol is able to protect effectively against QUIN-induced neurotoxicity but does not improve the state of these cells in comparison to intact females that have not undergone ovariectomy.

4.3.   CONCLUSION

From the results obtained in this chapter, it is evident that 17β-estradiol attenuates QUIN insult in the rat hippocampus. This protection elicited by estrogen as well as that shown against the increase in lipid peroxidation, could be due to only its antioxidant property, in this way preventing further damage induced by the free radicals formed during the initial QUIN damage. However, considering the extent of the protection that estrogen has against this potent neurotoxin, it is more likely that the protection involves the NMDA receptor. If estrogen were able to bind to the receptor to the same site at which QUIN binds, the hormone would compete with QUIN thus preventing neuronal damage.

CHAPTER 5

RADIOLIGAND BINDING STUDIES

5.1.   INTRODUCTION

Radioligand receptor binding assays provide a convenient and reliable method of studying the interactions between a given ligand and a receptor complex. For years, neuroscientists have investigated the properties of neurotransmitter receptors, which involved observing electrical, behavioral and biochemical changes after systemic administration of drugs, or direct application of drugs to the brain. Bioassays were also employed but this method, while substantiating the existence of receptors for drugs, cannot reliably reveal the direct interaction between the ligand and the receptor (Titeler, M, 1981).

The major neurotransmitter system in the hippocampus is the glutamatergic system with the highest concentration of NMDA sensitive L-[H^3]-glutamate binding sites located in the CA1 region of the hippocampus (Monaghan & Cotman, 1986). The development of radiolabeled NMDA agonists with high affinity and specificity for NMDA receptors has played a key role in providing biochemical information such as direct measurement of NMDA sites. This provides data regarding quantitation, distribution, pharmacological specificity and modulation of NMDA receptors. A non-competitive antagonist that has been derived is dizocilpine, which binds to activated NMDA receptors.

The receptor binding assay is one procedure that has helped revolutionize neuroscience research and has become an important tool in many disciplines in the biological sciences (Bylund & Yamamura, 1990). The radioreceptor assay has distinct advantages over other methods like bioassays. Radioreceptor assays provide:

1) direct quantitative information about the affinity of a drug for a receptor
2) the ability to directly measure the amount of receptors present

3) a fair amount of leeway in tissue preparation and storage owing to the stability of the receptor

4) interchangeability by switching from one receptor assay to another, since tissue contains more than one receptor and can be stored for many months (Titeler, 1981).

However, one downfall to this method is that it does not predict whether a tested ligand acts as an agonist or antagonist, or the action of indirectly acting drugs. In certain circumstances this can be overcome by employing an assay that allows one to predict the action of a drug due to its influence on the binding of another compound acting at the same receptor. Although this does not directly indicate whether the ligand is an agonist as such it is useful in showing whether activation of the receptor occurs. Activation could be the result of either an agonist or positive modulator.

5.2.   THE COMPARATIVE EFFECTS OF 17β-ESTRADIOL AND QUIN ON GLUTAMATE BINDING *IN VIVO* AND *IN VITRO*

5.2.1.   INTRODUCTION

The action of glutamate is exerted through stimulation of at least three pharmacologically distinct classes of ionotropic glutamate receptors, distinguished by their sensitivity to the specific synthetic agonists NMDA, AMPA and kainate (Weaver *et al*, 1997). The NMDA receptor is a complex molecular entity with a number of distinct recognition sites. One of these sites is a transmitter binding site that binds L-glutamate and related agonists (Cooper *et al*, 1996).

 The results of the previous chapters have indicated a role for estradiol in protecting against QUIN-induced neurotoxicity. This may be due to estradiol competing with QUIN to bind to the NMDA receptor, of which QUIN is known to be an agonist. Several reports favour the ability of estradiol to activate this subtype of glutamate receptor. NMDA receptors are implicated as mediators of effects of estradiol on morphological plasticity and related physiological and cognitive processes in the brain (Gazzaley *et al*, 1996). Estradiol has recently been shown to increase agonist binding sites on the NMDA receptor complex in the CA1 region of the hippocampus (Weiland, 1992) as well as increasing dendritic spine density (Gould *et al*, 1990) via a mechanism dependent on NMDA receptor activation (Woolley & McEwen, 1994). However, Weaver, 1997 reported that NMDA-induced excitotoxicity is decreased by estradiol via the direct inhibition of NMDA receptors.

The mechanism by which estradiol is neuroprotective may extend beyond that of an antioxidant. The present study aims to investigate the effect of 17β-estradiol on glutamate binding and MK-801 binding to the NMDA receptor in comparison to QUIN.

5.2.2. MATERIALS AND METHODS

5.2.2.1. Chemicals and Reagents

Quinolinic acid (2,3-pyridinedicarboxylic acid), 17β-estradiol and N-methyl-D-aspartate (NMDA) were purchased from Sigma (St. Louis, USA). [H^3]Glutamate (33.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Scintillation cocktail was purchased from Packard (USA). All reagents were of the highest quality available.

5.2.2.2. Animals

As described in section 2.2.2.2.

5.2.2.3. Surgical Procedures and Treatment Regime

Animals were subjected to the procedures outlined in sections 2.2.2.3. and 2.3.2.5. The dosing of the animals was exactly as in previous studies and is described in section 2.3.2.4. with the exception that the animals were separated into three groups. The Premarin group was not included.

5.2.2.4. Dissection of the Hippocampus

On the eighth day following the intrahippocampal injection of QUIN, the brains were removed as described in 2.2.2.4. and the hippocampi rapidly dissected according to a modified method of Glowinski and Iversen, 1966. Briefly, the rhombencephalon is separated by a transverse section from the rest of the brain (Figure 5.1., section 1). Then a transverse section is made at the level of the optic chiasma, which delimits the anterior part of the hypothalmus and passes through the anterior commissure (section 2). This section separates the cerebrum into two parts, B and C. Part B is divided into five fractions. The easiest way to reach the hippocampus is to first dissect the hypothalamus and the striatum from section B.

The midbrain is then gently separated from the remaining part of the brain. The hippocampus is then dissected.
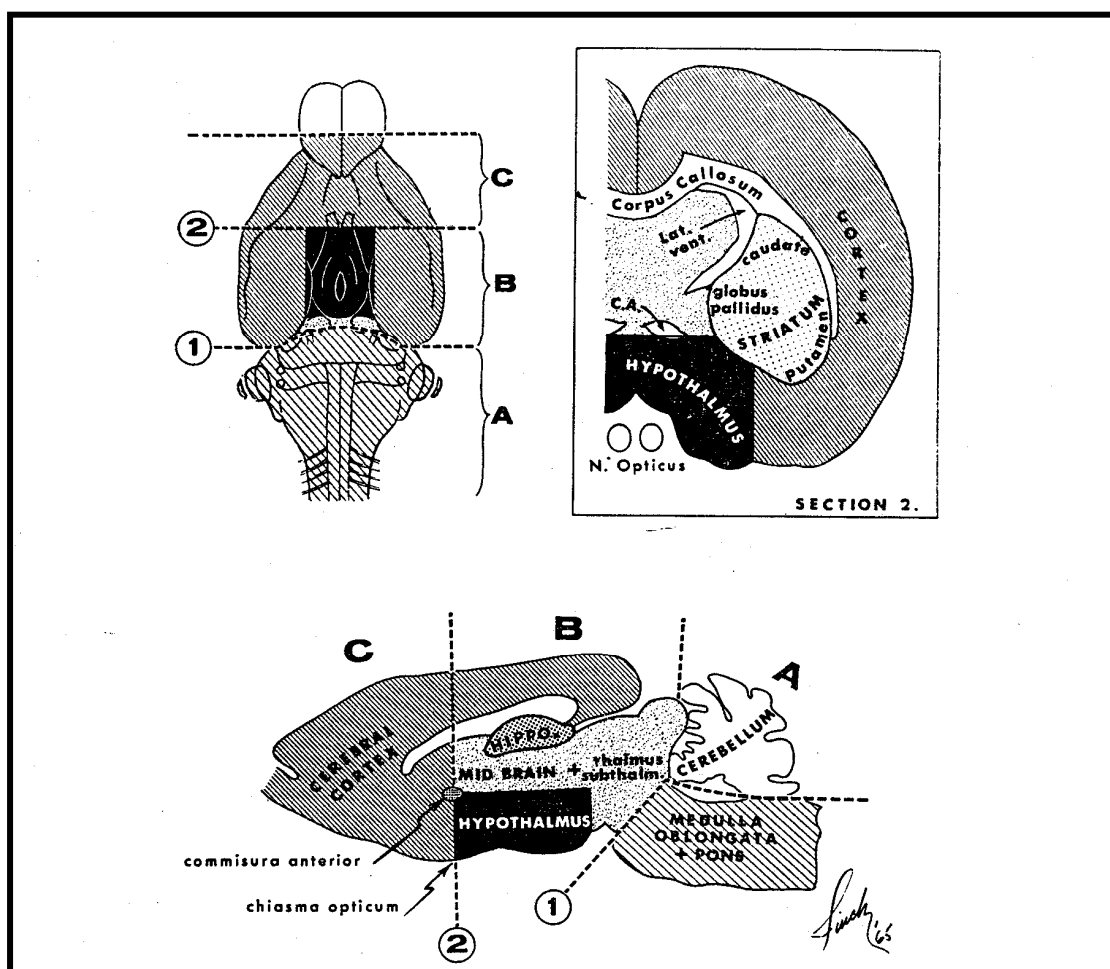
Figure 5.1. Diagrammatic representation of the dissection procedure for rat brain (Glowinski & Iversen, 1966).

5.2.2.5. Preparation of Synaptic Membranes

Hippocampal neuronal membranes were prepared using the method of Bylund & Yamamura, 1990. Hippocampi were homogenized in 50 volumes 50 mM Tris-HCl buffer (pH 7.4) containing 1mM EDTA. This was centrifuged at 50 000 x g for 15 min. The pellets were resuspended in 50 volumes 50mM Tris-HCl buffer (pH 7.4) and centrifuged as before. The centrifugation step was repeated once

more and the final pellets were resuspended in 15 volumes 50mM Tris-HCl buffer (pH 7.4). Protein estimation was performed as in 2.2.2.7.

5.2.2.6.   Saturation Glutamate Binding Studies

In a saturation experiment the radioligand concentration is variable, whereas the receptor concentration, the drug concentration and time are all constant. The synaptic membranes prepared from the three brains in each treated group were used for these studies. Thus this study was considered to be *in vivo* investigation.

