AN INVESTIGATION INTO THE NEUROPROTECTIVE EFFECTS OF ESTROGEN AND PROGESTERONE IN A MODEL OF HOMOCYSTEINE-INDUCED NEURODEGENERATION

THESIS

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Wing Man Wu

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'The brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brain's woven feltwork can be mapped. In short, the brain can be studied, just as the kidney can.'

By David H. Hubel
(1981 Nobel Prize Winner)

ABSTRACT

Homocysteine (Hcy) is a sulfur containing amino acid and is a potent neurotoxin. It has been shown that elevated levels of Hcy, termed hyperhomocysteinemia, plays a role in the pathologies of Alzheimer's disease (AD) and age-related cognitive decline. Hcy is a glutamate agonist, which causes in increase in Ca^{2+} influx via the activation of NMDA class of excitatory amino acid receptors, which results in neuronal cell death and apoptosis.

Estrogen and progesterone are female hormones that are responsible for reproduction and maternal behaviour. However, in the last decade, it is evident that both female hormones have neuroprotective properties in many animal models of neurodegeneration. Collectively, both estrogen and progesterone reduce the consequences of the oxidative stress by enhancing the antioxidant defence mechanisms, reducing excitotoxicity by altering glutamate receptor activity and reducing the damage caused by lipid peroxidation. However, the mechanisms by which estrogen and progesterone provide such neuroprotection probably depend on the type and concentration of hormone present. Moreover, numerous studies have shown that hormone replacement therapy (HRT, estrogen and progestins) or estrogen-only replacement therapy (ERT) may prevent or delay the onset of AD and improve cognition for women with AD. Clinical trials have also shown that women taking HRT may modify the effects of Hcy levels on cognitive functioning.

Oxidative stress increases in the aging brain and thus has a powerful effect on enhanced susceptibility to neurodegenerative disease. The detection and measurement of lipid peroxidation and superoxide anion radicals in the brain tissue supports the

involvement of free radical reactions in neurotoxicity and in neurodegenerative disorders.

The hippocampus is an important region of the brain responsible for the formation of memory. However, agents that induce stress in this area have harmful effects and could lead to dementia. This study aims to investigate and clarify the neuroprotective effects of estrogen and progesterone, using Hcy-induced neurodegenerative models. The initial studies demonstrate that estrogen and progesterone have the ability to scavenge potent free radicals. Histological studies undertaken reveal that both estrogen and progesterone protect against Hcy-induced neuronal cell death. In addition, immunohistochemical investigations show that Hcy-induced apoptosis in the hippocampus can be inhibited by both estrogen and progesterone. However, estrogen also acts at the NMDA receptor as an agonist, while progesterone blocks at the NMDA receptor. These mechanisms reduce the ability of Hcy to cause damage to neurons, since Hcy-induced neurotoxicity is dependent on the overstimulation of the NMDA receptor.

SOD and GPx are important enzymatic antioxidants which can react with ROS and neutralize them before these inflict damage in the brain. Hcy can increase oxidative stress by inhibiting expression and function of these antioxidants. However, it has been shown that the antioxidant abilities of both estrogen and progesterone can up-regulate the activities of SOD and GPx. These results provide further evidence that estrogen and progesterone act as antioxidants and are free radical scavengers.

The discovery of neuroprotective agents is becoming important as accumulating evidence indicates the protective role of both estrogen and progesterone in

Hcy-induced neurodegeneration. Thus further work in clinical trials is needed to examine whether reducing Hcy levels with HRT can become the treatment of neurodegenerative disorders, such as Alzheimer's disease.

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LIST OF SYMBOLS AND ABBREVIATIONS

AD -- Alzheimer's Disease
AMPA -- α-2-amino-3-hydroxy-5-methylisoxazole-4-proprionate
ANOVA -- Analysis of variance
AP5 -- 2-amino-5-phosphonovalerate
ApoE -- Apolipoprotein E
APP -- Aβ precursor protein
ATP -- Adenosine tri-phosphate
Aβ -- Amyloid β peptides
BHT -- Butylated hydroxytoluene
B_{max} -- Maximum Number of Binding Sites
BSA -- Bovine Serum Albumin
Ca^{2+} -- Calcium (II)
CA1 -- Cornu Ammonis *1*
CA3 -- Cornu Ammonis *3*
CAT -- Catalase
Cl^- -- Chloride
CNS -- Central nervous system
CPM -- Counts per minute
CSF -- Cerebrospinal fluid
Cu^{1+} -- Copper (I)
Cu/Zn-SOD -- Copper-zinc superoxide dismutase
CβS -- Cystathionine β-synthetase
DNA -- Deoxyribonucleic Acid
EAA -- Excitatory amino acid
EDTA -- Ethylenediaminetetraacetic acid
ER -- Endoplasmic reticulum
ERs -- Estrogen receptor receptors
ERT -- Estrogen replace therapy
Fe^{2+} -- Iron (II)
Fe^{3+} -- Iron (III)
GABA -- γ-aminobutyric acid
GPx -- Glutathione peroxidase
GSH -- Reduced Glutathione
GSSG -- Oxidized Glutathione
H_2O -- Water

H_2O_2 -- Hydrogen peroxide
Hcy -- Homocysteine
HCl -- Hydrochloric acid
HRT -- Hormones replace therapy
K^+ -- Potassium
K_D -- Dissociation constant
LDL -- Low-density lipoprotein
M -- Molar
MAPK -- Mitogen activated protein kinase
MDA -- Malondialdehyde
mg/Kg -- Mlligram per kilogram
Mg^{2+} -- Magnesium (II)
min --Minute
MK-801-- dibenzocycloheptеneimine
ml -- Millilitre
mM -- Millimolar
mRNA -- Messenger ribonucleic acid
Na^+ -- Sodium
NADP -- Nicotinamide Adenine dinucleotide phosphate
NADPH -- Reduced nicotinamide adenine dinucleotide
NBD -- Nitro-blue diformazan
NBT -- Nitro-blue tetrazolium
NGF -- Nerve growth factor
NMDA -- N-methyl–D-aspartate
$NO^{\cdot}$ -- Nitric oxide
NSAID -- Nonsteroidal anti-inflammatory drugs
O_2 -- Oxygen
$O_2^{\cdot}$ -- Superoxide radical
$O_2^{\cdot-}$ -- Superoxide anion radical
$^{\cdot}OH$-- Hydroxyl radical
$ONOO^{\cdot}$ -- Peroxinitrite
P5P -- Pyridoxal 5'-phosphate
PARP --Poly-(ADP-ribose) polymerases
PBS -- Phosphate buffered saline
PCP -- Phencyclidine
PD -- Parkinson Disease
PUFA -- Polyunsaturated fatty acids
ROS -- Reactive oxygen species

SAH -- S-adenosylhomocysteine hydrolase
SAM -- S-adenosylmethionine
SOD -- Superoxide dismutase
TBA --Thiobarbituric acid
TBRAS -- Thiobarbiturate reactive substrances
TCA -- Trichloroacetic acid
TdT -- Terminal deoxynucleotidyl transferase
Tris -- Tris (hydroxymethyl)-aminomethane
TUNEL -- TdT-mediated dUTP nick end labeling
USA -- United States of America
UV/Vis -- Ultraviolet/Visible Spectroscopy
WHI -- Women's Health Initiative
Zn^{2+} -- Zinc
µg -- Microgram
µM -- Micromolar
µmol/L -- Micromole per litre
µl -- Microlitre
˚C -- Degrees celcius

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CHAPTER 9

CONCLUSION

The results of this study illustrate the neuroprotective effects of pre- and post-treatments of 17β-estrogen and progesterone against Hcy-induced neurodegeneration. Initial studies were conducted to investigate the effects of estrogen and progesterone of both regimes on lipid peroxidation induced by Hcy. These results demonstrate that estrogen and progesterone significantly decrease lipid peroxidation *in vitro* and *in vivo.* The effects of lipid peroxidation may be harmful to plasma membrane of the cells, resulting in damage to membrane proteins and disturbance of membrane fluidity. However, it has been shown that high Hcy concentrations, is responsible for the production of oxygen free radicals and a consequent increase in lipid peroxidation (Heinecke, 1988), which is an implication in several neurodegenerative disorders. Estrogens and progesterone have been shown to be potent inhibitors of lipid peroxidation; therefore, both hormones may be a therapeutic advantage to Hcy-induced neurodegeneration.

Hcy is a sulphur-containing amino acid and auto-oxidation of Hcy results in generation of $O_2^{\bullet -}$ in the brain and which initiates lipid peroxidation. Therefore, the neuroprotective effects of estrogen and progesterone as antioxidants against Hcy-induced $O_2^{\bullet -}$ were investigated. The antioxidant properties of estrogen and progesterone allow them act as $O_2^{\bullet -}$ scavengers and thus cause a significant decrease in Hcy-induced $O_2^{\bullet -}$. However, these results correlate with the abilities of both

hormones to reduce of Hcy-induced lipid peroxidation, shown in the first part of this study.

Hcy is an endogenous ligand and an agonist at the glutamate site of the NMDA receptor and causes Ca^{2+} influx via the stimulation of NMDA receptor, this results in neuronal cell death and apoptosis. Histological investigation of hippocampal neurons demonstrates that Hcy causes neuronal cell swelling and both estradiol and progesterone are able to protect these neurons. Moreover, the Hcy-induced neuronal cell damage was investigated further by the detection of apoptotic cell death. These photomicrographs demonstrate that estradiol and progesterone of both treatment regimes protect the neurons against Hcy-induced apoptosis in both CA1 and CA3 regions of the hippocampus. However, the cell damage induced by Hcy is dependent on the NMDA receptor. Hcy results in a reduction in the number of binding sites. However, treatment with either 17β-estradiol or progesterone prior to and after the intrahippocampal injection of Hcy results in up-regulation in the number of binding sites of the receptor. Therefore, the protection of either hormone against Hcy-induced damage is mediated by an interaction with the NMDA receptor.

Prevention of apoptosis by antioxidants indicates that oxidative stress plays an essential role in the neurotoxic effects of Hcy. Hcy can increase oxidative stress by inhibiting expression or function of key antioxidant enzymes such as SOD and GPx, which might potentiate the toxic effects of oxidative stress. However, both estradiol and progesterone are able to increase both SOD and GPx activities which are suppressed by Hcy. Thus, the ability of both hormones to modify the antioxidant defence mechanism of SOD and GPx, in the presence of Hcy provides further evidence that 17β-estradiol and progesterone act as antioxidants and free radical

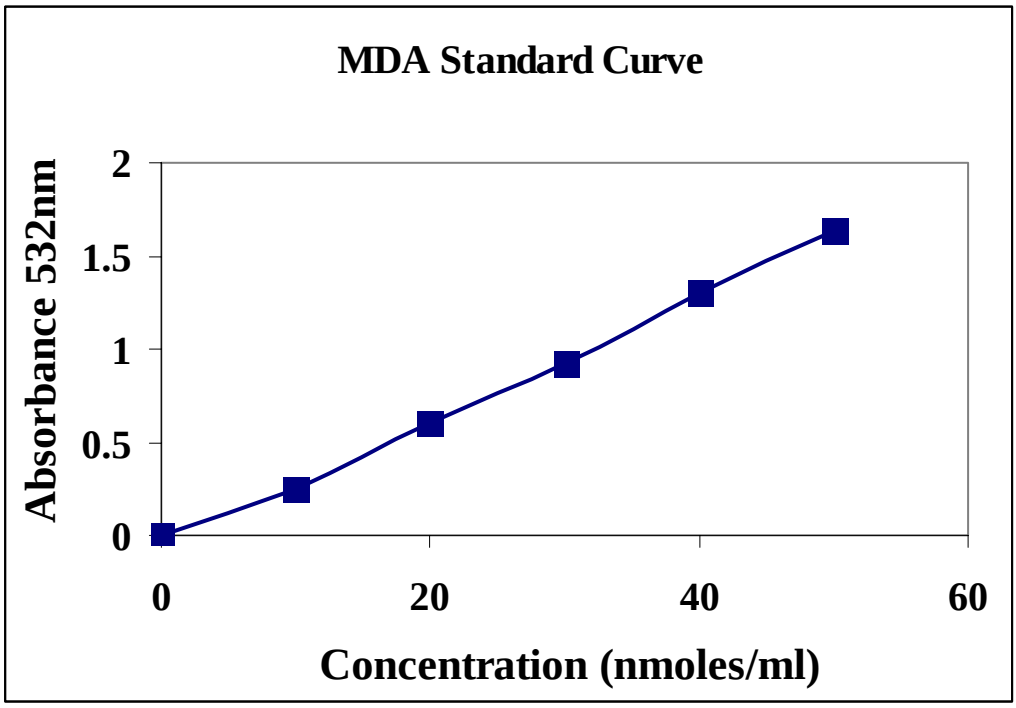
scavengers.

17β-estradiol and progesterone are potent antioxidants and free radical scavengers, and are able to reduce Hcy-induced oxidative stress significantly. However, the neuroprotective mechanisms of 17β-estradiol and progesterone can be multifactorial. This includes the ability of both hormones to reduce lipid peroxidation and formation of superoxide anion radicals induced by Hcy. Finally, both hormones act on the NMDA receptors thus up-regulating the number of binding sites. It is evident that these hormones appear to be effective at protecting neurons from Hcy-induced neuronal damage investigated in the histological studies in chapter 4.