For total binding, incubation mixtures consisted of 425μl membrane suspension, stock solution of [H^3] Glutamate ranging from 0.5nM to 5nM final concentration, and 50mM Tris-HCL buffer pH 7.4 to give a final incubation volume of 500μl. A series of uncapped scintillation vials with GF/A filters on top were prepared. Aliquots (20μl) of each concentration of hot glutamate was pipetted directly onto the filter paper to determine total added radioactivity.

Non-specific binding was determined by adding NMDA (1mM final concentration) to a separate set of tubes prepared as before. The tubes were incubated at 25°C for 100min and the reaction was terminated by the addition of 4ml ice cold Tris buffer followed immediately by rapid filtration through Whatman glass fibre (GF/A) filters under negative pressure. The filters were washed twice with 4ml cold buffer and placed into scintillation vials containing 3ml scintillation cocktail. The vials were left overnight and the radioactivity measured using a Beckman Liquid Scintillation Counter. Radioactivity was expressed as pM tritiated glutamate bound.

5.2.2.7.   Glutamate Receptor-Displacement Binding Studies

 In this experiment, the receptor concentration, the radioligand concentration and time are all constant, whereas the concentration of the tested agent/drug is variable. Brains were removed from untreated, intact female rats and membrane suspensions prepared in the same way as in 5.2.2.4. Eight tubes were set up in

triplicate. Tube 1 (total binding) contained 25μl [H^3]-Glutamate to give a final concentration of 5nM, 50μl of 50mM Tris-HCL buffer pH 7.4, and 25μl of 100% ethanol. Tubes 2 through 7 contained 25μl of 17β-estradiol dissolved in ethanol or quinolinic acid dissolved in MilliQ water ranging from 0.05nM to 5μM final concentration, 25μl [H^3]-Glutamate to give a final concentration of 5nM, and 50μl of buffer. Tube 8 (non-specific binding) contained 25μl [H^3]-Glutamate to give a final concentration of 5nM, 25μl ethanol and 50μl NMDA to give a final concentration of 1mM. 400μl of membrane suspension was added to all the tubes. Results were expressed as percent glutamate bound in the presence of 17β-estradiol or QUIN.

5.2.3. RESULTS

5.2.3.1. Saturation Binding Studies – calculations and data analysis

The radioactivity measured by the liquid scintillation counter is in CPM (counts per minute). Once the mean of the triplicate CPM values are calculated, the non-specific binding values are subtracted from the total binding values, to give specific binding data. A saturation plot can be constructed by plotting the specific binding of the radioligand versus the ligand concentration. The specific binding can be plotted in units of fmol/mg protein, which is calculated by multiplying the specific binding by a factor and dividing by the protein concentration. The factor calculated in this experiment was as follows:

f = specific activity^{-1} x volume^{-1} x efficiency^{-1} x Ci/ 2.2 x 10^{12}dpm

Efficiency refers to the efficiency of the scintillation counter (65%), volume is the incubation volume ie. 0.5ml and the final number is the definition of a curie in terms of dpm (disintegrations per minute). The units of this factor is pM/cpm (Bylund & Yamamura, 1991)

It is difficult to determine the K$_D$ and B$_{max}$ values from a saturation plot since the relationship is nonlinear. Therefore a scatchard plot is constructed in order to calculate these values. This is a plot of bound/free versus bound radioligand. Firstly, the specific binding (in CPM) is subtracted from the total added (in CPM) to give free

radioligand values. Both the specific binding and the free just calculated is multiplied by the factor f to give values in pM concentrations. Then the bound/ free values are calculated by dividing the specific binding by the free radioligand (Bylund & Yamamura, 1991).

5.2.3.2.   Receptor-Displacement Studies – calculations and data analysis

The first step as before is to calculate the mean of the cpm values. An inhibition curve can be generated by plotting the percentage of total binding in the presence of the different concentrations of drug versus drug concentration. This plot is not well suited to determining the IC_{50} value. Thus a logit-log plot is constructed where the data is first calculated in terms of percent bound (P) where 100% is the amount specifically bound in the absence of drug. This was done by taking the mean of the diluent tube (tubes 1 in this case) and subtracting the mean of the non-specific values (NSB; tube 8). For tubes 2 through 7 P was calculated by subtracting the non-specific binding from the amount bound (B), dividing by B_0 and multiplying by 100:

P = B-NSB / B_0 x 100

The logit transformation is the natural logarithm (ln) of the ratio of percent bound to 100 minus the percent bound.

Logit = ln[P/(100-P)]

The IC_{50} is 50% binding, and the logit of 50% [ln(1)] is 0. Thus the IC_{50} can be calculated by direct correlation or as in these studies by extrapolating the values from the logit of 0. The Ki was determined by the following equation:

Ki = IC_{50} / 1 + F/K_D

The K_D is determined from the saturation experiment and F is the free radioligand (Bylund & Yamamura, 1991).

Saturation binding studies performed on the same treated animals showed that both treatment with QUIN, and treatment with QUIN in the presence of 17β-estradiol, reduces glutamate binding to the NMDA receptor, as evident in the saturation plot (Figure 5.2.).

Scatchard plots of the data were generated, (Figure 5.3. (a), (b), and (c)) and the K_D and B_{max} values determined.
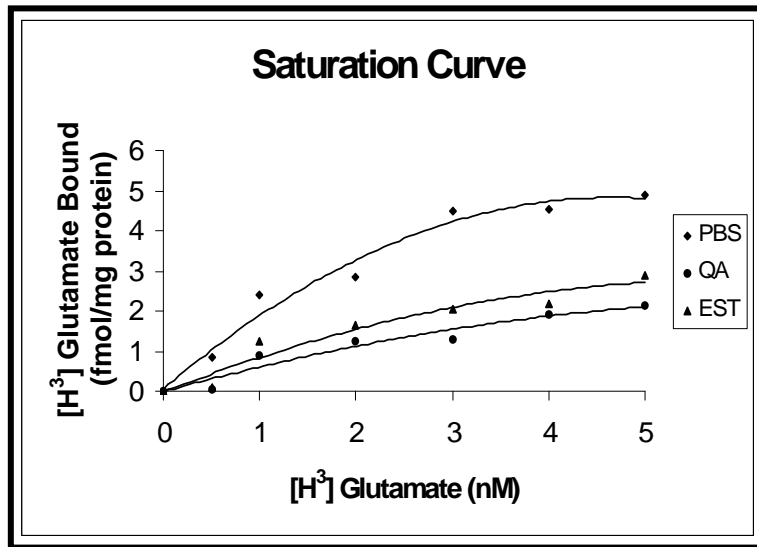
Figure 5.2. Saturation binding plot indicating [H[3]]-Glutamate binding vs added [H[3]]-Glutamate concentration of the control (PBS) animals, the QUIN treated animals (QA) and the animals treated with both QUIN and 17β-estradiol (EST). Three animals from each group were used in saturation binding experiments
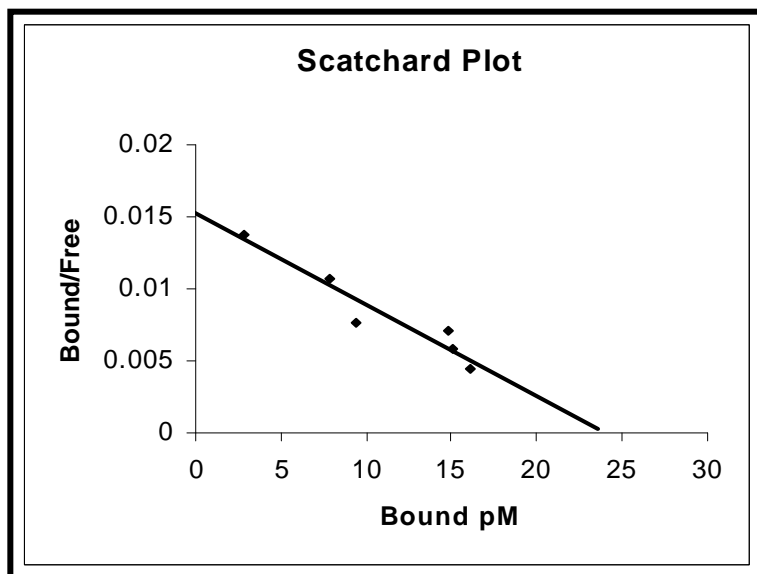
(a)
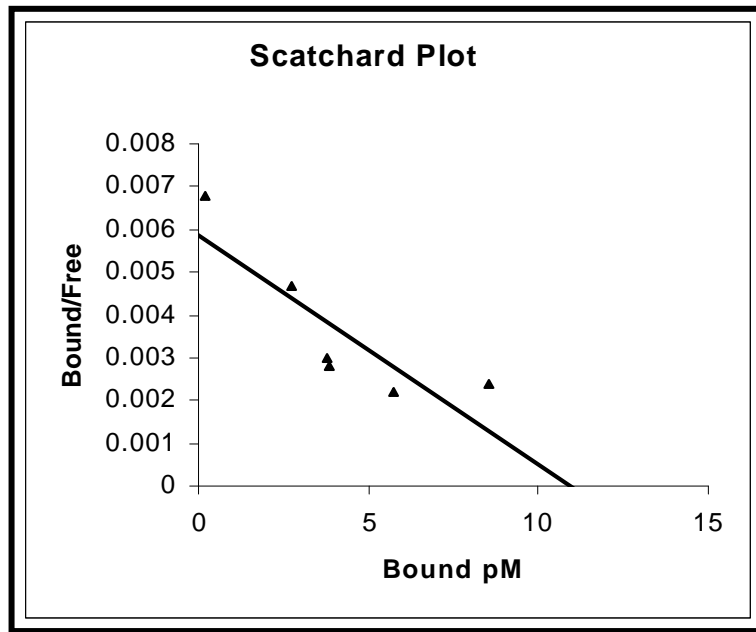
(b)
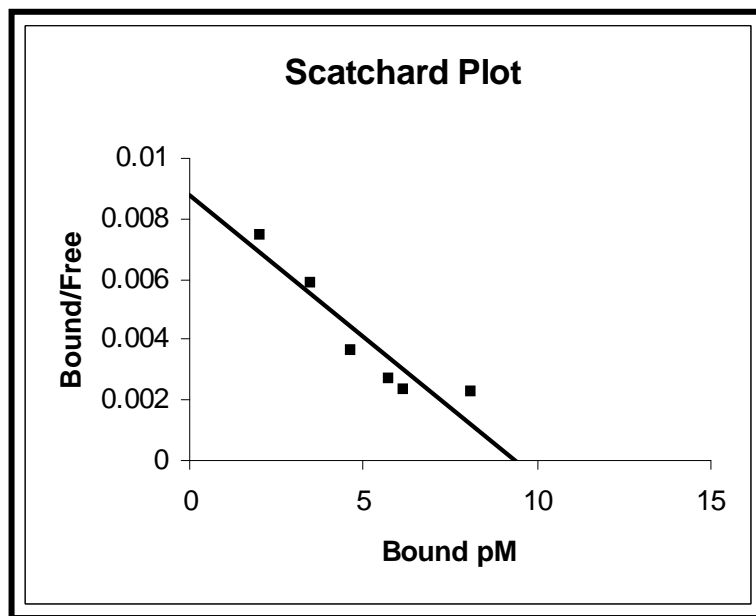
(c)

Figure 5.3. Scatchard plots generated from $[H^3]$-Glutamate saturation binding studies of the control (a), treated with PBS; the QUIN treated animals (b); and the animals treated with QUIN and 17β-estradiol (c)

The intrahippocampal injection of QUIN resulted in a reduction of the density of receptor sites and glutamate binding to the NMDA receptor. As a result of QUIN administration, the affinity of glutamate for these receptor sites is lower than that for the control (Table 5.1.).