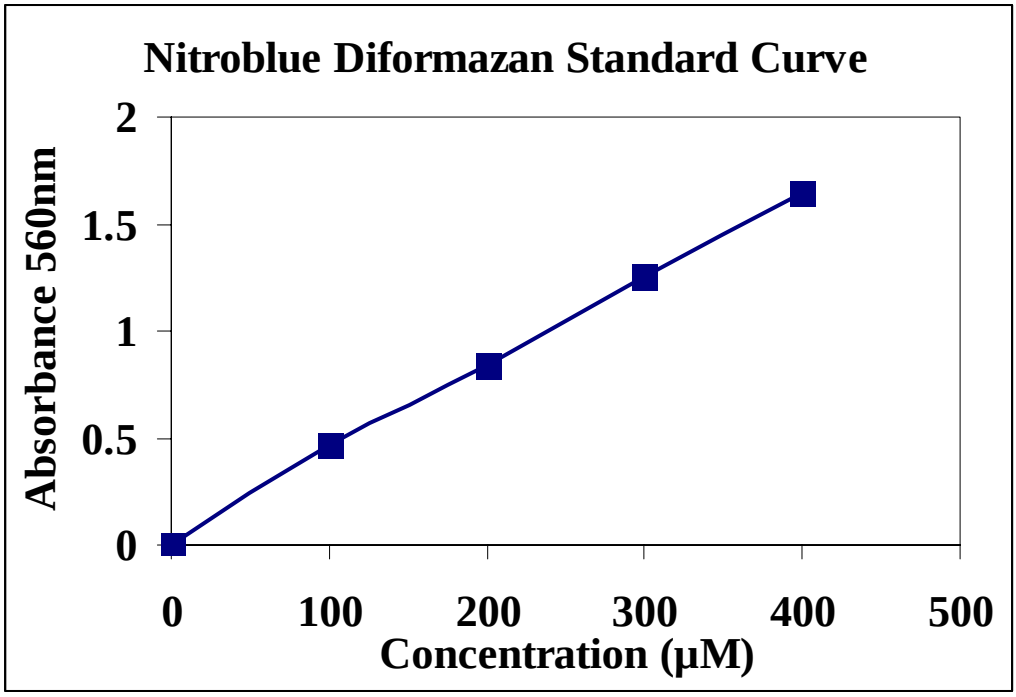
The neuroprotective effects of estradiol and progesterone may lead to an improved understanding of the mechanisms of several neurodegeneration diseases, such as Alzheimer's disease, and to the identification of novel pharmacological neuroprotective strategies. As a result of this thesis from the following areas have been identified as possible questions which need to be understood and future studies:

1. Further research is needed to determine the clinical efficacy of both female hormones in neuroprotection.
2. Reduction of the level of Hcy by estradiol, either indirectly by its effect on gene expression or directly by effects on Hcy synthesis might account for the neurodegeneration induced by Hcy.
3. Age is associated with a decreased antioxidant capacity of the brain. The possibility of a combination of female hormone therapy with antioxidant therapy, such as Vitamin C and E, may lead to a new therapeutic implication for AD.

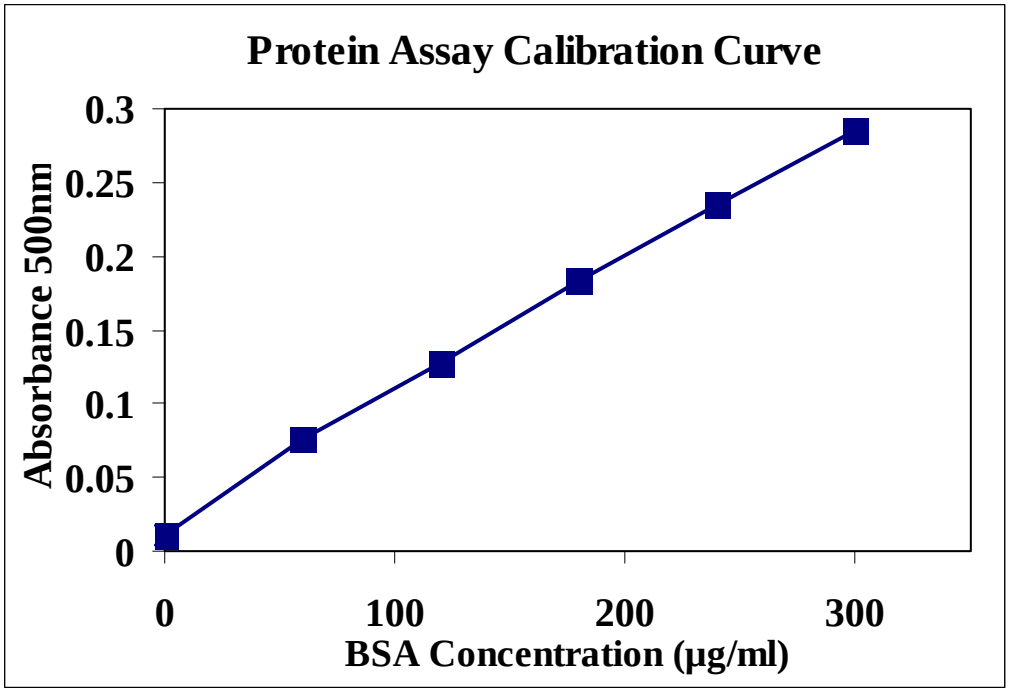
APPENDICES



Appendix 1 MDA (malondialdehyde) standard curve
($y=0.0333x - 0.0484$, $R^2=0.9972$)



Appendix 2 Nitroblue Diformazan Standard Curve
($y=0.0041x + 0.0256$, $R^2=0.9988$)



Appendix 3 Protein Standard Curve generated from BSA ($y=0.0009x + 0.0171$, $R^2=0.9986$)

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CHAPTER 1

LITERATRUE REVIEW

1.1. Neuroanatomy

1.1.1. Introduction

Neuroscience is a field of study which deals with the structure, function, development, genetics, biochemistry, physiology, pharmacology and pathology of the nervous system. The study of behavior and learning is also a division of neuroscience. Over the years, neuroscientists have shown increasing interests into the pathophysiology and etiology of neurodegenerative disorders. This has led to the current research of neuroprotective agents for therapeutic intervention.

The population of the world is aging. By the year 2010, the proportion of people aged more than 65 years will have increased by 30%. Thus, an increasing number of people will experience significant age-related cognitive decline, stroke and late-onset neurodegenerative disorders, such as Alzheimer's disease (AD).

Increasing evidence has demonstrated striking sex differences in the pathophysiology of these age-related disorders. Lesser susceptibility to post-ischemic and post-traumatic brain injury in females has been observed in experimental models. Additional evidence suggests this gender difference extends to human as well. The greater neuroprotection afforded to females is likely due to the effects of circulating female hormones, estrogen and progesterone. However, most patients being treated

for menopausal symptoms will be receiving progesterone in addition to estrogen. The exogenous administration of both hormones has been shown to improve outcome after cerebral ischemia and traumatic brain injury in experimental models. The mechanisms by which estrogen and progesterone provide such neuroprotection are likely multifactorial. There is a large body of literature documenting the effects of gonadal hormones, estrogen and progesterone, on brain structure, function and metabolism. An understanding of the effects of both female hormones is therefore of central importance.

In order to understand the mechanisms of action that both hormones have on the brain, it is necessary to give a brief outline of the processes involved in the structural features of the brain and the role of oxidative stress in the brain, particular the hippocampus.

1.1.2. The structure and function of the hippocampus

The hippocampus is a medial temporal lobe brain structure that is thought to contribute to learning and memory. The hippocampus is one of several brain structures classified as being part of the limbic system, which is involved in various emotions such as fear, aggression, pleasure, and also in the formation of memory. Although the precise role of the hippocampus remains controversial, it is generally agreed that it is involved in certain forms of learning and for acquiring new memories.

The hippocampus is essential in memory consolidation — a process in which short term memory is converted into long-term memory (Cohen & Eichenbaum, 1993; Kim & Fanselow, 1992). Long-term memory allows people to recall information, days,

weeks, or years after learning. In addition, damage to the hippocampus can cause anterograde amnesia, which prevents a person from forming new declarative memories, yet leaves procedural memories intact. Procedural memory (implicit knowledge) refers to skills and procedures essential for human motor performance. Yet, unlike procedural memory, declarative memory stores events and facts that can explicitly be recalled.

The rat hippocampus is located approximately 1mm anterior to the starting point of the cerebellum (dorsal view) and extends about 4mm posterior and approximately 2.5mm lateral of the midline separating the two hemispheres (figure 1.1.).

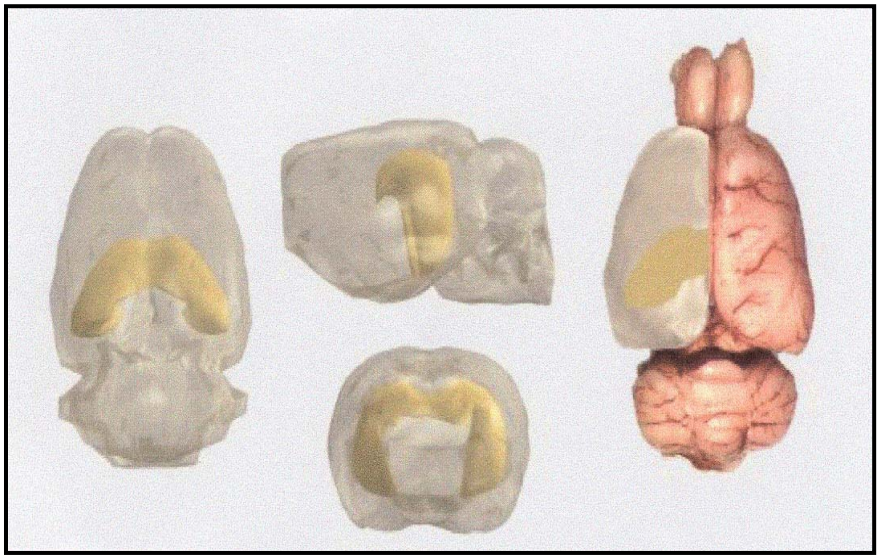


Figure 1.1. A range of sections of the rat brain displaying the hippocampus shown in brown. (Left and Right) A dorsal view of the rat brain; (Middle, Top) A midsaggital section through the brain; (Middle, bottom) A coronal section through the brain (Heron, 2002).

The hippocampus is made of two thin sheets of neurons named, the dentate gyrus and the Ammon's horn. There are four divisions in the Ammon's horn, of which the most important are the CA1 and CA3. CA stands for *Cornu Ammonis* which means Ammon's horn in Latin. The entorhinal cortex sends information to the hippocampus

via the perforant path, which is a bunch of axons. In the CA3 region, the dentate gyrus neurons give rise to axons, called mossy fires. 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 1.2).

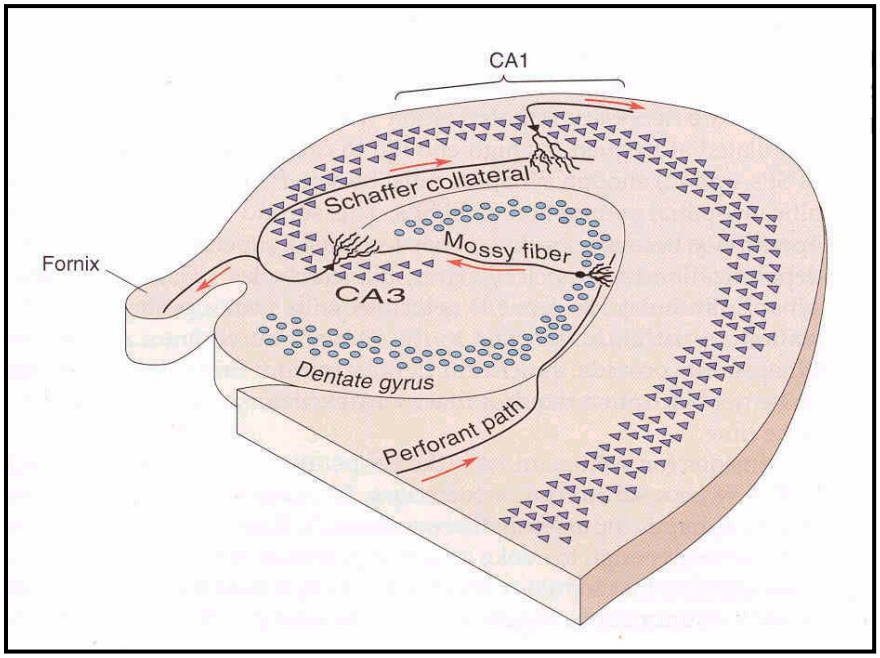


Figure 1.2. Illustration of microcircuits of the hippocampus (Bear *et al.*, 2001).

The hippocampus has long been thought to play a key role in the formation of new memories (Scoville *et al.*, 1957) and has been extensively studied for both its role in learning and memory (Silva, 2003) and its susceptibility to excitotoxicity (Ben-Ari *et al.*, 1980; Golarai *et al.*, 2001; Grady *et al.*, 2003; Olsson *et al.*, 2003). 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. However, the hippocampus is one of the first regions of the brain to be affected by AD where disorientation and memory problems are the primary symptoms (Rossler *et*

al., 2002; Kauffman *et al.*, 1998).

1.2. Excitotoxicity

1.2.1. Introduction

Excitotoxicity, which was first described by Olney *et al.*, (1972) in the nineteen-seventies, involves the activation of glutamate receptors in the central nervous system (CNS). Excitotoxicity is the process by which excess or prolonged stimulation of neurons by excitatory amino acids (EAA), glutamate, through postsynaptic receptors, which leads to neuronal cell death (Schinder *et al.*, 1996).

Glutamate, an excitatory amino acid, activates different types of ion channel-forming receptors (ionotropic) and G-protein-coupled receptors (metabotropic) to develop their essential role in the brain. However, high concentrations of glutamate or neurotoxins acting at the same receptors, cause cell death through the excessive activation of these receptors. In physiological conditions, the presence of glutamate in the synapse is regulated by active, adenosine triphosphate (ATP)-dependent transporters in neurons and glia. 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. 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. Glutamate exerts its physiologic actions on several distinct

families of glutamate receptors, located principally on postsynaptic neurons. The actions of glutamate are terminated when glutamate is removed from the synaptic cleft by sodium-dependent, high-affinity glutamate uptake carriers, which are located primarily on glial cells (Rothstein *et al.*, 1996; Schousboe *et al.*, 1981), but presynaptic terminals also sequester glutamate directly from the synaptic cleft (Savolainen *et al.*, 1995). Within the glial cells, glutamate is converted to glutamine via the enzyme glutamine synthetase (Savolainen *et al.*, 1995; Yudkoff *et al.*, 1993). Glutamine can then be recycled back to the glutamatergic nerve terminals, where it is converted to glutamate by the mitochondrial enzyme, phosphate-activated glutaminase (Bradford, 1986; Nicklas *et al.*, 1986). 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).

However, 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 (figure 1.3.). 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).

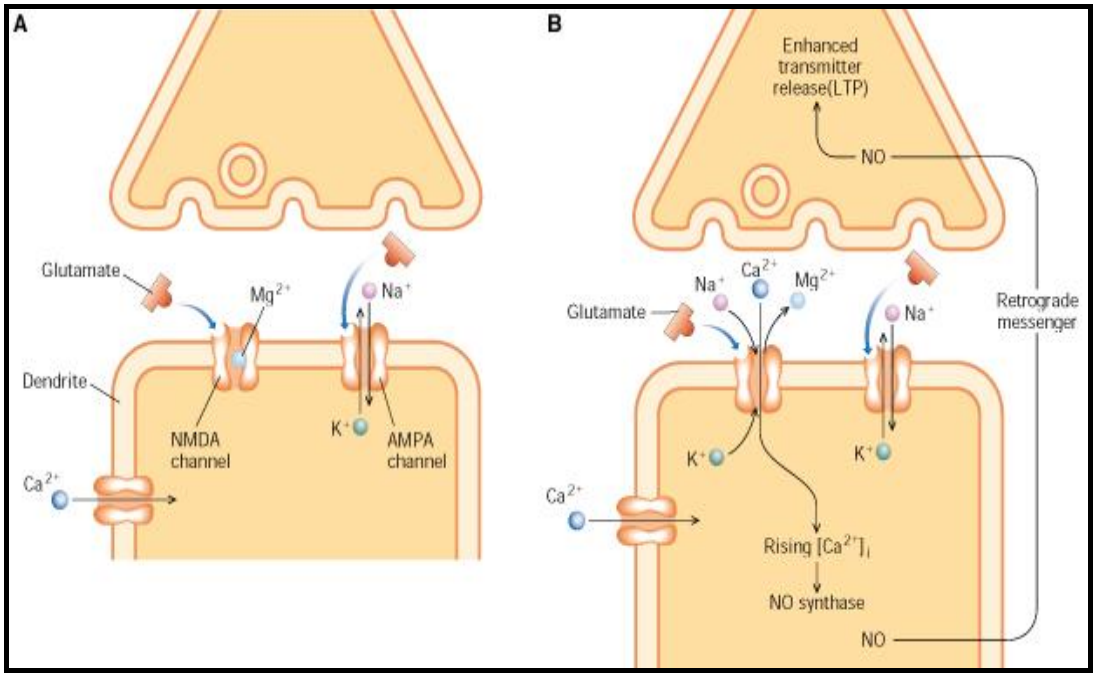


Figure 1.3. The mechanism of glutamate raises the intracellular calcium through the activation of several types of receptors and allows excessive amounts of Na^+, K^+ and Ca^{2+} to flow across the membrane. (soma.npa.uiuc.edu/ courses/bio303/Image129.jpg).

1.2.2. N-methyl-D-aspartate Receptors

The NMDA receptors play a critical role in excitotoxicity and are classified as ionotropic glutamate receptors. The NMDA receptor channel is a complex molecular entity consists of a number of distinct recognition sites for endogenous and exogenous ligands, each has distinct binding domains (Cooper *et al.*, 1996). 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 co-agonist, glycine, to a modulatory site (Carfagno *et al.*, 2000).

Activation of the NMDA receptor is modulated by polyamines, such as spermine and spermidine (Williams *et al.*, 1991). The polyamine site needs not be occupied for

receptor activation, but the presence of polyamines increases the ability of glutamate and glycine to open the NMDA-receptor ion channel (Yoneda *et al.*, 1991). The voltage-dependent blockade of the receptor ion channel by Mg^{2+} is one of the most important factors of the NMDA receptor (Greenamyre *et al.*, 1994; Mayer *et al.*, 1984; Monaghan *et al.*, 1986; Nutt, 1993; Couratier *et al.*, 1996). When the glutamate and glycine sites are occupied, at resting potential (about -70mV), normal extracellular concentrations of Mg^{2+} block the NMDA-receptor ion channel and prevent current flow. However, because the Mg^{2+} blockade is voltage-dependent, when a neuron becomes depolarised, the degree of Mg^{2+} block is reduced (Dodd *et al.*, 1994), allowing more movement of ions.

Besides allowing intracellular influx of Ca^{2+}, the NMDA receptor also permits exchange of Na^{+} and K^{+} across the cell membrane (Tilson *et al.*, 1995; Yudkoff *et al.*, 1993; Gonzales, 1990). However, the Ca^{2+} permeability of NMDA receptor is important to the initiation of long term potentiation as the increase of Ca^{2+} concentration activates a number of cellular and second messenger systems (Mayer *et al.*, 1990; Collingridge *et al.*, 1990; Madison *et al.*, 1991).

Extracellular Zn^{2+} acts as an inhibitory divalent cation at the site near the "mouth" of the ion channel, to produce a voltage-independent block. There are a number of competitive and non-competitive inhibitors present for the NMDA receptor. The most common competitive inhibitor is 2-amino-5-phosphonovalerate (AP5), which competes directly with glutamate for the binding site (Tilson *et al.*, 1995), while the non-competitive inhibitors dibenzocycloheptеneimine (MK-801), phencyclidine (PCP) and remacemide act within the ion channels to block current flow (Greenamyre *et al.* 1994, Tilson, 1995). High concentrations of the NMDA receptors can be found in the

cerebral cortex and CA1 regions of the hippocampus (Monaghan *et al.*, 1986). Figure 1.4 shows that structure of NMDA receptor.

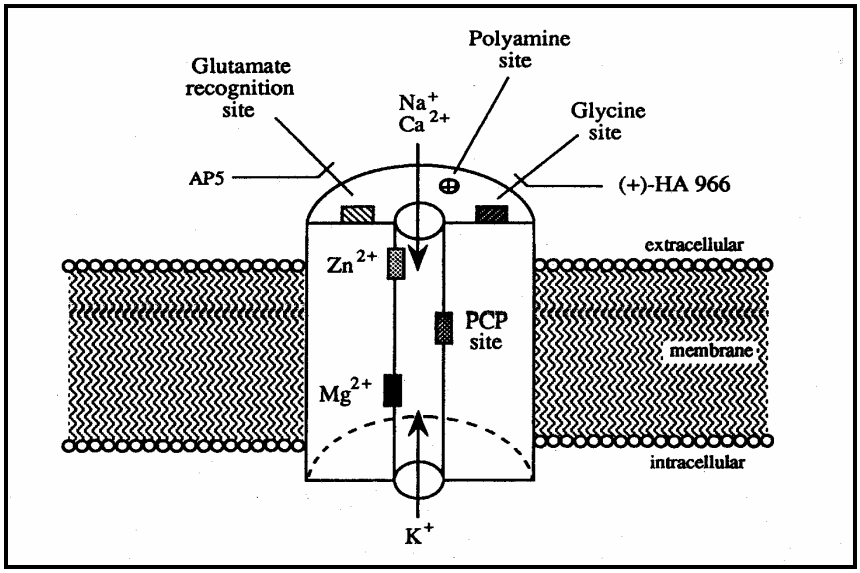


Figure 1.4. Diagrammatic representation of NMDA receptor (Cooper *et al.*, 1996).

1.2.3. NMDA Receptor Activation and Cell death

1.2.3.1. Acute NMDA Toxicity

Acute toxicity occurs due to the rapid influx of Na^+ into the neuron. This causes passive Cl^- and water entry into the cell via osmotic pressure. This toxic process occurs within minutes and depends on the presence of extracellular Na^+ and Cl^- ions. It may be associated with abnormalities in membrane permeability and may be lethal, via osmotic lysis. Under certain circumstances, the neuron may be able to restore osmotic pressure and thus survive. This process can be mimicked by depolarizing agents like veratridine (Churchwell *et al.*, 1996) or by raising extracellular K^+ concentrations. Activation of non-NMDA receptors can cause such neurodegeneration, with both kainic acid and α-2-amino-3-hydroxy-5-methylisoxazole-4-proprionate

(AMPA) being examples of potent neurotoxins (Collingridge *et al.*, 1989). The process that takes place may be direct, by over-excitation of the neuron leading to prolonged depolarization and depletion of energy reserves, or indirect, for example by excessive Na^+ ion influx resulting in Ca^{2+} build up via Na^+ - Ca^{2+} exchange.

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

Ca^{2+} influx and subsequent calcium overload following NMDA receptor activation triggers cytotoxic processes responsible for neuronal degradation. In most cases Ca^{2+} influx does not actively initiate cell death, but rather activates a number of enzymatic and metabolic processes that set in motion a process that can prove neurotoxic. In part, these changes are reversible, as shown by the ability of glutamate receptor antagonists to confer neuroprotection several hours after an excitotoxic insult (Leigh *et al.*, 1996).

However, in most cases, once the process has begun, neuronal cell death results.

Among the processes triggered by increased Ca^{2+} concentrations, is the activation of proteases calpain-1. The enzyme can degrade major neuronal structural proteins, and induce cytoskeletal breakdown (Seubert *et al.*, 1988). Calpain also cleaves xanthine dehydrogenase to convert it to xanthine oxidase (Meldrum & Garthwaite, 1990; Atlante *et al.*, 1997) enzyme converts hypoxanthine to uric acid while also producing superoxide radical ($O_2^{\cdot}$) (Wie *et al.*, 1997).

Phospholipases (Meldrum & Garthwaite, 1990; Himmelseher *et al.*, 1996) are capable of breaking down cell membranes and liberating arachidonic acid may also be activated by Ca^{2+}. Protein kinase C is an enzyme responsible for a prolonged phase of Ca^{2+} influx following EAA receptor activation (Mayer & Miller, 1990). Phospholipase 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). However, phospholipase C generates IP_3 (inositol 1,4,5-triphosphate), which raises cytosolic Ca^{2+} release from internal stores, and DAG (diacylglycerol), which activate protein kinase C and enhances glutamate release (Mayer & Miller, 1990).

Ca^{2+} can also bind to calmodulin to form Ca^{2+}-calmodulin complexes. The role of this complex in mammalian cells is to modulate the activity of a large number of enzymes (Ross, 1980). Among the processes activated are Ca^{2+}-calmodulin-linked protein kinases, which disrupt the cytoskeleton (Wang *et al.*, 1996), cause mitochondrial dysfunction and activate lipases (Wang *et al.*, 1996). Ca^{2+}-calmodulin complexes can

also activate nitric oxide synthase (NOS) (Scatton *et al.*, 1994), which produces nitric oxide ($NO^{\cdot}$), a toxin that has been implicated in a number of neurological diseases (Dodd, 1992). Activation of Ca^{2+}-sensitive kinases (proteases) could also result in over-phosphorylation of cytoskeletal proteins such as tau, hyperphosphorylated forms of which are found in neurofibrillary tangles (Mattson *et al.*, 1992a, b).

Dykens *et al*., (1994) reported that isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+}. Mitochondria play an important role in the removal of $Ca^{2+}$from cells after a glutamate insult (White *et al.*, 1997; Khodorov *et al*., 1996a). $Ca^{2+}$is sequestered to the mitochondrial matrix (Budd & Nicholls, 1996; Wang & Thayer, 1996; Schinder *et al.*, 1996), driven by the proton electron chemical gradient generated by the electron transport chain (Ankarcrona *et al.*, 1996). The reduction in the electrochemical gradient (Ankarcrona *et al.*, 1996) caused by the Ca^{2+} influx decreases ATP synthesis (Khodorov *et al.*, 1996b), at a time when there is great demand for ATP by the plasma membrane Ca^{2+} pump, and indirectly by the Na^{2+} / Ca^{2+} exchanger (Schinder *et al.*, 1996). Mitochondrial Ca^{2+} accumulation and the subsequent permeability transition may therefore be a critical early event specific to the NMDA receptor mediated excitotoxic cascade (White & Reynolds, 1996; Isaev *et al.*, 1996).

1.2.5. NMDA Receptor Antagonists

NMDA receptor antagonists have potential therapeutic benefits in a number of neurodegenerative disorders such as stroke and trauma; chronic neurodegeneration such as Alzheimer's disease and symptomatic treatment such as epilepsy, Parkinson's disease, depression, anxiety. Neuroprotective agents which completely block NMDA

receptors also impair normal synaptic transmission and thereby cause numerous side effects. Therefore, it is a challenge to develop antagonists that prevent the pathological activation of NMDA receptors but allow their physiological activity.

However, memantine is a clinically well tolerated uncompetitive NMDA receptor antagonist with strong voltage-dependency and rapid blocking/unblocking kinetics and is effective in the treatment of dementia. The action of memantine has been showed potential profile in animal models of chronic neurodegenerative disease (Parsons *et al.*, 1999). Since then, clinical research has focused on uncompetitive NMDA receptor antagonist in the treatment of dementia (Ditzler *et al.*, 1991).

(+)-MK-801(Dizocilpine,(+)-5-methyl-10,11-dihydro-5H-dibenzocyclo-hepten-5,10-imine maleate) is the most potent NMDA antagoinst, which binds to the receptor when the receptor channel is open. The binding of MK-801 is dependent on the existence of NMDA receptor agonist. Therefore, 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 1.5. By combining with the transmitter recognition site, an agonist such as NMDA activates the receptor. This causes a transformation of the receptor to the open state. The opening state of the receptor allows the conductance of Na^{+}, Ca^{2+} and K^{+}, which initiate the neuronal response. In contrast, the non-competitive antagonists act upon the ion channel such as Mg^{2+}. Mg^{2+} blocks the channel directly and prevents ionic conductance. When the channel is in the open state, MK-801 binds to sites within the channel. However, the binding site that MK-801 binds to is separated from but interacts with those for divalent cations, thus preventing the passage of ions (Kemp *et al.*, 1987).