As a result of both QUIN and 17β-estradiol treatment, more glutamate bound to the receptor in comparison to both the control and the QUIN treated groups. However, a reduction in the density of glutamate binding sites were observed in comparison to the control and QUIN treated groups.

Table 5.1. Summary of the data obtained for saturation binding studies of the three treated groups of animals

Group	K_D (nM)	B_{Max} (pM)
PBS	1.66	25.33
QUIN	2	11.6
QUIN + EST	1.11	9.66

Glutamate receptor-displacement binding studies show that 17β-estradiol is slightly more effective at reducing the amount of glutamate bound compared to QUIN. 17β-estradiol exhibits a slightly greater inhibition of glutamate binding as opposed to QUIN as evidenced by the sharper initial decrease in percent glutamate bound at low concentrations of 17β-estradiol (Figure 5.4.). However, the IC_{50} value obtained is approximately equal to that obtained for QUIN at the same concentrations, calculated from the plots in Figure 5.4.. Furthermore, the K_i for 17β-estradiol in comparison to that for QUIN is roughly the same, indicating equal inhibition of glutamate binding (Table 5.2.) by 17β-estradiol.
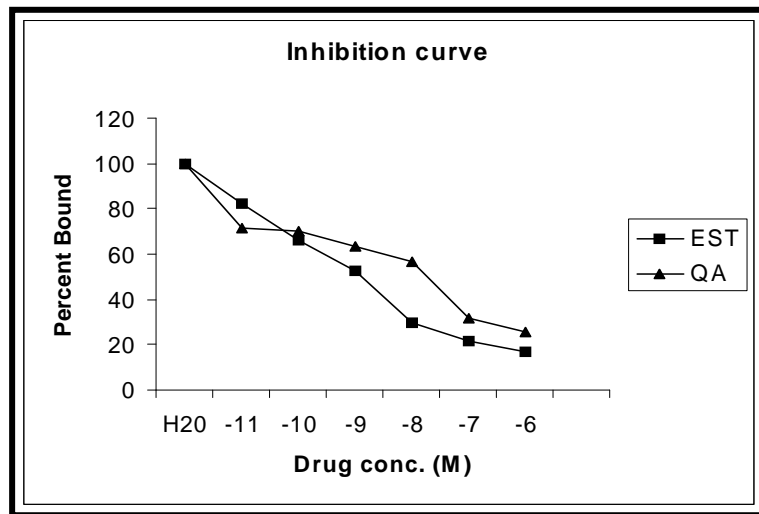
Figure 5.4. Inhibition curves of [H^3]-Glutamate binding in the presence of 17β-estradiol (EST) or QUIN (QA). The graph indicates a slightly greater inhibition of glutamate binding with 17β-estradiol as opposed to QUIN as shown by the sharper decrease in percent glutamate binding with 17β-estradiol

(a)

112

(b)
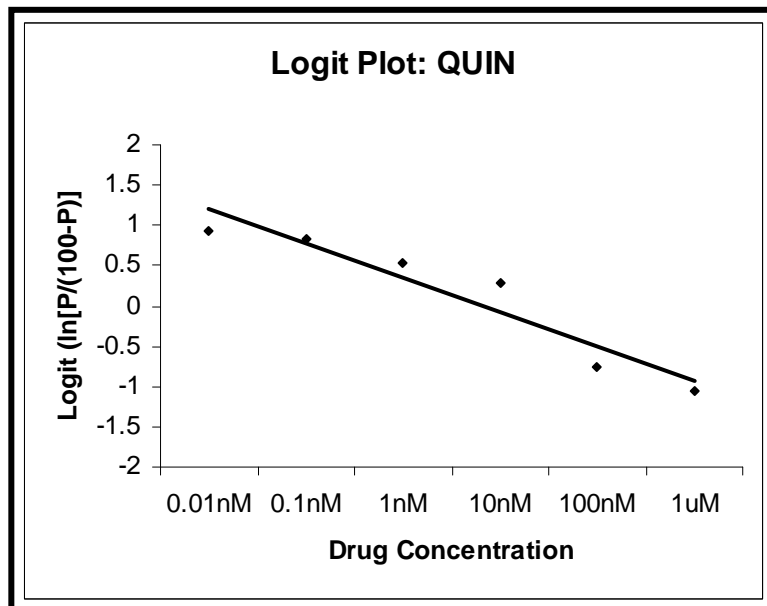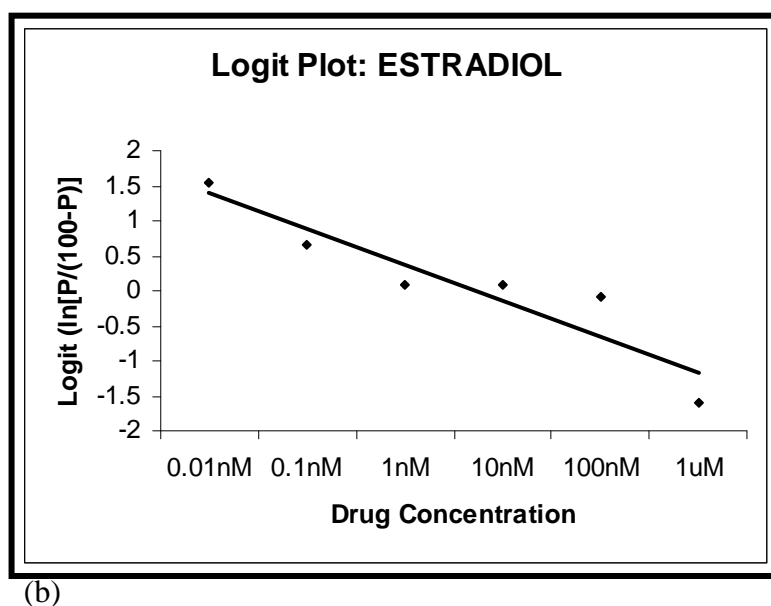
Figure 5.5. Logit plots generated from inhibition binding studies. [H^3]-Glutamate binding was decreased in the presence of 17β-estradiol or QUIN

Table 5.2. Summary of the data obtained for inhibition binding studies. [H^3]-Glutamate binding was observed in the presence of 17β-estradiol or QUIN

Tested Ligand	IC$_{50}$ (nM)	K$_i$ (nM)
QUIN	9.5	3.546
EST	9.325	2.286

5.2.4. DISCUSSION

The results of the saturation binding studies demonstrate the effect of treatment with QUIN alone or in combination with estradiol on the ability of glutamate to bind to the receptor. The K_D and B_{max} values determined (Table 5.1.) show that the intrahippocampal injection of QUIN results in a decrease in the number of binding sites and as a result, a decrease in the capacity of glutamate to bind. This could be explained by the histology results of the previous chapter, which demonstrate the neurotoxic effects of QUIN and the ultimate destruction of the neuronal cells thus causing a decrease in the number of membrane bound receptor sites. QUIN is known to be an endogenous agonist of the NMDA glutamate receptor subtype (Santamaria *et al*, 1996).

Treatment with estradiol prior to and after the intrahippocampal injection of QUIN results in an increase in the affinity of glutamate for the receptor in comparison to the control and to treatment with QUIN alone. However, the number of binding sites decrease. It is not clear why this should be the case, since estradiol clearly protects hippocampal neurons against QUIN-induced degeneration. One explanation could be that the decrease in receptor sites is due to the effect of QUIN while estradiol is able to increase the affinity of glutamate for the receptor, thus preventing, in part, QUIN from binding to the NMDA site.

Thus it would appear that estradiol causes more glutamate to bind to the NMDA site. It has also been previously reported that estradiol increases the number of agonist binding sites (Weiland, 1992). The mechanism by which the combination of estradiol and QUIN alters the binding of glutamate to the receptor is unclear. However, the displacement binding studies show that estradiol is as effective as QUIN at reducing the amount of glutamate bound. The K_i obtained for 17β-estradiol is not significantly different than for QUIN.

Whatever the mechanism by which estradiol acts on the NMDA receptor, the results of the histological studies demonstrate that estradiol protects hippocampal neurons against QUIN-induced damage. This damage is a direct result of QUIN acting on the NMDA receptor. Therefore the protection of estradiol against QUIN-induced damage, requires an interaction with the NMDA receptor, thus reducing the amount of QUIN binding to this receptor. The results from the displacement studies support the hypothesis that estradiol binds to the receptor with an equal affinity to QUIN, thus competing with QUIN for the receptor and protecting against neuronal damage.

5.3. THE COMPARATIVE EFFECTS OF CONJUGATED ESTROGEN AND QUIN ON MK-801 BINDING *IN VITRO*

5.3.1. INTRODUCTION

The results of the previous experiment indicated that 17β-estradiol offers protection against QUIN-induced neurotoxicity via a NMDA receptor-dependent mechanism. However, the results obtained did not differentiate whether estradiol was acting as an agonist or an antagonist. Both an agonist and an antagonist would decrease the binding of glutamate to the receptor. It is not likely that estradiol is acting as an antagonist to reduce glutamate binding since, this contradicts previous reports stating that estradiol is able to enhance NMDA receptor-mediated excitatory postsynaptic potentials (Foy et, al., 1999).