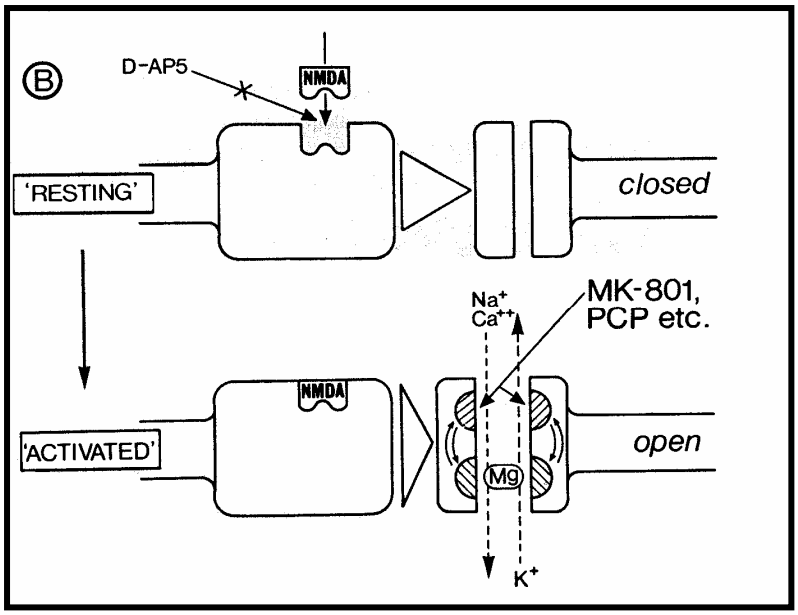


Figure 1.5. Model of the NMDA receptor complex and propsed sites of action of non-competitive antagonists (Kemp *et al.*, 1987). Once an agonist (D-AP5) has bound the channel opens allowing antagonists such as MK-801 and PCP to bind.

1.3. Oxidative Stress

1.3.1. Introduction

For years researchers have known that free radicals can cause cell degeneration, especially in the brain. Scientists have implicated the unstable molecules as a cause of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease. Now researchers are unraveling the pathways of free radical destruction in order to create targeted therapies for these disorders.

Oxygen, because of its bi-radical nature, readily accepts unpaired electrons to give rise to a series of partially reduced species collectively known as reactive oxygen species (ROS) including, superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) (Hoyt *et al.*, 1997),

hydroxyl radical (Cheesman & Slater, 1993), peroxyl radical, nitric oxide ($NO^{\cdot}$) and peroxynitrite, until it is itself completely reduced to water. Most of the superoxide radicals are formed in the mitochondrial and microsomal electron transport chain. However, not all of these species are particularly active in aqueous biological soloutions (Dawson & Dawson, 1996). These reactive oxygen species cause an oxidative stress that could lead to cell damage or death (Hoyt *et al.*, 1997).

Oxygen (O_2) can cause damage to DNA, proteins, essential or protective enzymes, provoke lipid peroxidation and dissolution of plasma and mitochondrial membranes via transformation to more reactive forms. However, about 1- 4% of the O_2 taken up into cells forms are partially reduced O_2 species, the ROS. Some of these reduced O_2 species contain unpaired electrons and are therefore classified as free radicals. Leigh et al., (1990) states that the word 'free' preceding 'radical' is unnecessary because all radicals are free. The diatomic oxygen molecule, O_2, possesses two unpaired electrons, each electron locates in a different orbital and both have the same spin quantum number and, thus qualifies as free radical itself. In living cells, there is a balance between oxidative stress and an antioxidative effect exists generally. When this balance is disturbed in the cells, either by defeat of reducing agents (e.g. glutathione) or protective enzymes (e.g. SOD and GPx) or by the increased production of reactive oxygen species (e.g. superoxide and hydrogen peroxide) or by both actions concurrently, the cells are then under oxidative stress (Fahn & Cohen, 1992). As a consequence, cell damage induced by free radicals or reactive oxygen species is referred as oxidative stress.

1.3.2. Free radicals

Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly ROS is an integral feature of normal cellular function

like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism. Their production however, multiplies several folds during pathological conditions. The release of oxygen free radicals has also been reported during the recovery phases from many pathological noxious stimuli to the cerebral tissues (Halliwell & Gutteridge, 1989).

There are different ROS found in living cells (Table 1.1). When two free radicals react, both radicals are eliminated; on the other hand, if a radical reacts with a non-radical, another free radical is produced. This characteristic allows free radicals to play a part in chain reactions, and cause thousands of actions (McCord, 1985).

Table 1.1. Formation of different types of ROS by reduction of molecular oxygen (Cui *et al.*, 2004).

(i) $O_2 + e + H^+ \rightarrow HO_2$ (hydroperoxyl radical)
(ii) $HO_2 \rightarrow H^+ + O_2^{\cdot}$ (superoxide radical)
(iii) $O_2^{\cdot} + 2H^+ + e \rightarrow H_2O_2$ (hydrogen peroxide)
(iv) $H_2O_2 + e \rightarrow OH^- + OH$ (hydroxyl radical)
(v) $OH + e + H^+ \rightarrow H_2O$
(vi) $O_2^{\cdot} + H_2O_2 \rightarrow OH^- + OH + O_2$ (Haber–Weiss reaction)
(vii) $Fe^{2+} + H_2O_2 \rightarrow OH^- + OH + Fe^{3+}$ (Fenton reaction)

The majority of superoxide radicals generated by mitochondrial electron transport chain are enzymatically dismutated to H_2O_2. The hydroxyl and alkoxy free radicals are very reactive species and rapidly attack the macromolecules in cells (Boveris *et al.*, 1972). The superoxide anion, lipid hydroperoxides, and nitric oxide are comparatively less reactive. A limited number of enzymes like xanthine-oxidase, tryptophan

dioxygenase, and indole-amine dioxygenase produce the superoxide free radical directly. Reactions catalysed by enzymes, such as monoamine oxidase and L-amino acid oxidase, also produce hydrogen peroxide, directly (Stohs *et al.*, 1995). Further, nitric oxide free radical ($NO^{\cdot}$) can react with superoxide radical to form highly toxic peroxynitrite ($ONOO^{\cdot}$). When peroxynitrite reacts with human body fluids and tissues, nitrotyrosines are generated, which have been detected in human brain and may be increased in neurodegenerative diseases, especially because glial cells and macrophages generate nitric oxide (Halliwell & Gutteridge, 1985). There has been speculation that nitric oxide is implicated in many of the neurodegenerative disorders.

1.3.3. Defense Mechanisms Against ROS Neurotoxicity

Biological systems have evolved with defence mechanisms to help protect against free radical linduced cell damage. (Machlin & Bendich, 1987) see table1.2. Glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutases (SOD) are antioxidant enzymes, which metabolize toxic oxidative intermediates. These require micronutrient as cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity and effective antioxidant defence mechanisms (Halliwell, 2001; Halliwell & Gutteridge, 1992). 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. Antioxidants usually refer to molecules that inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxy radicals (Halliwell, 1992a). The toxicity of free radicals can be mitigated by direct free radical scavengers and by indirect antioxidants (Sies *et al.*, 1993). These protective mechanisms can be classified into two main categories (Dawson & Dawson, 1996), namely the enzymatic defence antioxidants and non-enzymatic cellular antioxidants.

SOD and GPx are among the most important members of the enzymatic defence antioxidants. Non-enzymatic cellular antioxidants, such as the lipophilic free radical scavenger α-tocopherol (Vitamin E), or the hydrophilic compound ascorbate (Vitamin C), are the most prominent antioxidants of their class, can directly interact with ROS at the molecular level (Sies *et al.*, 1993).

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	

SOD, catalase, and glutathione peroxidase are three primary enzymes, involved in direct elimination of active oxygen species (hydroxyl radical, superoxide radical, and hydrogen peroxide). Glutathione is the most significant component which directly quenches ROS such as lipid peroxides and plays a major role in xenobiotic metabolism. When an individual is exposed to high levels of xenobiotics, more glutathione is utilised for conjugation making it less available to serve as an antioxidant. It also maintains ascorbate (vitamin C) and alpha-tocopherol (vitamin E), in their reduced

form, which also exert an antioxidant effect by quenching free radicals. Table 1.3 shows a list of neutralising antioxidants against ROS (Anderson, 1996; Meister *et al.*, 1994a; Sies & Stahl 1995).

Table 1.3. Reactive oxygen species and their corresponding neutralising antioxidants.

ROS	Antioxidants
Hydroxyl radical	Glutathione peroxidase and vitamin C
Lipid peroxide	Glutathione peroxidase and vitamin E
Superoxide radical	Superoxide dismutase and vitamin C
Hydrogen peroxide	Catalase and vitamin C

The breakdown of by SOD yields hydrogen peroxide and oxygen (reaction 1). There are two distinct SOD's in eukaryotes; the manganese-containing SOD found and copper-zinc-containing SOD, which found in the mitochondrial matrix and the cytoplasm, respectively.

$$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (1)$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (2)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad (3)$$

$$2H_2O_2 \rightarrow O_2 + 2H_2O \qquad (4)$$

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).

1.3.4. Superoxide Radical

Acceptance of a single electron by an O_2 molecule forms the superoxide radical, $O_2^{\cdot}$, has one unpaired electron. McCord *et al*., (1985) shows that $O_2^{\cdot}$ is a major agent responsible for O_2 toxicity and that SOD appears to have evolved specially to scavenge $O_2^{\cdot}$. Superoxide is formed *in vivo* in a variety of ways. A major source is the activity of electron transport chains in mitochondria and endoplasmic reticulum (Halliwell, 1992a). Some of the electrons passing through these chains "leak" directly from intermediate electron carriers onto O_2. Because O_2 accepts electrons one at a time, $O_2^{\cdot}$ is formed (Halliwell, 1992a). The rate of leakage at physiological O_2 concentrations rise as O_2 concentration is increased (Fridovich *et al.*, 1989). Some leakage of $O_2^{\cdot}$ can occur, however, mitochondria normally contain high levels of the protective enzymes, SOD and GPx to remove toxic species (Jesberger & Fichardson, 1991). Hence the toxicity of excess O_2 may be due to increased formation of $O_2^{\cdot}$, beyond the ability of antioxidant defenses to cope.

It is generally held that $O_2^{\cdot}$ is not highly reactive in an aqueous environment. Moreover, once formed, $O_2^{\cdot}$ quickly undergoes dismutation to generate H_2O_2; this reaction is markedly accelerated by a family of enzymes, the SOD (Fridovich *et al.*, 1989). Since SOD removes an oxidant, $O_2^{\cdot}$, from the cell, it is generally considered as an important

antioxidative enzyme (Touati, 1989). The reduction of cytochrome c by xanthine and xanthine oxidase is mediated by $O_2^{\cdot}$ (McCord & Freidovich, 1968) and this reduction is used as the basis of assays for both $O_2^{\cdot}$ and SOD. The SOD enzymes are catalysts that have evolved a surface charge arrangement to facilitate the specific use of $O_2^{\cdot}$ as a substrate (Benovic *et al.*, 1983).

The evidence supporting the superoxide theory of O_2 toxicity is important, but the exact mechanism by which the excessive $O_2^{\cdot}$ generation at increased O_2 levels could exert toxic effects is not completely clear. The reduction of molecular oxygen to form $O_2^{\cdot}$ 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 and 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, 1992a).

1.3.5. Hydrogen Peroxide

SOD removes $O_2^{\cdot}$ by converting it into H_2O_2. H_2O_2 can act as an oxidizing agent, although it is poorly reactive. However, unlike $O_2^{\cdot}$, H_2O_2 crosses cell membranes

easily. H_2O_2 is not itself a free radical and does not contain unpaired electrons. In the presence of transition metals, most often Fe^{2+} but also $Cu^{1+,}$ H_2O_2 is reduced to the hydroxyl radical (˙OH) via either Haber-Weiss or Fenton reactions (Halliwell & Gutteridge, 1990). The ultimate fate of H_2O_2 is not always the ˙OH. H_2O_2 can be removed within human cells by catalases and glutathione peroxidases (Halliwell & Gutteridge, 1990).

In the brain GPx are considerably more important than catalase because of the low activity of the latter enzyme in most parts of the central nervous system (CNS) (Jain, 1991). GPx utilizes H_2O_2 and hydroperoxides as substrates during the conversion of reduced glutathione (GSH) to its disulfide oxidized glutathione (GSSG) (Griffith & Meister, 1985).

1.3.6. Hydroxyl Radical

Hydroxyl radical (˙OH) is formed when H_2O_2 reacts with Fe^{2+} ions, by the Fenton reaction, which can be represented by the overall reaction:

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

The ˙OH radical is probably the most reactive of the ROS species (Poeggler *et al.*, 1993; Dawson & Dawson, 1996). It will react with almost all molecules in living cells, including DNA (causing strand breakage), membrane lipids and carbohydrates. (Fridovich, 1996). Most of the DNA damage may be mediated by reaction of H_2O_2 with iron and /or copper ions, bound close to DNA to form ˙OH (Aruoma *et al.*, 1991).

However, another devastating effect of ˙OH radical is its action on membrane lipids. Various reactive species that are generated on mixing $O_2^{\cdot}$, H_2O_2 and iron of copper ions are capable of initiating the process of lipid peroxidation (Halliwell, 1992a). Alternatively, during excitatory neurotransmitter stimulation of neurons, the large increases in intracellular Ca^{2+} activate nuclease enzymes in the nucleus which results in the formation of ˙OH leading to DNA damage (Orrenius *et al.*, 1989).

1.3.7. Nitric oxide and Peroxynitrite Anion

Nitric oxide ($NO^{\cdot}$) is another free radical released by several cell types, especially vascular endothelial cells and phagocytes (Moncada *et al.*, 1991). $NO^{\cdot}$ reacts with $O_2^{\cdot}$ at physiological pH to yield a nonradical product, peroxynitrite ($ONOO^-$) (Radi *et al.*, 1991). Under normal physiological conditions this nitrogen-centered radical has important functions as a neuronal messenger molecule and is responsible for relaxation of the pyloric sphincter in the gastrointestinal tract (Yun *et al.*, 1996) and vasodilation (Holscher *et al.*, 1997); however, when $NO^{\cdot}$ increases intracellularly to unusually high concentrations it initiates a toxic cascade of events which can lead to the death of neurons (Zhang *et al.*, 1994). A common example of $NO^{\cdot}$ toxicity is seen in glutamate neurotransmission in the CNS where N-methyl-D-aspartate (NMDA) receptor activation leads to large rises in intracellular calcium concentration followed by the stimulation of neuronal nitric oxide synthase (NOS) (Dawson *et al.*, 1991) leading to the generation of $NO^{\cdot}$.

$ONOO^-$ is cytotoxic by oxidizing thiol groups, and also decompose to form ˙OH (Beckman, 1991; Radi *et al.*, 1991). Although $ONOO^-$ is a simple compound, it is chemically highly complex. Its reactivity is similar to that of $OH^{\cdot}$. The toxicity of

$ONOO^-$ derives from its ability to directly nitrate and hydroxylate the aromatic rings of amino acid residues (Beckman, 1992), with zinc-thiolate moieties (Crow, 1995) as well as with lipids (Radi *et al.*, 1991), proteins (Moreno & Pryor, 1992) and DNA (King *et al.*, 1992). This ubiquitous activity makes the $ONOO^-$ a molecule that can have devastating effects on neuronal physiology as well as viability.

1.3.8. The Involvement of Metal Ions

Ions of transition metals such as copper and iron, in tissues of animals, including humans (Choi *et al.*, 1994), are involved in many free radical reactions, and lead to generation of highly reactive species (Halliwell & Gutteridge, 1989). Transition metals promote lipid peroxidation in two ways: (1) by catalyzing the formation of oxygen free radical species capable of initiating lipid peroxidation and (2) by catalyzing the decomposition of preformed lipid peroxides to propagate lipid peroxidation.

Evidence suggests that iron is involved in lipid peroxidation (Lauffer *et al.*, 1992). However, 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 iron (III) (reaction 5). The Fenton reaction can be augmented by the reduction of ferric ion by superoxide thus regenerating ferrous ion. However, the net result is the production of hydroxyl radicals as in the iron-catalyzed Haber-Weiss type of reaction.

The hydroxyl radical is the most reactive of all oxygen radicals, which is iron dependent. The hydroxyl radical is harmful to the cell and no enzyme system uses it as

a substrate. Cells attempt to prevent hydroxyl radical formation by removing hydrogen peroxide and transition metals to inactive sites (Fahn & Cohen, 1992). However, hydrogen peroxide crosses the blood brain barrier and is converted to the hydroxyl radical in the brain causing severe damage to brain neurons.

Evidence shows that iron stores increase with age in humans and animals (Leggett, 1990). It has been suggested that iron accumulated in human and rat brain during aging can accelerate the degeneration in brain functions that occur during aging and in certain pathologies of CNS degenerative disorders (Viani *et al.*, 1991). However, the endogenous iron stores are available for participating in peroxidative process in tissue (Halliwell & Gutteridge, 1986).

1.3.9. Iron Source for Fenton Chemistry

Iron is a remarkably useful metal in nature. It is essential for the transport of haemoglobin, the storage of myoglobin and oxygen for cytochromes and cytochrome oxidase in respiration. In addition, it is also an essential component in the active sites of many enzymes and antioxidant defense enzyme catalase. In mammals, iron is absorbed from the gut and originates from food and beverages we consume that are prepared with iron. Iron not required for these is stored as ferritin, which can hold up to 45 000 ions of iron (Halliwell & Gutteridge, 1992). Transferrin enters cells by endocytosis in a vacuole, which is acidified to release the iron. The unloaded transferrin is ejected from the cell and the iron ions released from it are used for the synthesis of intracellular iron proteins. Excess iron is stored in the protein ferritin. 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 $OH^{\bullet}$ *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 cerebrospinal fluid (CSF) has no significant iron-binding capacity, and mechanical disruption of the brain releases iron that can stimulate radical reactions such as lipid peroxidation.

1.3.10. Lipid Peroxidation

Lipid peroxidation is one of the major outcomes of free radical–mediated injury to tissue. Membrane phospholipids are biomolecules which are the most susceptible to oxidative attack by free radicals, because of the high levels of polyunsaturated fatty acids (PUFA) present in these macromolecules. Lipid peroxidation can be extremely damaging because self-perpetuating chain reactions are caused by ROS attack. Table 1.4 illustrates the three well-described phases which characterise the lipid peroxidation process: initiation, propagation, and termination. During the initiation step (reaction 1) the free radical attacks a methylene group in the PUFAs ($In^{\bullet}$), leading to a rearrangement of the double bonds to the conjugated diene form, and simultaneously producing a carbon-centered alkyl radical. The alkyl radical reacts with molecular oxygen to give rise to a peroxyl radical (RH) and the formation of a lipid-derived radical ($R^{\bullet}$). The propagation step (reaction 2) the peroxyl radical, in its turn, starts a self-perpetuating chain reaction in which most of the membrane lipids are converted to a variety of lipid peroxyl radical ($ROO^{\bullet}$) and cyclic peroxides. The lipid hydroperoxides (ROOH) can be further degraded to hydrocarbons, alcohols, ether, epoxides, and aldehydes. Of these products, malondialdehyde and

4-hydroxynonenal have the additional ability to inactivate phospholipids, proteins, and DNA by bringing about cross-linking between these molecules (Esterbrauer *et al.*, 1990). A self perpetuating autocatalytic reaction then follows with reactions 2 and 3 undergoing a number of cycles (Biziere *et al.*, 1980). The last step is a termination step, which results when two $ROO^{\cdot}$ radicals react together to form a non-radical product (Krinsky, 1992). An important aspect of these reactions is that lipid peroxidation will proceed until substrate is consumed or termination occurs. There are two broad outcomes to lipid peroxidation, viz., structural damage to membranes and generation of secondary products. Membrane damage derives from the generation of fragmented fatty acyl chains, lipid-lipid cross-links, endocyclization to produce novel fatty acid esters, and lipid-protein cross-links (Farber *et al.*, 1995). In total, these processes combine to produce changes in the biophysical properties of membranes that can have profound effects on the activity of membrane-bound proteins. Secondary products from lipid peroxidation may be divided conceptually into those generated by fragmentation or rearrangement of oxygenated lipid. However, lipid peroxidation is an indiscriminate process and formation of both types of products occurs simultaneously.

Table 1.4. Chain sequence for free radical auto-oxidation (Krinsky, 1992).

Initiation	$In^{\cdot} + RH \rightarrow InH^{\cdot} + R^{\cdot}$	(1)
Propagation	$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$	(2)
	$ROO^{\cdot} + RH \rightarrow R^{\cdot} + ROOH$	(3)
Termination	$2\ ROO^{\cdot} \rightarrow$ Non-radical products	(4)

Fragmentation of lipid peroxidation liberates a number of diffusible products, which are potent electrophiles (Esterbauer *et al.*, 1991). Thus, the detection and

measurement of lipid peroxidation is the evidence cited to support the involvement of free radical reactions in toxicology and in human disease. Lipid peroxidation is defined as the oxidative deterioration of lipids that contain more than two carbon-carbon double covalent bonds (Halliwell, 1992a). Cell membranes are rich in polyunsaturated lipids and together with saturated fatty acids which give rise to membrane fluidity. Damage to these PUFA side chains reduces membrane fluidity and as a result the biological membrane is not able to function properly. The most abundant diffusible product of lipid peroxidation is malondialdehyde (MDA). Reactive aldeyhdes from lipid peroxidation are biologically active. Most studies have focused on their reactivity with a number of cellular nucleophiles, including proteins, nucleic acids and some lipids (Esterbauer *et al.*, 1991). Indeed, many of the cytotoxic effects of lipid peroxidation can be reproduced directly by electrophilic lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) (Farber *et al.*, 1995). These include depletion of glutathione, dysfunction of structural proteins, reduction in enzyme activities, and induction of cell deaths.

The removal of ROS by various antioxidant systems is therefore essential to limit lipid peroxidation occurring in cells. Numerous antioxidant mechanisms have evolved, including several metabolic routes to detoxify fragmentation products from lipid peroxidation. The diffusible reactive aldehydes generated from lipid peroxidation are excellent substrates from glutathione transferases and a number of oxidoreductases that act to detoxify these molecules (Esterbauer *et al.*, 1991).

However, there is compelling evidence that the magnitude of lipid peroxidation in the brains of AD patients examined postmortem exceeds that in age-matched control individuals without neurologic disease (Montine *et al., 2002*). These authors

demonstrate that there is significantly increased thiobarbiturate reactive substrances (TBRAS) in disease regions of AD brain.

1.3.11. Free radicals and Disease

1.3.11.1. Introduction

Free radicals have been reported to be involved in aging as well as many diseases such as arthritis, neurological damage, Down's syndrome and diabetic cataract. The mammalian brain is especially vulnerable to oxidative stress (Hall & Braughler, 1989) as it consumes 20% of total body oxygen, contains large amounts of PUFA, which is relatively deficient in protective mechanisms, and readily accumulates iron (Dawson & Dawson, 1996). It is believed that approximately up to 5% of the oxygen taken up by an organism may become damaging oxygen-based radicals (Reiter *et al.*, 1995; Chan *et al.*, 1993). Therefore, in the human, there could be an equivalent of 2 kg $O_2^{\cdot}$ produced each year (Reiter *et al.*, 1995). As a consequence, it is substantial to measure the molecular carnage and cytotoxicity that is in the brain after toxin exposure and during aging. This oxidative damage has been considered as a common link in the pathogenesis and neuropathology of a variety of neurodegenerative disorders, such as AD.

1.3.11.2. Aging

Aging is a natural phenomenon and there are many physiological factors that can either advance or slow the aging process. In mammal, aging could be result from normal developmental and metabolic processes responsible for the decreases in the

rate of wound healing or increases in susceptibility to disease and death. Brain aging has become an area of intense research and a subject of much speculation fueled largely from the widely recognized fact that age is the biggest risk factor in most neurodegenerative diseases.

There are two ideas as to how aging and stress are related (Selye & Tuchweber 1976). The first is that as an organism ages so it is less able to adapt to stress. This idea is supported by the many physiological systems that function normally in younger life, and yet do not adequately respond to the challenge in old age. The second idea is that by impairing hippocampal functions, stress can accelerate the aging process like learning and memory (Endo *et al.*, 1996).

ROS are believed to be responsible for many of the ageing processes. These molecules affect the integrity of the macromolecules in the cell. Although most of the ROS produced are rapidly removed from the cell by the antioxidant defense system, there is a slow accumulation of damage that occurs during an animal's life. It is estimated that 10 000 oxidative hits occur on DNA in the human per day (Ames *et al.*, 1993). These damages are repaired by enzymes that remove the lesion; however there is a steady increase in the number of lesions as the animal ages. Viani *et al.,* (1991) compared young, adult and old rat brains. The group found that old brains were more susceptible to lipid peroxidation, demonstrating that peroxidation injury can have even more dramatic consequences when it takes place in the aged brain.

1.3.11.3. Alzheimer's Disease

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disorder

that occurs gradually and results in memory loss, unusual behavior, personality changes, and a decline in thinking abilities. AD is a dementia characterized by neurodegeneration; in particular, a loss of acetylcholine in the entorhinal cortex, hippocampus, ventral striatum, basal forebrain, and cerebral cortex (Bartus *et al.*, 1982; Gilman, 1997; Kasa *et al.*, 1997) and behavioral pathologies, such as wandering behavior (Ryan *et al.*, 1995). AD accounts for more than 70 percent of all cases of dementia, so it is important to identify modifiable risk factors for the disease (Brookmeyer *et al.*, 1998). During the past decade, there has been growing interest in vascular factors that may underlie AD. It is now recognized that subjects with cardiovascular risk factors and a history of stroke have an increased risk of both vascular dementia and AD (Hofman *et al.*, 1997; Breteler, 2000; Snowdon, 1997).

In AD, the death of neurons in brain regions critical for learning and memory is believed to result from increased production and accumulation of insoluble forms of amyloid β peptides (Aβ). It may endanger and kill neurons by inducing oxidative stress and disrupting cellular ion homeostasis (Mattson, 2000). Aβ can induce oxidative stress and DNA damage in cultured neurons and both oxidative stress and DNA damage have been documented in neurons associated with Aβ-containing plaques in the brains of AD patients (Mattson, 1997; Mattson, 2000).

A clinical diagnosis of AD is confirmed by observing numerous neuritic (amyloid) plaques and neurofibrillary tangles in the hippocampus, amygdala, and cortex (Yamaguchi *et al.*, 1988; Tagliavini *et al.*, 1988). The plaques (extracellular) are composed of the 42- and 40-residue Aβ proteins (Skovronsky *et al.*, 1998; Walsh *et al.*, 2000), whereas the tangles (intraneuronal) are composed of modified forms of the microtubule-associated protein, tau. Aβ protein is generated normally throughout life

from a large, receptor-like precursor, Aβ precursor protein, (APP) through proteolytic cleavages by the β- and γ-secretases. Mutations in 3 genes (APP, presenilin 1, and presenilin 2) that cause AD increase cerebral Aβ production (Berezovska *et al.*, 2001), whereas inheritance of the apolipoprotein E4 (Apo E4) polymorphism enhances its stability, leading to Aβ accumulation. Figure 1.6 shows the sequence of the pathogenetic steps of AD based on currently available evidence (Dennis & Selkow, 2004).