Several other reports have supported the notion that estradiol activates the NMDA receptor (Gazzaley, *et al*., 1996 ; McEwen, 1998), however none to date have shown estrogen to bind directly to the NMDA receptor. To test the hypothesis that estradiol may possibly be acting as an agonist, a radioligand binding stusy was performed using tritiated MK-801.

MK-801 (dizocilpine) is a non competitive NMDA receptor antagoinst that binds to the receptor when the receptor channel is open. Thus an agonist or positive modulator would result in an increase of MK-801 binding (Foster & Wong, 1987) The binding of this antagonist is illustrated in Figure 5.7. An agonist such as NMDA activates the receptor by combining with the transmitter recognition site, which transforms the receptor to the open state allowing conductance of Na^+, Ca^{2+} and K^+ to initiate the neuronal response. Non-competitive antagonists act upon the ion channel. Mg^{2+} blocks the channel directly by physically preventing ionic conductance. MK-801 binds to sites within the channel that are separate from but interact with those for divalent cations when the channel is in the open state,

preventing the passage of ions (Kemp, *et al.*, 1987). Furthermore, the binding of MK-801 is entirely dependent on the presence of an NMDA receptor agonist.
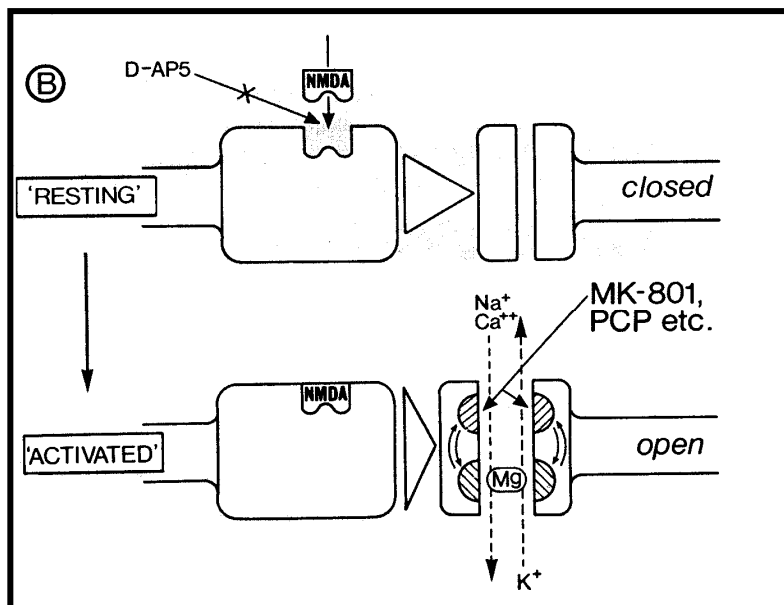
Figure 5.6. Model of the NMDA receptor complex and propsed sites of action of non-competitive antagonists (Kemp, *et al.*, 1987). Once an agonist (D-AP5) has bound the channel opens allowing antagonists such as MK-801 and PCP to bind.

5.3.2.   MATERIALS AND METHODS

5.3.2.1.   Chemicals and Reagents

Quinolinic acid (2,3-pyridinedicarboxylic acid), 17β-estradiol, N-methyl-D-aspartate (NMDA), glycine, unlabelled MK-801 and unlabelled glutamate were purchased from Sigma (St. Louis, USA). [H^3]Glutamate was purchased from Amersham Pharmacia Biotech (Buckinghamshire, England), while [H^3]-(+)-MK-801 (23.9 Ci/mmol) was purchased from NEN Laboratories (Boston, MA, U.S.A.) Scintillation cocktail was purchased from Packard (USA). All reagents were of the highest quality available.

5.3.2.2.   Animals

Adult male rats of the Wistar strain were used for this *in vitro* experiment. The animals were housed 4 per cage and cared for as described in section 2.2.2.2. In

terms of estrogen concentration, male rats are equivalent to ovariectomised females.

5.3.2.3. Preparation of Synaptic Membranes

Rats were sacrificed and their brains removed as described in section 2.2.2.4. Tissue preparation was performed according to Parsons, *et al*., 1999. The hippocampi were dissected rapidly as done before, and homoenized in 20 volumes of ice cold 0.32M sucrose using a glass-Teflon homogenizer. The homogenate was centrifuged at 1000 x g for 10 min.

The pellet was discarded and the supernatent centrifuged at 20000 x g for 20 min. The resulting pellet was re-suspended in 20 volumes of distilled water and centrifuged for 20 min at 800 x g. Thereafter the supernatent and the 'buffy coat' were centrifuged at 48 000 x g for 20 min in the presence of 50mM Tris-Hcl, pH 8.0. The pellet was then re-suspended and centrifuged four more times at 48 000 x g for 20 min in the presence of the same tris buffer. After re-suspension in 5 volumes of buffer, the membrane was frozen rapidly at -70°C.

On the day of the assay, the membranes were thawed and washed 5 times by re-suspension in 50mM Tris-HCl, pH 8.0 and centrifugation at 48 000 x g for 20 min and finally resuspended in 50mM tris-HCl, pH 7.4. The amount of protein in the final membrane preparation (250-500µg/ml)was determined as in section 2.2.2.8.

5.3.2.4. Receptor Binding Studies

5.3.2.4.1. Glutamate Binding Studies

Glutamate receptor-displacement studies were repeated in a different way to that described in the previous experiment and were modified from Parsons, *et al*., 1999. For these binding assays, vials contained 5nM [H^3]-Glu, glycine (100µm) and 0.1-0.25 mg protein (total volume 0.5ml), and various concentrations of the agents tested ie. Premarin or QUIN. Non-specific binding was defined by the addition of 1mM unlabeled glutamate.

The tubes were vortexed and left to stand at room temperature for 60 min. Thereafter, the reaction was terminated by rapidly adding 5ml of ice-cold 50mM Tris-HCl, pH 7.4 buffer to the vial and then rapidly filtering through Whatman GF/C filters under negative pressure. A millipore sampling manifold was linked to a vacuum pump to ensure that the sample was filtered very rapidly. The filters were washed a further three more times with 5ml of the same ice cold buffer under a constant vacuum. The filters were placed into scintillation vials containing 5ml Hewlett Packard scintillation cocktail and left overnight before measuring the radioactivity in a Beckman Liquid Scintillation Counter. Results were expressed as % specific [H^3]-Glu bound.

5.3.2.4.2. Functional MK-801 Binding Assay

Incubations were started by adding 5nM [H^3]-(+)-MK-801 to vials with glycine (10μM), glutamate (10μM), and 125-250 μg protein (total volume 0.5ml) and various concentrations of the agents tested ie. Premarin or QUIN. The incubations were continued at room temperature for 120 min (equilibrium was achieved under these conditions).

Non-specific binding was defined by the addition of unlabeled (+)-MK-801 (10μM). Incubations were terminated using the same millipore filter system described in 5.3.2.4.1. The samples were rinsed twice with Tris-HCl, pH 7.4 buffer over GF/C filters under a constant vacuum. The filters were placed into scintillation vials containing 5ml scintillation cocktail and the radioactivity measured after an overnight incubation period.

5.3.3.   RESULTS

The results indicate that both Premarin and QUIN decrease glutamate binding in the concentration range 10nM – 1mM (Figure 5.7.). Furthermore, the Premarin appears to increase the MK-801 binding up to a concentraiton of 1µM, while QUIN increases MK-801 binding up to a concentration of 10µM (Figure 5.8.).
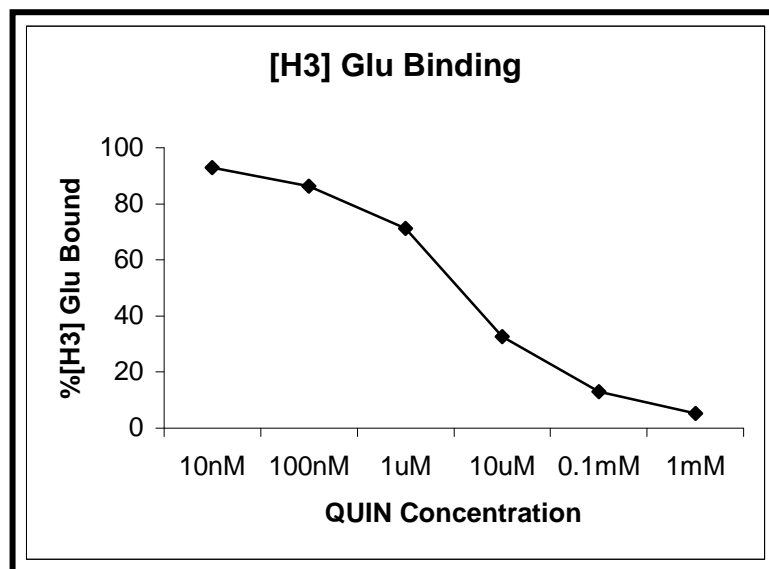
(a)
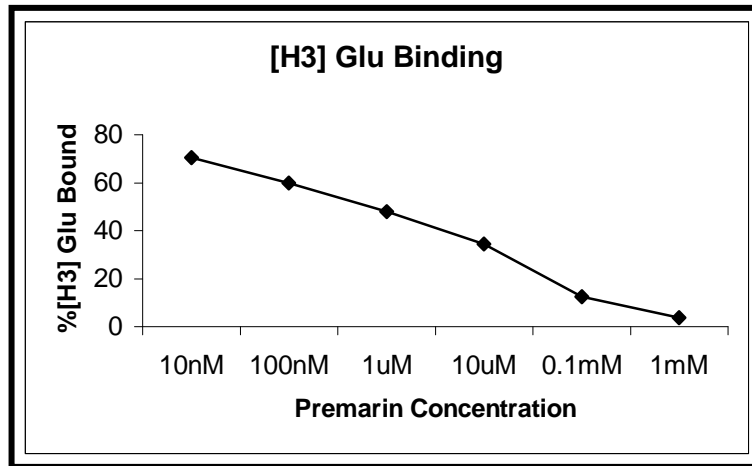
(b)

Figure 5.7. Decrease of [H3]-Glu binding to rat hippocamal membranes by a) QUIN and b) Premarin

(a)