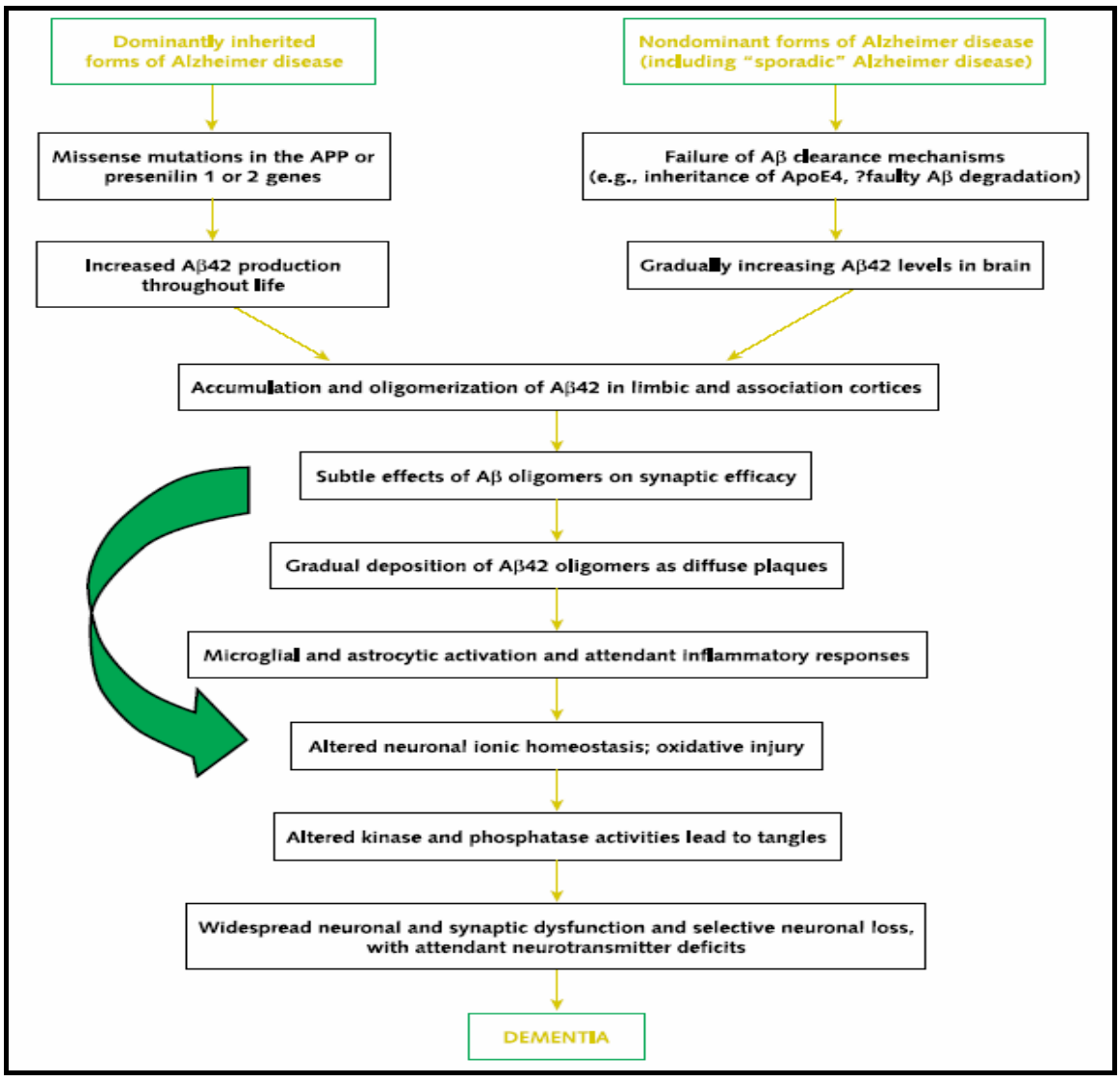


Figure 1.6. The sequence of the pathogenetic steps of AD (Dennis & Selkoe, 2004).

Currently, the pharmaceutical treatment of the dementia symptoms of AD is restricted

to 2 types of drugs: acetylcholinesterase inhibitors (such as donepezil, galantamine, and rivastigmine) (Mayeux & Sano, 1999) and the recently approved *N*-methyl-D-aspartate antagonist, memantine (Zajaczkowski *et al.*, 2000; Jain, 2000). These agents temporarily relieve some symptoms for a period of time for a subset of patients, but they do not address the underlying pathologic process or substantially slow clinical progression. Prospective mechanism-based treatments include inhibitors of the β- and γ -secretases (Petersen *et al.*, 1999) and immunotherapeutics (anti-Aβ protein monoclonal antibodies and Aβ protein vaccines) (Schenk, *et al.* 1999).

Another Aβ protein–based approach to treating AD is to administer anti-inflammatory drugs that could interfere with aspects of the microglial, astrocytic, and cytokine responses found in the brain of a patient with Alzheimer disease. The epidemiologic evidence that prolonged use of certain nonsteroidal anti-inflammatory drugs (NSAIDs) (specifically inhibitors of cyclooxygenase-1) is associated with a lower risk for AD could be explained on this basis (in t'Veld *et al.*, 2001). However, exciting studies in cultured cells and APP transgenic mice suggest that some NSAIDs associated with protection from AD, such as ibuprofen, can modulate processing of APP to decrease Aβ protein 42 productions without lowering overall Aβ protein levels (Weggen *et al.*, 2001).

Finally, various antioxidants, free radical scavengers, calcium-channel blockers, metal chelators, or modulators of certain signal transduction pathways might protect neurons from the downstream effects of the accumulation of protein. One problem with such approaches may be that neurons respond to Aβ protein and its associated inflammatory process in several ways. Blocking cyclooxygenase-1 or -2 of these response pathways might not substantially decrease overall neuronal dysfunction and

loss. A chelator of copper and zinc ions that may decrease cerebral Aβ protein levels (Cherny *et al.*, 2001) is being tested in patients with AD.

In the future, individuals who are nearly 50 years of age or older will probably be offered a specific risk-assessment profile to determine their likelihood of developing AD. Such an assessment, modeled on that used to predict the risk for serious atherosclerotic disease, could include inquiry about a family history of AD; identification of specific predisposing genetic factors; structural and functional brain imaging to detect evidence of presymptomatic lesions; and measurement of Aβ protein 42, tau, and other markers of the neuropathology in cerebrospinal fluid and perhaps (in the case of Aβ protein) in blood. Therefore, those individuals who are at high risk of developing AD might be offered preventative treatments with 1 or more of the agents. Although the achievement of an integrated diagnostic and therapeutic approach to this devastating disorder may seem remote, the current rate of scientific progress and the recent initiation of further clinical trials suggest that some level of practical success may come soon.

1.3.12. Antioxidant Therapy

The term “antioxidant” is frequently used in the literature to mean a chain-breaking antioxidant inhibitor of lipid peroxidation. Antioxidants inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals. This would prevent the peroxyl radicals from attacking adjacent fatty acid side chains or membrane proteins. In human brain, α-tocopherol is an important antioxidant. Muller & Goss-Sampson *et al.,* (1990) shows that severe and prolonged deprivation of α-tocopherol cause severe neurological derangements. However, it takes considerable

time (weeks) to increase the α-tocopherol content of brain tissue in mammals supplemented with vitamin E.

Antioxidant enzymes, especially human SOD, are now another approach as an antioxidant therapy. Endogenous SOD has an important protective role. SOD diminishes reperfusion injury after spinal cord ischemia (Lim *et al.*, 1986) and oedema induced by low brain temperatures (Chan *et al.*, 1987). Moreover, GPx is another antioxidant enzyme which is able to remove H_2O_2 and inhibit lipid peroxidation.

1.3.13. Molecular Targets of Oxidative Stress

Ischemia/reoxygenation injury and traumatic damage to the brain can produce liberation of catalytic metal ions and an increase in the formation of reactive radicals, thus causing further cellular damage. Several groups have found evidence that increased oxidative stress can be the result of CNS lipid peroxidation after ischemia (Michel *et la.*, 1987); trauma (Hall & Braughler, 1988).

However, oxidative damage does not always lead to the formation of lipid peroxides in injured nervous tissue. DNA and protein damage *in vivo* are of equal or greater importance (Halliwell *et al.*, 1987). Human cells subjected to oxidative stress show DNA damage and consequent activation of poly-(ADP-ribose) synthetase and decreases in ATP content as well as increases intracellular free Ca^{2+} (Orrenius *et al.*, 1989). In addition, generation of excitatory amino acids such as glutamate produced by ischemia in brain can cause oxidative stress (Meldrum *et al.*, 1990). However, protein damage is also an important consequence of oxidative stress (Halliwell *et al.*,

1987). Free radicals can also damage brain proteins (Oliver *et al.*, 1990), including the enzyme glutamine synthetase. This enzyme is responsible for glutamate removal inactivated by oxidative stress (Oliver *et al.*, 1990). There is evidence suggests that protein damage is important in a variety of normal and pathological process, such as aging and oxygen toxicity (Oliver *et al.*, 1990).

1.4. Homocysteine

1.4.1. Introduction

Homocysteine (Hcy) is a nonessential sulfur-containing amino acid and an intermediary metabolic product derived from the demethylated essential amino acid methionine. It is first discovered in 1932 by the 1955 Nobel Prize recipient Vincent DuVigneaud (DuVIgneaud *et al.*, 1952) and its association with premature arteriosclerotic (fibrotic) vascular disease is then described by Kilmer McCully in 1969 (McCully, 1969), Hcy has emerged as a novel marker of risk for cardiovascular disease.

Hcy is an important metabolite of methionine playing a key role in *trans*-sulphuration and *trans*-methylation pathways. Hcy can be re-methylated to methionine, and this reaction catalysed by Hcy; methionine methyltransferase requires folic acid and Vitamin B12. Moreover, Hcy can be catabolised to cystathionine by cystathionine β-synthase using Vitamin B6 (Yudkoff *et al.*, 1999; Molloy and Weir, 2001).

An elevated plasma level of Hcy, termed hyperhomocysteinemia, is recognized as an independent risk factor for myocardial infarction, coronary artery disease, strokes,

genetic disorders, AD and loss of cognitive functions (Schwartz *et al.*, 1997; Whincup, 1999; Stehouwer *et al.*, 1998). Normal Hcy concentration in human blood does not exceed 14μM (Seshadri *et al.*, 2002). Inborn errors of its metabolism or disturbances caused by dietary deficiency in folic acid and/or Vitamins B6 and B12, may lead to hyperhomocysteinemia in the range of 50-200μM or even higher (Scott and Weir, 1998; Yudkoff, 1999; Lipton *et al.*, 1997). Elevated plasma Hcy levels are also associated with an increased risk of neural tube defect, placental infarcts and eclampsia (Wenstrom *et al.*, 2000; Wang *et al.*, 2000; Ray *et al.*, 1999; Sohda *et al.*, 1997; McCully, 1994), and some reports suggest that hyperhomocysteinemia might be a manifestation of an underlying common biologic disorder which is directly associated with the process of DNA methylation (Kramer *et al.*, 1990; Yi *et al.*, 2000). These observations led to the hypothesis that elevated plasma Hcy may be a risk factor for dementia and AD (Seshadri *et al.*, 2002). Direct support for this hypothesis was provided recently by Clark et al., (1998), who compared serum Hcy levels in AD patients and age-matched controls without neurodegenerative disease. Several studies (Andersson, 1992; Joosten *et al.*, 1997; Rasmussen *et al.*, 1996) have shown increased plasma Hcy levels in healthy elderly people, which could increase the risk of suffering degenerative disease by the impairment of redox processes and the increase of oxygen-free radicals during aging. Hcy has been shown to promote low-density lipoprotein (LDL) oxidation *in vitro* (Heinecke *et al.*, 1993; Hirano *et al.*, 1994; Lynch *et al.*, 1997), potentiate glutamate excitotoxicity (Kruman *et al.*, 2000), causes changes in redox thiol status (Ueland *et al.*, 1996), serves as a source of hydrogen peroxide, a harmful free radical (Dimitrova *et al.*, 2002) and together with Cu^{2+} (Hultberg *et al.*, 1997) induces a decrease in the intracellular levels of glutathione in HeLa culture cells. There is reported evidence implicating Hcy promotion of *in vivo* lipid oxidation (Roberts & Morrow, 2000) and in men with mild

hyperhomocysteinemia (Voutilainen *et al.*, 1999).

1.4.2. Biochemistry of Homocysteine

Hcy is a thiol-containing amino acid derived from methionine, an essential amino acid. Hcy is produced entirely from the methylation cycle as it is totally absent from any dietary source (Finkelstein *et al.*, 1974; Finkelstein *et al.*, 1998). Hcy occurs in blood in three forms, whereby most of it (80-90%) is bound to protein; a smaller part is available as free amino acid, and traces are present as disulphide. Normal fasting plasma levels (total homocysteine) are between 5 and 15μmol/L. Moderate elevated Hcy levels range between 16 and 30μmol/L, intermediate elevated Hcy levels between 31 and 100μmol/L, and severe hyperhomocysteinaemia is present at over 100μmol/L (Graeme & Eikelboom, 1999). In populations without evidence of a nutritional deficit (vitamin B12 deficiency, malnutrition), a reference interval of between 4.9 and 11.7μmol/L was determined (Ubbink *et al.*, 1995). Under these conditions, higher Hcy values probably increase the cardiovascular risk (Andreotti *et al.*, 2000). The frequency of re-stenoses in coronary vessels is significantly elevated at plasma Hcy levels between 11 and 12μmol/L, but is significantly reduced upon lowering of the Hcy values through folic acid substitution (Schnyder *et al.*, 2002). Recent data from a meta-analysis has shown that an increased folic acid intake (approximately 200μg/day) reduces Hcy levels by approximately 4μmol/L which significantly decreases coronary artery disease mortality (Boushey *et al.*, 1995).