(b)
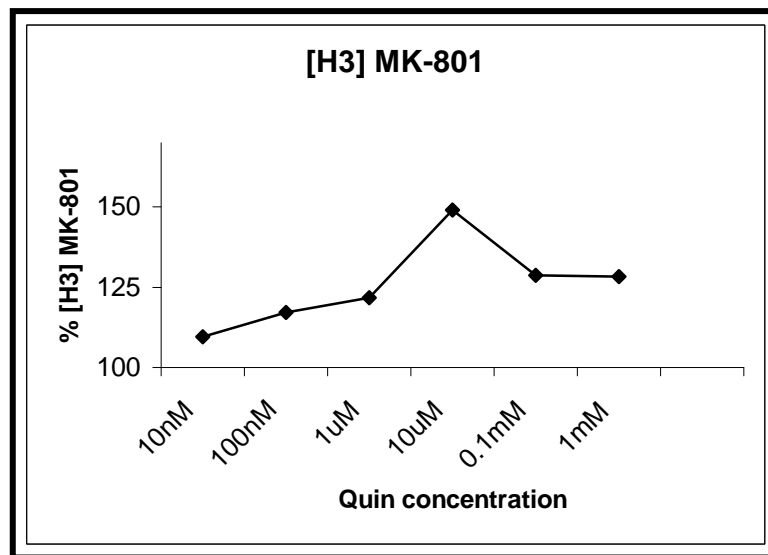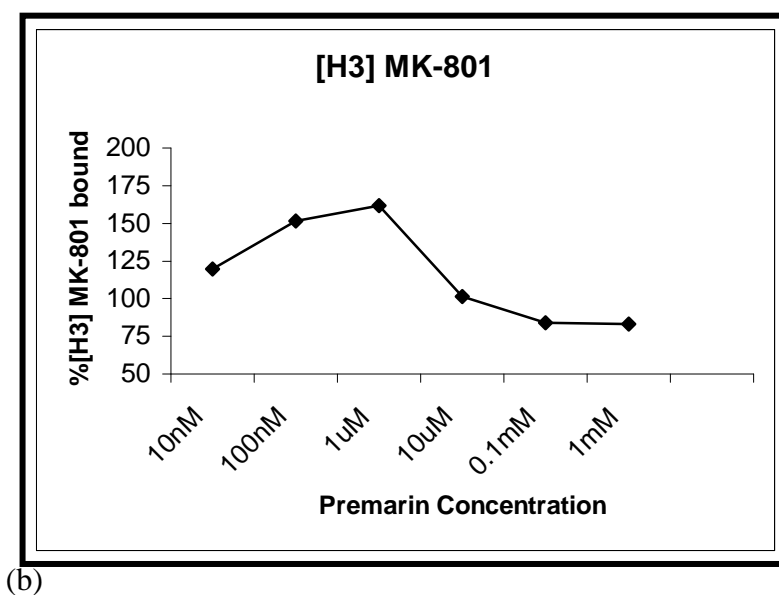
Figure 5.8. The enhancement of [H[3]]-MK-801 binding to rat hippocampal membranes by a) QUIN and b) Premarin

5.3.4.   DISCUSSION

The gluatmate binding studies were repeated in this study for two main reasons. Firstly, the receptor displacement studies performed in experiment 5.2. were performed with 17β-estradiol as a test compound. This form of estrogen is not water soluble at all, and only dissolves in very high concentrations of ethanol. It has been reported that ethanol acts as an antagonist at the NMDA receptor. Secondly, even though the presence of ethanol was taken into account in the previous study by including it in the control, it was decided that it would be interesting to test the effect of a different form of estrogen, namely Premarin, which so many postmenopausal women worldwide are taking. This drug is also easy to work with since it is highly water soluble. It should be noted that the form of Premarin® that is used is the injectable form. This preparation, unlike the tablets, is suitable to use for *in vitro* studies since no enzymes are needed to break it down to form an active compound.

From the results, conjugated estrogens cause a significant increase in MK-801 binding. Since this agent only binds to the receptor when the channel is open, this compound could possibly be acting as a competitive agonist or positive modulator

at the NMDA receptor. In addition, this estrogen preparation increases the amount of MK-801 bound to roughly the same level as that by QUIN but at a ten fold lower concentration. This would indicate that Premarin® is more effective at increasing the MK-801 binding than QUIN and it is possible that estrogen binds with a higher affinity that QUIN to the recptor.

5.4.   CONCLUSION

The results of the experiments presented in this chapter show that 17β-estradiol offers protection against QUIN-induced neurotoxicity via a NMDA receptor-dependent mechanism. It appears that estradiol is acting as a competitive agonist and causes a reduction in QUIN binding to the receptor. In this way estradiol protects against QUIN-induced neuronal degeneration.

These results are in accordance with numerous reports linking a role for estrogen through the NMDA receptor in the brain. Gazzaley *et al*, 1996 have shown that 17β-estradiol increases dendritic spine density and synapses on pyramidal cells via a mechanism dependent on NMDA receptor activation. From the present report, estradiol appears to act directly on NMDA receptors. Previously it was not known whether estrogen acts directly on the receptors or indirectly via second messenger processes that in turn influence NMDA receptor/channel processes.

Weaver *et al*, 1997, reported that 17β-estradiol protects against NMDA-induced excitotoxicity by direct inhibition of NMDA receptors. However, the effects of estradiol on NMDA receptor-mediated activity are excitatory and at high concentrations induced seizure activity (Foy *et al*, 1999). Thus estradiol appears to activate the receptor. Furthermore, in the hippocampus, estradiol treatment may influence learning behaviours and seizure activity by altering the sensitivity of hippocampal neurons to glutamate activation (Weiland, 1992). This is supported by the findings of the present study which demonstrates that 17β-estradiol is able to displace [H^3]-Glutamate from the NMDA receptor.

The induction of new spine synapses on CA1 pyramidal neurons is blocked by concurrent administration of NMDA receptor antagonists such as MK-801 (McEwen, 1998). Estradiol has been reported to enhance NMDA receptor-mediated currents and promotes an enhancement of Long-term potentiation (LTP) magnitude (Foy *et al*, 1999). NMDA antagonists will prevent LTP induction without interfering with previously potentiated transmission (Cooper *et al*, 1996).

CHAPTER 6

HEAT SHOCK PROTEIN STUDIES

6.1.   INTRODUCTION

Heat shock proteins (Hsp's) are induced by various environmental stresses, including heavy metals, ethanol, amino acid analogues and anoxia. Hsp's have basic and indispensable functions in the life cycle of proteins as molecular chaperones as well as protecting cells from deleterious stresses (Bechtold et al., 2000; Fink & Goto, 1998; Lindquist, 1986). Molecular chaperones are able to inhibit the aggregation of partially denatured proteins and facilitate their refolding to the native state. Amongst these chaperones, heat shock protein 70 (Hsp70) has received a lot of attention due to its role in the central nervous system.

Hsp70 has been shown to play a major role in protein folding of nascent chains as well as membrane transport of proteins (Hartl, 1996). Hsp70 has two major members, Hsp70, an inducible form and the heat shock cognate protein Hsc70, a constitutively expressed form. Hsp70 inducible is not expressed under unstressed conditions (Suzuki, *et al*., 1999), but is induced after brain hyperthermia. In contrast, Hsc70 expression remains unchanged. Recently, Hsc70 was found to play a role in the CNS and has been found to be localized to the synapse, indicating a synaptic role for this molecular chaperone (Ohtsuka & Suzuki, 2000).

Furthermore, Hsp70 has been shown to protect the brain and heart from severe ischemia (Carroll & Yellon, 1999; Chopp, et al., 1989). This together with its role in protein refolding, has resulted in several reports implicating Hsp70 for protection against and therapeutic treatment of inherited diseases caused by protein misfolding. Recently, the cytoprotective functions of Hsp's in stress tolerance and neurodegenerative diseases are being investigated (Ohtsuka & Suzuki, 2000).
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6.2   THE EFFECT OF CONJUGATED ESTROGEN AND QUIN ON THE LEVEL OF Hsp70 IN THE RAT HIPPOCAMPUS

6.2.1.   INTRODUCTION

Quinolinic acid has been shown to induce lipid peroxidation in the rat brain *in vivo* and *in vitro*, and as a result destroys biological membranes. Subsequently, membrane proteins are also affected. Since heat shock proteins, and in particular, Hsp70 are induced in response to any stress that perturbs protein structure, it follows that the level of Hsp70 should increase in response to QUIN-induced neurodegeneration.

The hippocampus is involved in memory formation and thus any damage to these neurons would have deleterious effects on learning and memory and could lead to various types of dementia. In view of the previous experiments, which have shown that estrogen protects against QUIN-induced neuronal damage, the present study was performed to investigate a further possible mechanism for this protection at the molecular level. The level of Hsp70 expression in hippocampal neurons, in response to treatment with QUIN alone or in combination with conjugated estrogen was analyzed using Western analysis.

Western analysis is more commonly referred to as immunoblotting. The principle of this technique is simple: protein, which is separated in a gel by means of electrophoresis, is extracted from the gel by means of diffusion, transport of solvent or electrophoresis and transferred to a solid surface. This is usually a membrane, which is permeable to molecules of the solvent and proteins. Despite the fact that the membrane is permeable, protein molecules will bind to the surface of the membrane until all binding sites are occupied. These can easily be reached by other molecules so that reactions, such as immune reactions, are possible.

6.2.2.   MATERIALS AND METHODS

6.2.2.1.   Chemicals and Reagents

Sodium dodecyl sulphate (SDS) was purchased from BDH Chemicals, Poole, England. Quinolinic acid, 17β-estradiol, N,N,N,N-tetramethylethylenediamine (TEMED), N,N-methylene-bis-acrylamide, acrylamide, Ponceau S, Monoclonal Anti-Heat Shock Protein 70 (Anti HSP70 antibody) were purchased from Sigma, St. Louis, U.S.A.   Broad range SDS-PAGE Molecular Weight Standards (pre-mixed), was purchased from BIO-RAD, California, U.S.A. Hybond-C Extra Nitrocellulose (for protein transfer) was purchased from Amersham. BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) was purchased from Roche, Indianapolis, U.S.A.

6.2.2.2.   Animals

The rats used in the experiment were cared for as described in section 2.2.2.2.

6.2.2.3.   Surgical Procedures and Dosing

Male rats were used for the experiment and were separated into 5 groups as shown in Table 6.1. The treatment regime was the same as that described in section 2.3.2.4. Stereotaxic procedures were followed as described in section 2.3.2.5.

Table 6.1.   Treatment regime

Group	Daily s.c. injection for 7 days	Intrahippocampal injection of:	Daily s.c. injection for 7 days
Untreated	-	-	-
Control	100μl PBS	2μl PBS	100μl PBS
PREM	100μg Premarin® in 100μl PBS	2μl PBS	100μg Premarin® in 100μl PBS
QUIN	100μl PBS	1μmol QUIN in 2μl PBS	100μl PBS
QA + PREM	100μg Premarin® in 100μl PBS	1μmol QUIN in 2μl PBS	100μg Premarin® in 100μl PBS

6.2.2.4. Tissue Preparation

The rats were sacrificed, the brains removed and the hippocampi dissected as described in sections 2.2.2.4. and 5.2.2.4. respectively. The hippocampi were homogenized in lysis buffer containing: 50mM Tris-HCl pH 8, 150mM Nacl, 0.02% Sodium azide, 100µg/ml phenylmethylsulphonylfluoride (PMSF), 1µg/ml Aprotinin and 1% Triton X-100. The lysates were centrifuged at 13000 x g for 20min and the supernatant carefully removed. Total protein concentration of the homogenate was determined as described in section 2.2.2.8. Equal volumes of the lysate and protein sample treatment buffer were mixed and heated for 3-5min at 95°C. Sample treatment buffer contained: 100mM Tris-HCl pH 6.8, 5% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol.

6.2.2.5. SDS-Polyacrylamide Gel Electrophoresis

The lysate was resolved on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using a Biorad PAGE apparatus. The gels were prepared according to Table 6.2. and allowed to set for 20 minutes at room temperature. Samples were loaded as in Table 6.3. and run in 1 x SDS electrophoresis buffer containing 3.02 g Tris-base, 18.8 g glycine, 2 g SDS in 1 litre. The sample was run at 80 V for 2 hours after one gel was stained with Coomassie brilliant blue stain (0.25% Coomassie Blue, 50% methanol, 7.5% glacial acetic acid) and the other gel was transferred onto nitrocellulose membrane. The gel was stained for 2-3 hours and destained in a solution containing 20% methanol and 7.5% glacial acetic acid.

Table 6.2.   Preparation of a 12% SDS PAGE Gel

Reagents	Running Gel (12%)	Stacking Gel (5%)
Distilled water	4.9 ml	4.1 ml
30% Acrylamide mix	6.0 ml	1.0 ml
1.5 M Tris-HCl, pH 8.8	3.8 ml	-
1.0 M Tris-HCl, pH 6.8	-	0.75 ml
10% SDS	0.15 ml	0.06 ml
10% Amm. Persulphate	0.15 ml	0.06 ml
TEMED	0.006 ml	0.006 ml

Table 6.3.   Loading of the samples for SDS-PAGE

Sample & Well No.		µg Protein	*Volume (µl)
MW marker	(1)	-	10
Untreated	(2)	300	10
Control	(3)	300	5
Premarin® alone	(4)	300	9
QUIN alone	(5)	300	9
Premarin® + QUIN	(7)	300	7
Neg. control	(9)	250	10
Pos. control	(10)	250	10

* The volume was calculated according to the protein concentration that was measured.

6.2.2.6.   Western Blotting Analysis

Transfer buffer containing 25mM Tris base, 192mM glycine and 20% methanol was prepared and allowed to chill until use. Sheets of 3MM Whatmann filter paper and the nitrocellulose membrane were cut to the size of the SDS PAGE gel and soaked in ice cold transfer buffer for 30min to eqilibrate. The SDS PAGE gel was carefully removed from the glass plates, the stacking cut off and the remaining gel placed in ice-cold transfer buffer to equilibrate. The sandwich for the western transfer was constructed as follows: 2 sheets of 3MM Whatmann filter paper placed on a Scotchbrite fibre pad, the nitrocellulose membrane placed on top followed by the gel and finally the other 2 sheets of Whatmann paper and the fibre pad respectively. The sandwich was placed into the transfer panel with the nitrocellulose facing the anode and the gel facing the cathode. The cooling block was inserted and the tank filled with transfer buffer. The transfer was done at 100 V for 1 hour.

At the end of the run, the sandwich was dismantled and the nitrocellulose membrane was stained with Ponceau S (0.5g Ponceau S, 1ml glacial acetic acid in 100ml water), to visualize the protein. The membrane was stained for 5min, scanned and thereafter destained by rinsing in two washes of Tris-buffered saline (TBS) pH 7.5.

The membrane was blocked with 5% non-fat milk powder in TBS and left at 4°C overnight. The block was removed and the membrane incubated with primary

antibody (Anti HSP70 antibody)(1:5000 in block) for 1 hour before washing twice in TBS each for 20 min. Thereafter, another two washes in block followed before incubating with the horseradish peroxidase conjugated secondary antibody (1:1250 in block) for 30 min. Finally, the membrane was washed four times with TBS-tween 20 for 15 min each before developing it.

All developing stages were performed in a dark room. After the last wash, the TBS-tween 20 was decanted and substrate added and left to incubate for 60 seconds. The membrane was transferred to a X-ray film cassette and cling wrap neatly placed on top. A piece of X-ray film was carefully placed on top of the cling wrap and the cassette closed tightly. The film was exposed to the blot for 15 min. Finally, the film was placed in developer for 3 min, then passed through the stop solution and transferred to the fix solution for 2min before rinsing under tap water and hung to dry.

6.2.3.   RESULTS

The Coomassie-stained gel is shown in Figure 6.1. It is evident from this that the SDS-PAGE was successful in separating out the various proteins present in the hippocampal homogenate within the molecular weight range shown.

The Ponceau S stain of the nitrocellulose membrane confirmed the successful transfer of proteins from the SDS PAGE gel to the nitrocellulose membrane.

Figure 6.3. shows the immunoblots of the western transfer. It is evident by the signals obtained, that all three treatments groups (ie. P, Q and PQ) displayed an increase in the amount of Hsp 70 in comparison to the control and the untreated group. The band obtained for PQ appears to be an added effect of both P and Q. The band for Q appears to be larger than that obtained for P. The stronger the signal, the higher the level of Hsp70 in the hippocampl tissue used.
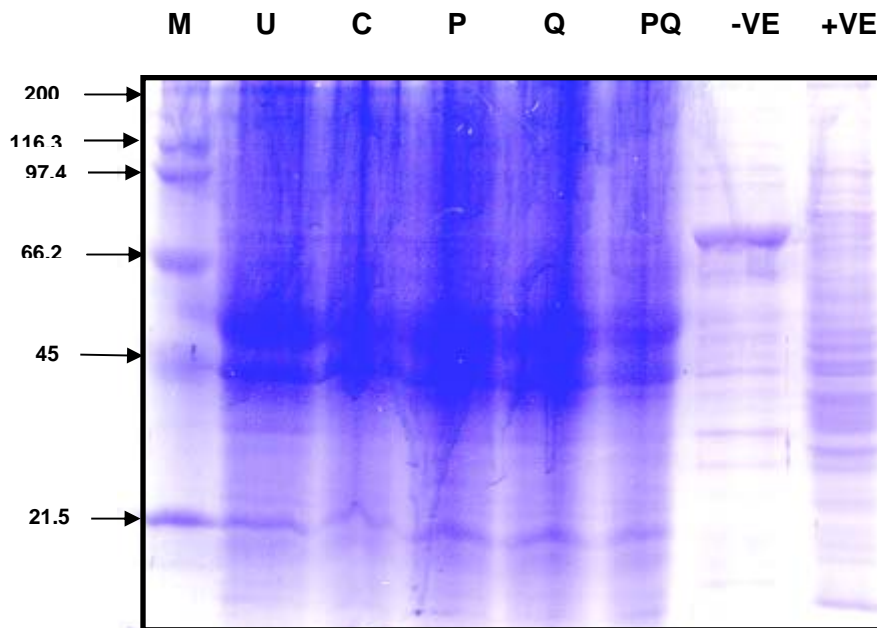
Figure 6.1. The gel stained with Coomassie stain, illustrating successful separation of proteins. (Molecular weights alongside are in kDa) M = Biorad broad range SDS PAGE enzyme molecular weight marker, U = Untreated group, P= Premarin® alone group, Q = QUIN alone group, PQ = Premarin® and QUIN combined, -VE = negative control (TC HSP70 lysate) and +VE = positive control (NIH 3T3 Mouse fibroblast cell extract)
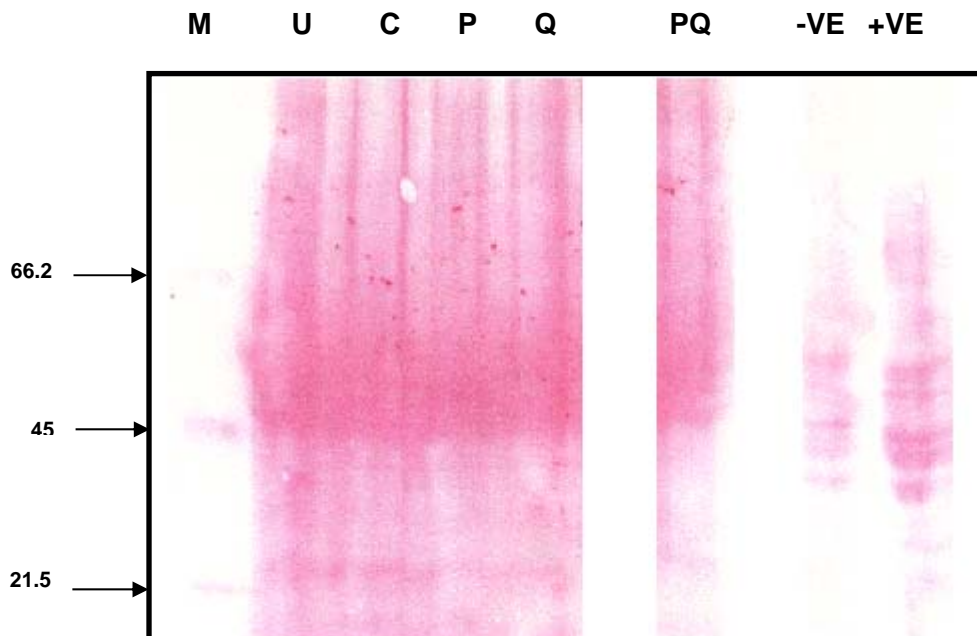
Figure 6.2. The membrane stained with ponceau stain (Molecular weights alongside are in kDa) M = Biorad broad range SDS PAGE enzyme molecular weight marker, U = Untreated group, P= Premarin® alone group, Q = QUIN alone group, PQ = Premarin® and QUIN combined, -VE = negative control (TC HSP70 lysate) and +VE = positive control (NIH 3T3 Mouse fibroblast cell extract)