1.4.3. Metabolism of Homocysteine

Hcy is an intermediate metabolite of methionine metabolism. Metabolism of the amino acid methionine, a limiting amino acid in the synthesis of many proteins, affects several biochemical pathways involving the production of nutrients which are essential to the optimal functioning of the cardiovascular, skeletal, and nervous systems. Hcy metabolized by two pathways: the *trans*-methylation pathway where Hcy is catalyzed by methionine synthase or betaine homocysteine methyl transferase, which regenerates methionine; the *trans*-sulfuration pathway, Hcy is catalyzed by cystathionine beta-synthase leading to cystathionine, which degrades Hcy into cysteine and then taurine. However, the intermediate metabolite Hcy is located at a critical metabolic crossroad, and therefore both directly and indirectly impacts all methyl and sulfur group metabolism occurring in the body. Studies have shown that if high enough levels of Hcy and adenosine accumulate in the cell, all methylation reactions are completely inhibited (Duerre *et al.*, 1981).

The *trans*-methylation pathway is comprised of two intersecting biochemical pathways, and results in the transfer of a methyl group (CH_3) to Hcy by either methylcobalamin or betaine (trimethyl-glycine). Methylcobalamin originally receives its methyl group from S-adenosylmethionine (SAM) (Figure 1.8) or 5-methyltetrahydrofolate (5-methylTHF), an active form of folic acid (Figure 1.7). Hcy can be reconverted into methionine by methylation, catalysed by the methionine synthase, a process requiring vitamin B12 and folic acid as cofactors. Alternatively, it can be transformed to cystathionine catalysed by cystathionine β-synthetase (CβS), a vitamin B6-dependent enzyme. However, methionine can also be re-utilized to produce SAM, the body's "universal methyl donor," which participates in several key metabolic pathways, including methylation of DNA and myelin, synthesis of carnitine, coenzyme Q10, creatine, epinephrine, melatonin, methylcobalamin, and

phospha-tidylcholine, as well as phase II methylation detoxification reactions. Moreover, in the liver, remethylation can also be catalysed by the enzyme betaine-homocysteine transmethylase. Cellular export represents an additional mechanism of Hcy removal. Excess intracellular Hcy leads to export of Hcy and may thus lead to increased plasma Hcy levels in the circulation (Refsum *et al.*, 1998; Ueland *et al.*, 1997).

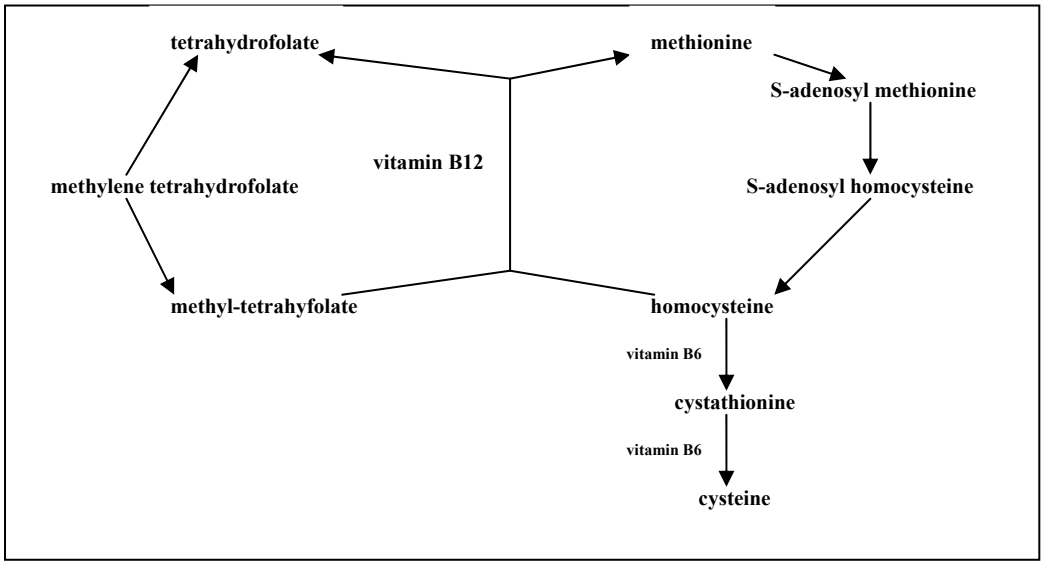


Figure 1.7. *Trans*-methylation cycle. Cofactors are represented in boxes (Teunissen *et al.*, 2002).

The *trans*-sulfuration pathway of methionine/Hcy degradation (Figure 1.8) produces the amino acids, cysteine and taurine, which are important nutrients for cardiac health, hepatic detoxification, cholesterol excretion, bile salt formation, and glutathione production. Cysteine is a precursor of glutathione (GSH). However, approximately half of the intracellular GSH pool in human liver cells is derived from Hcy via the *trans*-sulfuration pathway (Mosharov *et al.*, 2000). The redox sensitivity of the *trans*-sulfuration pathway can be rationalized as an auto-corrective response that leads to an increased level of GSH synthesis in cells challenged by oxidative stress. The importance of the Hcy dependent *trans*-sulfuration pathway is the maintenance of the

intracellular GSH pool, and the regulation of this pathway under oxidative stress conditions. This pathway is dependent on adequate dietary intake and hepatic conversion of vitamin B6 into its active form, pyridoxal 5'-phosphate (P5P). Also the amino acid serine is necessary, which is a metabolite generated from betaine via the homocysteine-remethylation pathway.

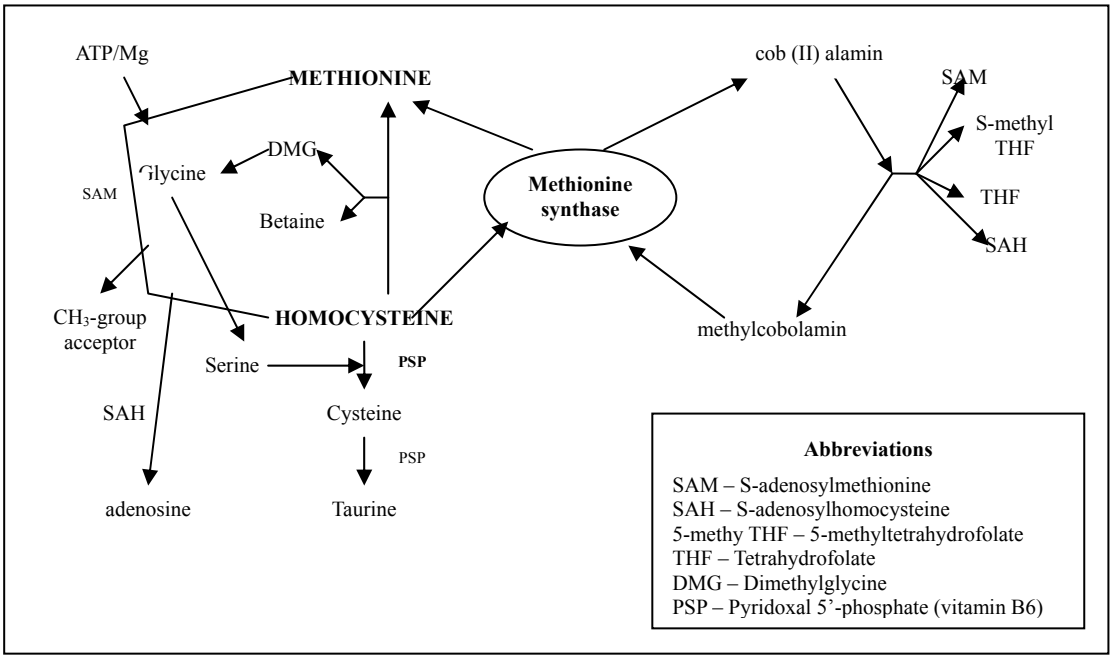


Figure 1.8. Homocysteine metabolism (Miller *et al.*, 1997).

1.4.4. The Mechanism of Neurotoxicity and the Role of Hcy

Elevated Hcy levels in the developing nervous system and adverse effects of folate/vitamin B12 deficiency, taken together with the fact that hyperhomocysteinemia, has prompted examination of potential roles for Hcy and deficiencies in one-carbon metabolism in age-related neurodegenerative disorders such as AD and PD. Hyperhomocysteinemia has been related to cerebral microangiopathy (Fassbender, 1999), endothelial dysfunction (Welch *et al.*, 1998), impaired nitric oxide activity (Chao *et al.*, 2000) and increased oxidative stress (Starkebaum *et al.*, 1986) and all factors associated with aging of the brain (McCann,

1887; Beal, 1995). Hcy also promotes copper-mediated and β-amyloid-peptide-mediated toxic effects in neuronal cell cultures (White *et al.*, 2001) and induces apoptosis in hippocampal neurons in rats (Kruman *et al.*, 2000). Hcy can damage and kill neurons in cell culture and can increase their vulnerability to being killed by various excitotoxic, oxidative and metabolic insults (Table 1.5).

Table 1.5. Examples of adverse effects of Hcy on neurons (Mattson & Shea, 2003).

Neurodegenerative agent or condition	Impact of Hcy	Neuroprotective agents
ROS	Increased	Vitamin E (Shea *et al.*, 2002)
Cytosolic Ca^{2+}	Increased	Chelators, channel blockers (Ho *et al.*, 2002)
Glutamate excitotoxicity	Increased	NMDA-receptor channel blocker (Ho *et al.*, 2002; Lipton *et al.*, 1997)
Tau phosphorylation	Increased	NMDA-receptor channel blocker (Ho *et al.*, 2002)
ATP levels	Depleted	PARP inhibitors (Ho *et la.*, 2002; Kruman, 2000)
Apoptosis	Increased	PARP inhibitors, SAM, antioxidants (Ho *et al.*, 2002; Kruman, 2000; Kruman, 2002; Ho *et al*, 2001)
Aβ-induced neurotoxicity	Increased	Vitamin E, 17β-estradiol (Kruman, 2002; Ho *et al.*, 2001)

Hcy induces DNA breakage in cultured neurons (Kruman, 2000) by a mechanism that may involve impaired *trans*-methylation of DNA, since folate and vitamin B12

deficiencies retard methionine regeneration, SAM levels are also reduced as a consequence of these vitamin deficiencies (Selhub *et al.*, 1992). In cultured neurons, Hcy depletes the ATP reserves of DNA, which is attempting to repair Hcy-induced DNA damage and thus induces apoptosis (Kruman., 2000). In this regard, depletion of cellular ATP is thought to be a key factor in neurodegeneration in AD and PD (Davey *et al.*, 1998; Mattson *et al.*, 1999). As with consideration of Hcy-induced oxidative stress, this maintains the possibility that moderate or slight hyperhomocysteinemia might foster a crucial reduction in ATP in neurons in aging brains in which ATP is already depleted.

Hcy acts as a partial antagonist of the glycine site of the *N*-methyl-D-aspartate (NMDA) receptor and therefore inhibits NMDA receptor mediated activity in the presence of normal concentrations of glycine. However, Hcy is also an agonist at the glutamate site of the NMDA receptor and is therefore a potential excitotoxin. Under normal conditions in the central nervous system, neurotoxicity would not occur because glycine levels are in the low micromolar range, whereas Hcy is 0.5μM in the cerebrospinal fluid (Hyland & Bottiglieri, 1992) and up to 10μM in certain brain regions (Broch & Ueland, 1984). Under conditions of normal glycine concentrations, the agonist (excitotoxic) action of Hcy at the glutamate site of the NMDA receptor would only prevail if levels approached millimolar concentrations. Indeed, this mechanism may contribute to the cognitive changes and markedly increased risk of cerebrovascular disease in children and young adults with this disorder. Elevated glycine levels synergize with Hcy to over-stimulate NMDA receptors and contribute to neuronal damage. Thus in conjunction with elevated glycine, micromolar concentrations of Hcy could contribute to neuronal injury if allowed access to the

extracellular space during tissue injury (Lipton *et al*, 1997) and enhance oxidative stress (Kim & Pae, 1996).

Oxidative damage resulting from Hcy is caused by increased cytosolic Ca^{2+} levels (Table 1.5) and DNA damage (Ho *et al.*, 2002; Kruman., 2000). In cultured neurons, Hcy treatment increases cytosolic Ca^{2+} and treatment with Ca^{2+}-channel blockers attenuates this increase, suggesting that the bulk of the increase results from influx across the plasma membrane (Ho *et al.*, 2002). Hcy potentiates glutamate neurotoxicity, and the toxicity of Hcy itself is attenuated by antagonisis of metabotropic glutamate receptors (Kruman, 2000; Ho *et al.*, 2001). Hcy also compromise glutathione peroxidase activity (Huang *et al.*, 2001a; Upchurch *et al.*, 1997) and reduce tissue levels of vitamins A, C and E (Henning *et al.*, 1997). Indeed vitamin C, vitamin E (Huang *et al.*, 2002) and estrogen (Dimitrova *et al.*, 2002) reduce Hcy-mediated apoptosis in non-neuronal cells by scavenging hydrogen peroxide. However, in the presence of increased Hcy, it causes an accumulation of S-adenosylhomocysteine hydrolase (SAH). SAH is a bi-directional enzyme that favours hydrolysis of SAH to homocysteine under normal conditions. Moreover, SAH is the demethylated product of SAM transmethylation, is itself a potent competitive inhibitor of SAM-mediated methylation reactions. Decreased intracellular methylation reactions can thus result from either a decrease in formation of SAM or an increase of SAH (Cantoni, 1986). Recent evidence demonstrates that Hcy induces DNA breakage and resultant apoptosis (Kruman, 2000; Kruman 2002), and that co-treatment with SAM prevents Hcy-induced apoptosis (Ho *et al.*, 2002).