M   U   C   P   Q   PQ   -VE +VE
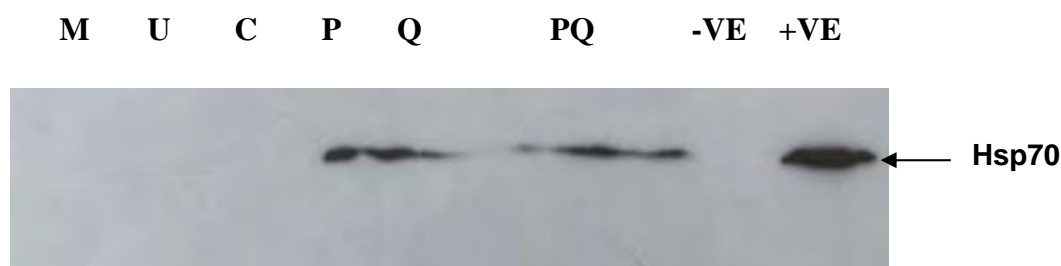
← Hsp70

Figure 6.3.   Western analysis to detect Hsp70/Hsc70 in the various treatment groups. M = Biorad broad range SDS PAGE enzyme molecular weight marker, U = Untreated group, P= Premarin® alone group, Q = QUIN alone group, PQ = Premarin® and QUIN combined, -VE = negative control (TC HSP70 lysate) and +VE = positive control (NIH 3T3 Mouse fibroblast cell extract)

6.2.4.   DISCUSSION

The results of the western blotting showed that the level of Hsp70 in the hippocampus increased in response to treatment with QUIN. This was expected since Hsp70 is expressed when the cell is under stress such as that resulting from free radical attack, which is initiated by QUIN-induced damage. However, the level of Hsp70 also increased in response to treatment with conjugated estrogen. Unfortunately it was not possible to quantify the exact level of expression of this chaperone protein, but from the thickness of the band it appears as though QUIN showed a higher level of expression that that induced by estrogen. Furthermore, the antibody that was used was unable to indicate whether the Hsp70 induced by the various groups, were that for Hsp70 inducible or the Hsc70 constitutive form, since the antibody detects both forms of this chaperone. The group treated with both QUIN and estrogen showed a level of expression of Hsp70 that was an added effect of both individually obtained.

It is surprising that the control animals did not show any expression of Hsp70 since stress can be induced from the handling of the animals and the operating procedures. However, an explanation for this could be that Hsp70 was indeed expressed during these stressful times for the animals, but that the levels declined from the time of operating to the time of sacrifice, which was 7 days.

From the results presented in this chapter, it could be argued that estrogen may induce the stress response through its action on a mitogen or carcinogen. Mitogenesis could

be deemed a stress to normally quiescent brain cells. Estrogen is involved in cancer, which is estrogen receptor-dependent (Yager & Liehr, 1996), and is a definite risk factor in breast cancer (Safe, 1998). Moreover, Hsp70 is induced in response to cellular growth and differentiation and Smith, et al, 1998 reported that an overexpression of Hsp's in breast and ovarian cancers occurs. Estrogen has also been reported to stimulate nerve regeneration (Tanzer, et al., 1999), in which case cells would be dividing and there would be an increase in protein synthesis. Thus a proposed mechanism for the involvement of nerve regeneration could be through the increased expression of Hsp70.

Another possibility for the increased expression of Hsp70 in response to estrogen treatment is that the result obtained actually represents Hsc70 expression. Hsc70 is the constitutively expressed form of Hsp70. Several lines of evidence support this hypothesis. Firstly, Krebs, et al, 1999 reported that Hsc70 is induced by ovarian hormones in the ventromedial hypothalamus. Secondly, Hsc70 chaperone protein has been reported to be involved in synaptic plasticity-related events; an electroconvulsive seizure induced Hsc70 mRNA but not Hsp70 (Kaneko, *et al*., 1993) and these authors also reported that Hsc70 could also be induced by synaptic activation. Chaperones localized at postsynaptic sites (including Hsc70) are involved in the mechanisms that activate AMPA receptors in the synapse during induction of LTP (Song, et al., 1998). Furthermore, Hsc70 is fundamental to synaptic transmission and plays a role in local protein synthesis at postsynaptic sites (Frydman, et al., 1994), which is essential for the maintenance of the already expressed synaptic plasticity, such as LTP and the consolidation process of memory (Tiedge & Brosius., 1996; Torre & Steward, 1992).

17β-estradiol increases synaptic excitability by enhancing the magnitude of AMPA receptor-mediated responses and the rapid onset of this increased excitability supported a postsynaptic membrane site of action for this hormone (Wong & Moss, 1992). Foy, et al., 1999 then reported that estradiol increases synaptic transmission in the hippocampus and enhances LTP in CA1 neurons of rats. In addition, the enhancement of LTP after acute 17β-estradiol application is due to the activation of NMDA receptor channels or an increase in AMPA receptor function.

6.3.   CONCLUSION

It may be possible that estrogen induces the expression of both forms of Hsp70, but that the level of Hsc70 that is expressed in response to estrogen is much higher than the inducible form. The reason for this rationale stems from the fact, that although estrogen is implicated in stress in the form of cancer, it is also a potent neuroprotectant, and thus if it was causing stress to such an extent then one wouldn't observe the protective effects against agents such as QUIN and the results seen in this chapter would then represent that for the inducible Hsp70.

 However, it is interesting that various reports have given evidence suggesting similar roles for both hsc70 and estrogen in the enhancement of synaptic transmission as well as the stimulation of LTP. Subsequently, one could postulate that estrogen's effects on both synaptic transmission and LTP could occur via an indirect mechanism, involving Hsc70. In other words, estrogen may increase the expression of Hsc70, which in turn stimulates LTP and enhances synaptic transmission.

CHAPTER 7

SUMMARY OF RESULTS

CHAPTER 2

The effect of 17β-estradiol on the levels of QUIN-induced lipid peroxidation was investigated in the rat brain *in vivo* and *in vitro*. In addition, the effect of a conjugated form of estrogen (Premarin®) on the level of QUIN-induced lipid peroxidation *in vivo* was studied. Both forms of estrogen were found to significantly reduce lipid peroxidation induced by QUIN *in vivo* and *in vitro*.

CHAPTER 3

The effect of Premarin® on the formation of the QUIN-iron (II) complex was studied by UV/VIS. Electrochemistry and NMR were techniques employed to further characterize any possible interactions between estradiol and iron (II). Finally, computer modelling was performed to illustrate the site that iron (II) would most likely form an association with estradiol. The results indicate an interaction between estradiol and iron (II), but that this is an unstable complex.

CHAPTER 4

Microscopic examination of hippocampal neurons was performed following treatment with QUIN alone or in combination with 17β-estradiol. Estradiol was shown to protect cells in both the CA1 and CA3 regions of the hippocampus from QUIN-induced neurodegeneration and swelling.

CHAPTER 5

Radiolabelled glutamate saturation binding studies were performed. QUIN was shown to decrease the affinity of glutamate to bind to the NMDA receptor as well as the number of binding sites. 17β-estradiol, however, increased the affinity of glutamate to bind. Receptor displacement studies showed that both estradiol and QUIN reduced glutamate binding to the NMDA receptor. By performing a functional MK-801 assay, estradiol was shown to act at the NMDA receptor as an agonist or positive modulator.

CHAPTER 6

Western blotting was performed to investigate the effects of both QUIN and Premarin® on the levels of expression of HSP70. From the results obtained it was not possible to differentiate between the inducible and the constitutive form of HSP70. However, increased expression of HSP70 (representing both forms of the heat shock protein) was found in response to both QUIN and Premarin®.

CHAPTER 8

CONCLUSION

The results of this study illustrate the neuroprotective effects of estrogen against QUIN-induced neurodegeneration. Initial studies conducted to investigate the effect of estradiol on lipid peroxidation induced by QUIN, demonstrated that estradiol significantly decreases lipid peroxidation. The significance of this is obvious when considering that damage to any plasma membrane, albeit the cell or mitochondrial membrane, results in a disruption of membrane fluidity and damage to proteins. The production of ATP may also be affected. Thus the effects of lipid peroxidation may be detrimental to the cell. Furthermore, neuronal damage due to oxidative stress has been implicated in several neurodegenerative disorders, in which case estradiol may be a therapeutic advantage.

Iron (II) plays an important role in accelerating lipid peroxidation and is itself a free radical. QUIN-induced lipid peroxidation has been reported to be dependent on iron (II). The mechanism of protection elicited by estradiol against this damage may be due to an interaction with iron (II) thus rendering this metal unavailable to cause further damage. Results demonstrate the existence of an interaction, however, the complex formed is not stable.

QUIN is an endogenous agonist at the NMDA receptor, which when activated results in excessive calcium influx into the cell resulting in neuronal swelling. Histological investigation of hippocampal neurons illustrates that estradiol protects these neurons against QUIN-induced damage. The photomicrographs also demonstrate the ability of estradiol to enhance axonal sprouting of hippocampal neurons. In addition, the mechanism behind this protection is NMDA receptor-dependent. Estradiol was shown to activate the NMDA receptor and thus it could be stated that it acts as an agonist or positive modulator. Estradiol's action on this receptor could explain the stimulation of LTP and the enhancement of dendritic spine density by estradiol treatment.

An explanation of the observed enhancement of expression of HSP70 by estradiol treatment could be twofold. Either estradiol increases the level of expression of Hsp70 (inducible), which may occur due to estrogen's known involvement in cancer. However, in light of the experiments showing estrogen's protective effects against QUIN-induced damage, it is not likely to be significantly large. It may be possible that the signal observed for the western blotting in response to estrogen treatment largely represents Hsc70. This cognate protein enhances synaptic transmission and stimulates LTP, which is also observed following administration of estrogen. Thus it may be possible that estrogen elicits these effects on the brain via a mechanism dependent on Hsc70 expression.

In conclusion, estrogen is a potent antioxidant, able to significantly reduce oxidative stress. However, further mechanisms are responsible for the protection against the neurotoxin, QUIN. This includes the ability of this hormone to interact with iron (II) as well as to act on the NMDA receptor thus competing with QUIN to bind. Finally, a different approach in the protective nature of estrogen involves the increased expression of Hsp70. Several actions elicited by estrogen in the brain could be via the actions of Hsc70.

APPENDICES
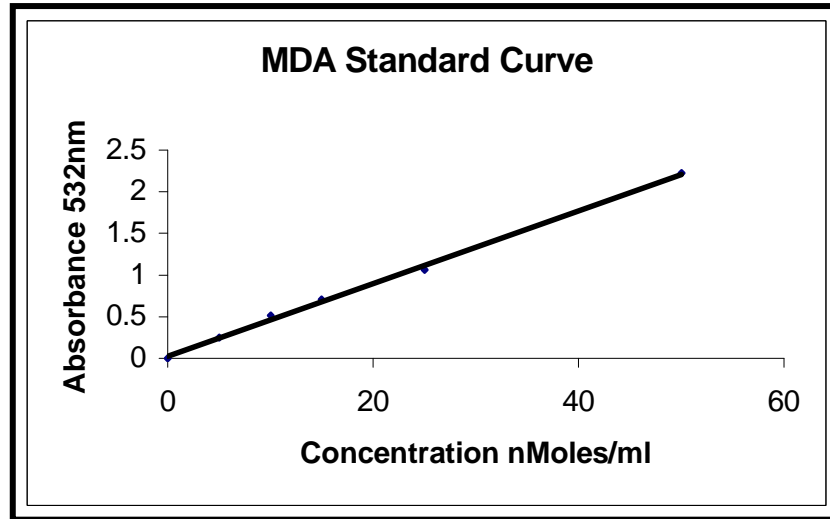
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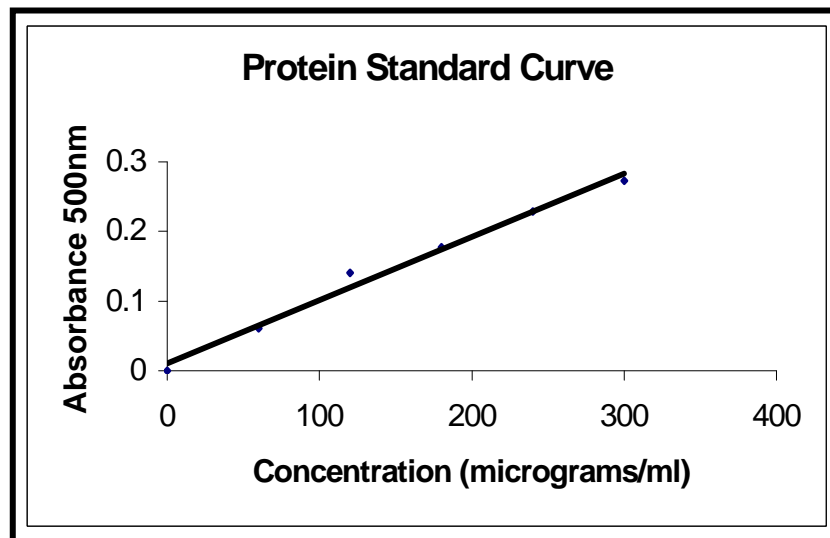
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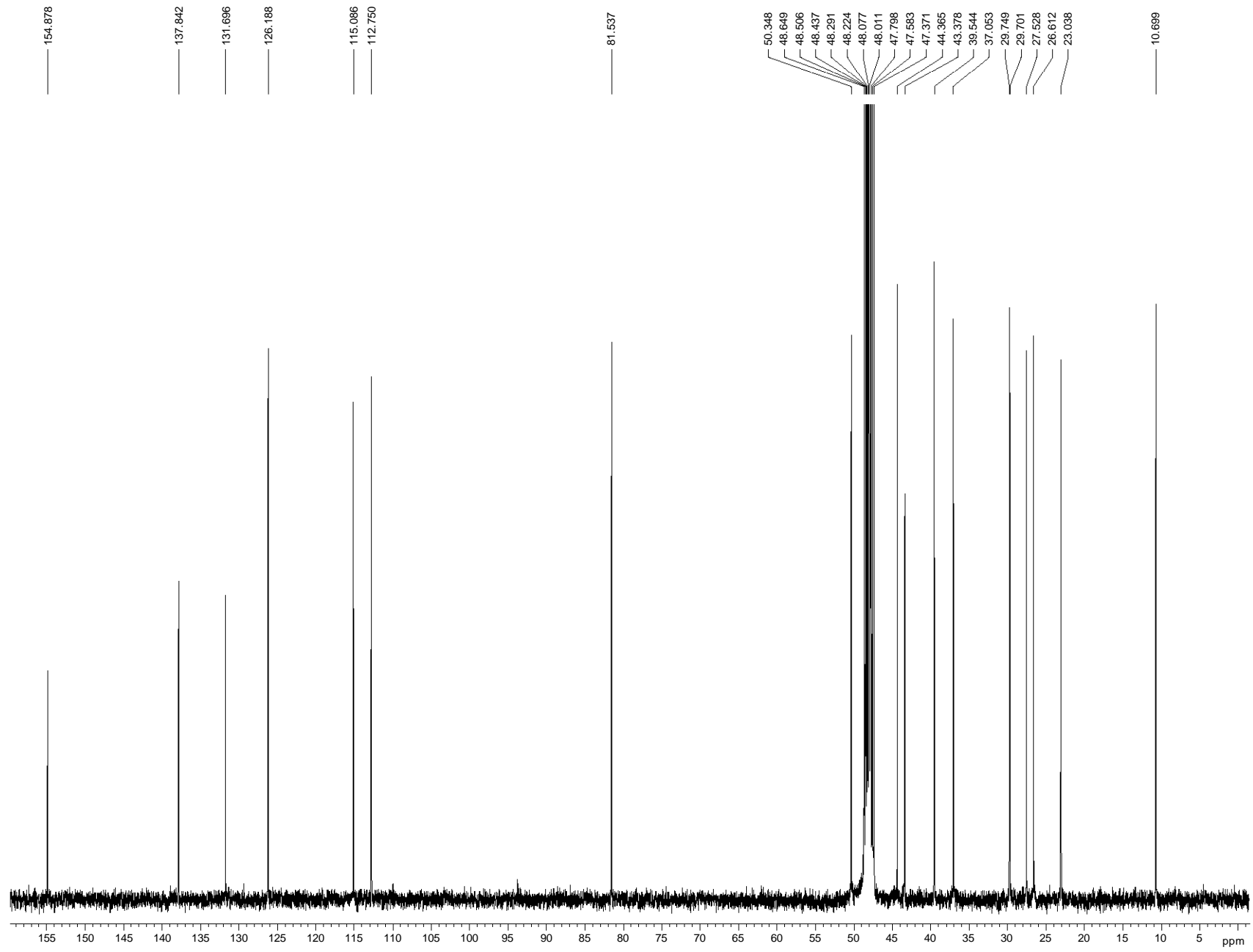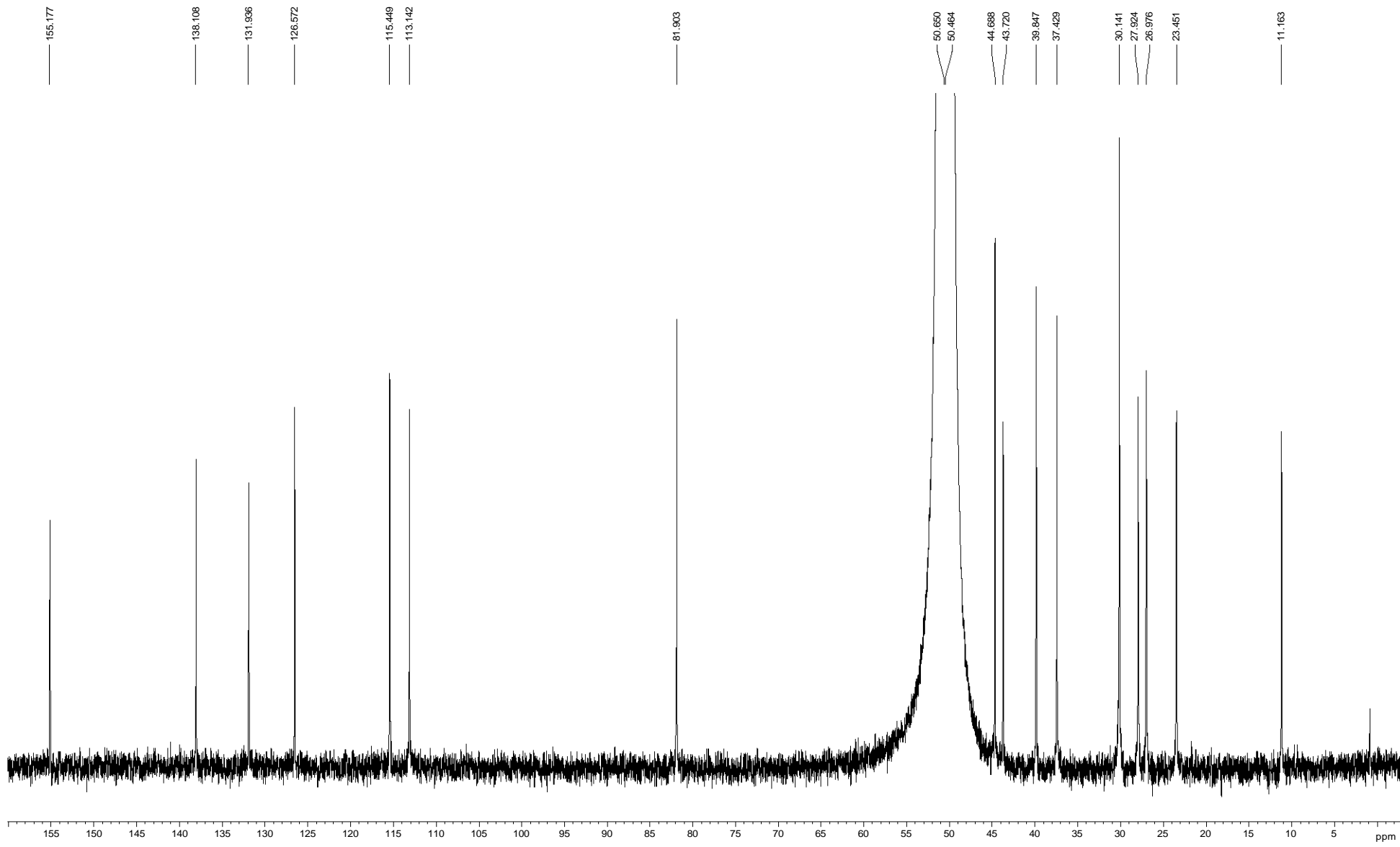
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