Potentiation of neurodegeneration induced by treatments such as glutamate excitotoxicity and Aβ toxicity by Hcy maintains the possibility that even moderate

hyperhomocysteinemia can exert a deleterious impact on neuronal health far beyond what might be anticipated. Figure 1.9 shows the mechanism by which Hcy induces the death of neurons, and cell cycle arrest in neural stem cells and glial cells. The pathway that has adverse effects on developing and mature neurons is shown in red. It involves methyl donor deficiency resulting in increased DNA damage due to deficient DNA repair. DNA damage activates poly-(ADP-ribose) polymerases (PARP), resulting in ATP depletion and activation of the tumor suppressor protein p53, which can trigger cell-cycle arrest of apoptosis. These adverse effects of Hcy can be enhanced by oxidative stress and perturbed cellular Ca^{2+} that occurs during aging and in age-related neurodegenerative disorders. The emerging impact of Hcy on the nervous system suggests that diagnostic and therapeutic approaches should include attention to conditions that promote neurodegeneration.

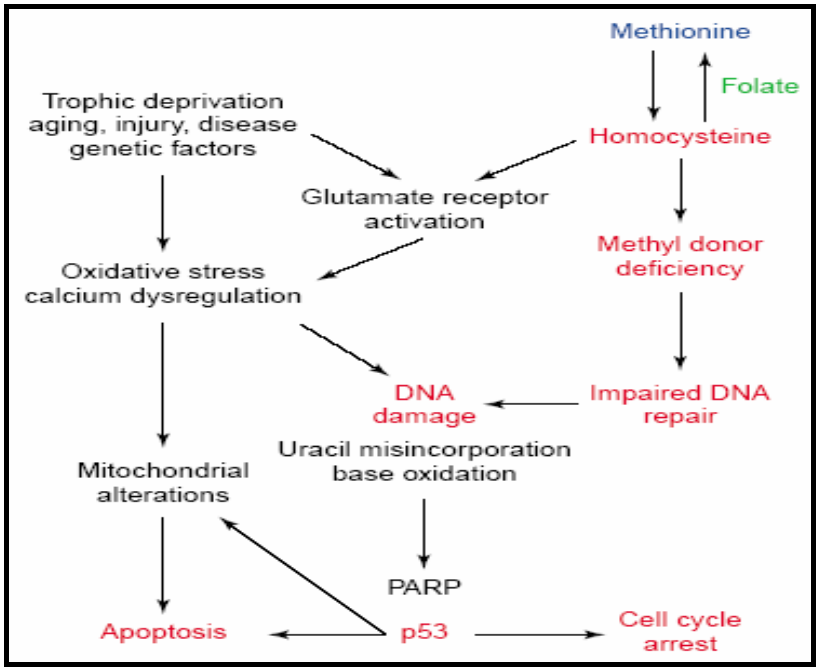


Figure 1.9. Mechanisms of Hcy-induced neuronal cell death (Mattson & Shea, 2003).

1.4.5. The Implication of Hcy in Neurodegenerative Diseases

Hcy metabolism associated with metabolic disorders resulting in Hcy accumulation suggests several possible mechanisms for the observed effects. Either a genetic defect in one of the enzymes of Hcy metabolism or a nutritional deficiency of one or more of the vitamins that participate in Hcy metabolism can lead to metabolic disruption and potentially to hyperhomocysteinemia. The results of clinical studies and experimental data accumulated during the last decade demonstrate that hyperhomocysteinemia may directly exert neurotoxicity. Even moderate hyperhomocysteinemia has been recognized as a strong, independent risk factor in the development of dementia and AD (Seshadri *et al.*, 2002). Moreover, increased oxidative stress, DNA damage, the triggering of apoptotic and excitotoxic cell death pathways are involved in the pathogenesis of each of the major neurodegenerative disorders including AD and Parkinson's disease (PD). The mechanisms occur despite the involvement of different disease-specific initiating factors – altered amyloid precursor protein (APP) processing in AD and accumulation of dopaminergic toxins in PD. Hcy levels increase during normal aging, and a high level of Hcy is an independent risk factor for stroke – the most prevalent age-related neurodegenative condition (Hankey *et al.*, 2001). In homozygous CβS metabolic defect, severely impaired Hcy *trans*-sulfuration and a diversion of the excess of Hcy toward the remethylation pathway to methionine has been observed (Figure 1.10). Methionine plays a key role in the generation of methyl groups required for the synthesis of DNA, and Hcy can be either remethylated to methionine by enzymes that require folate or cobalamin (vitamin B12) or catabolized by CβS, a pyridoxine (vitamin B6)-dependent enzyme, to form cysteine (Finkelstein *et al.*, 1990; Scott and Weir, 1998). The increased rate of methionine synthesis can lead to a temporal magnification of S-adenosylmethionine (SAM) concentrations and diminished levels of cysteine (Mudd *et al.*, 1985). Consequently, lack of cysteine availability causes impaired formation of glutathione (GSH), the

principal antioxidant in cells (Wang *et al.*, 1997). In such hyperhomocysteinemic conditions, the combination of the intensified free radical generation and diminished GSH formation is the main cause for AD. Hcy levels vary considerably among individuals, and reduced dietary intake of folate is associated with increased Hcy levels and increased risk for heart disease and stroke (Giles *et al.*, 1995). In addition, folate deficiency can cause DNA damage that may result from hypomethylation. As shown in figure 1.8, Hcy can also cause synaptic dysfunction and neuronal death by promoting DNA damage and activation of apoptotic signaling cascades involving p53, Bax, mitochondrial alterations, release of cytochrome c and caspase activation.

Hcy may also have direct actions on glutamate receptors that result in enhanced Ca^{2+} influx. Finally, Hcy induces endoplasmic reticulum stress, which might contribute to its pathogenic actions. Patients with severe hyperhomocysteinemia exhibit a wide range of clinical manifestations including neurological abnormalities such as mental retardation, cerebral atrophy, and seizures (Watkins & Rosenblatt, 1989).

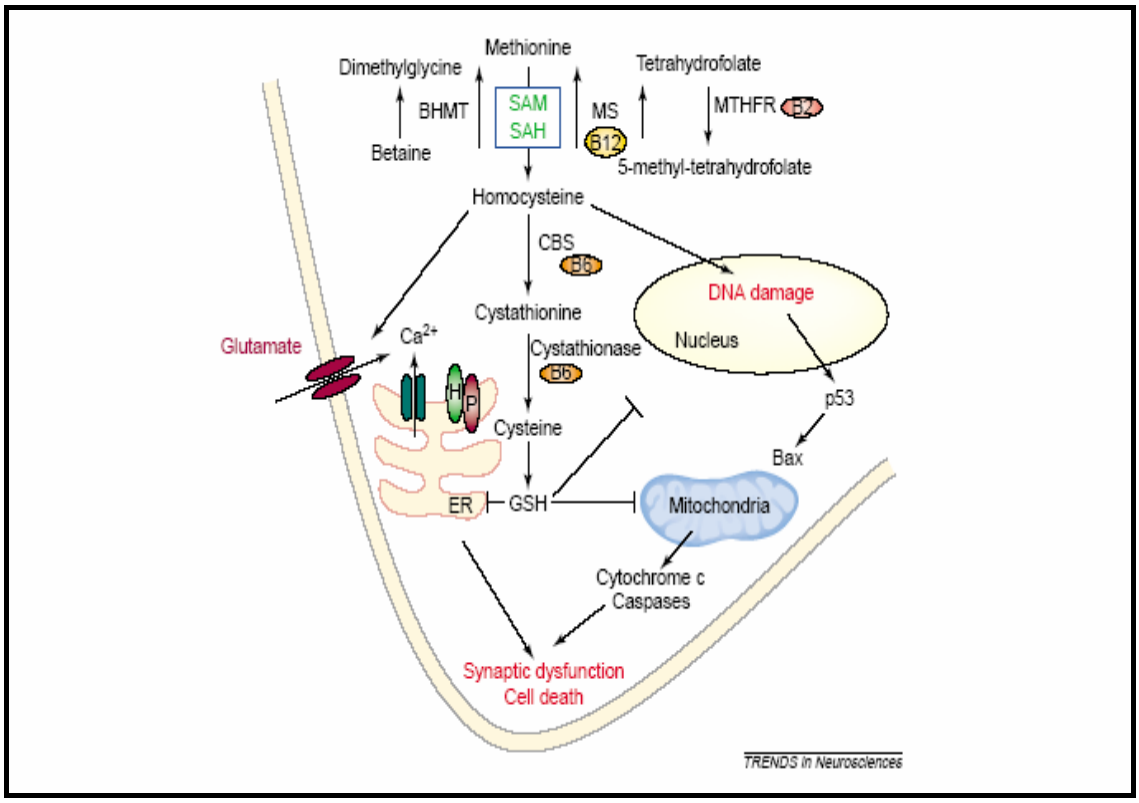


Figure 1.10. The roles of Hcy in one-carbon metabolism and neuronal survival and death (Mattson & Shea, 2003).

1.4.6. The Involvement of Hcy in Alzheimer's Disease

Hcy is reversibly formed and secreted during metabolism, is a potent neurotoxin (Lipton *et la.*, 1997; Kruman, 2000). An elevated plasma level of Hcy is recognized as an independent risk factor for cardiovascular, peripheral vascular and cerebrovascular disease (Refsum *et al.*, 1998). Because of the emerging evidence supporting a role for vascular disease in the etiology of AD, it has been hypothesized that elevated plasma Hcy also plays a role in the pathogenesis of AD and age-related cognitive decline. Studies of human populations and experimental models of AD suggest roles for Hcy in the disease process (Kruman, 2002; Seshadri *et al.*, 2002). Plasma Hcy has been reported to be elevated in some (Joosten *et al.*, 1997; Clark *et al.*, 1998), but not all (Fekkes *et al.*, 1998) confirmed cases of AD and represents an early marker of

cognitive impairment in the elderly (Lehmann *et al.*, 1999). However, individuals with AD exhibit elevated levels of Hcy (Clark *et al.*, 1998; Miller *et al.*, 1999; Gottfries *et al.*, 1998; McCaddon *et al.*, 1998), which can occur before disease onset (Joosten *et al.*, 1997). In addition to the increased risk of AD, hyperhomocysteinemia is also associated with poor performance on objective tests of cognitive function in both case-control studies of patients with AD and cross-sectional, population-based studies of community-dwelling elderly people (Riggs *et al.*, 1996; McCaddon *et al.*, 1998).

Factors contributing to the onset and development of AD include genetic predisposition, Aβ accumulation, increased oxidative stress, and excitotoxicity (Mattson, 1997; Growdon, 2001). Hcy can potentiate Aβ neurotoxicity in cultured neurons (Kruman, 2002; White *et al.*, 2001) and in transgenic mice over-expressing APP that develop amyloid deposits in the brains (Kruman, 2002). Ho *et al.,* (2001) demonstrates that concurrent exposure of cultured neurons to Aβ and Hcy induces oxidative stress and apoptosis at concentrations which when Aβ and Hcy are given independently. Moreover, increased oxyradical production occurs in cultured neurons exposed to Hcy (Kruman, 2000; Ho *et al.*, 2001). The resultant levels of oxyradicals and apoptotsis of either Aβ or Hcy alone, indicate a synergistic effect of Hcy and Aβ on neurodegeneration (Ho *et al.*, 2001). Since oxidative stress is at the core of neuronal degeneration in AD, increased oxidative stress represents one mechanism which contributes to neurodegeneration.

Moreover, elevated Hcy levels in subjects with cognitive impairment of dementia could be a result of poor nutrition and vitamin deficiencies (Diaz-Arrastia *et al.*, 1998). Low serum levels of folate, vitamin B6 and vitamin B12 have been associated with

elevated Hcy levels in several studies and with an increased risk of dementia in a few investigations (Cole *et al.*, 1984; Karnaze *et al.*, 1987; Ikeda *et al.*, 1990; Levitt *et al.*, 1992; Wang *et al.*, 2001). Kruman *et al.,* (2000) and Ho *et al.,* (2001) demonstrate that abnormal accumulation of Hcy due to vitamin deficiencies promote neurodegeneration in AD.

The full range of causes of AD remains unclear, but has been considered to be multifactoral (Holscher, 1998). Clinical studies implicate homocysteinemia in neurodegeneration, including that related to AD (Clark *et al.*, 1998; Fekkes *et al.*, 1998; Gottfries *et al.*, 1998; Joosten *et al.*, 1997; Lehmann *et al.*, 1999). Ho *et al.*, (2002) demonstrates that Hcy increases: cytosolic Ca^{2+}, generation of ROS, activity of calcium-dependent kinases, tau phosphorylation and apoptosis in cell culture. All of these pheonomena induced by Hcy in cultured neurons are directly elucidating the neuropathyology in AD (Ho *et al.*, 2002).

1.4.7. Neuroprotection Against Hcy

Hcy can impart multiple neuropathological effects, including cytosolic accumulation of Ca^{2+}, induction of oxidative stress, hyperphosphorylation of tau and apoptosis, all of which are characteristic of affected neurons in AD (Holscher *et al.*, 1998). Hcy is an agonist for NMDA channels, therefore co-treatment with NMDA channel blocker, MK-801, can prevent Hcy-induced Ca^{2+} influx (Kim, 1999). Inhibition of Ca^{2+} influx via co-treatment with MK-801 prevents tau phosphorylation and ROS generated in response to Hcy treatment. In addition to this finding, there are prior reports (Kruman, 2000; MacDonald & Wojtowicz, 1980) which suggest that Hcy exerts deleterious effects on neurons by acting as an excitotoxin, causing glutamate excitotoxicity.

Therefore, Hcy induced Ca^{2+} influx can be attenuated by antagonists of non-NMDA glutamate receptors (Ho *et al.*, 2002). However, the pivotal role of ROS in Hcy toxicity can be prevented by superoxide dismutase and/or catalase (Kim & Pae, 1996) and by the glutathione precursor N-acetyl cysteine (Ho *et al.*, 2001).

1.5. Estrogen

1.5.1. Introduction

The gonadal steroid hormone 17β-estradiol is a greatly under-appreciated neural growth and trophic factor for the mammalian brain of all ages. It is a "traditional" estrogen and is responsible for many of the reproductive effects associated with it. 17β-estradiol influences neurogenesis, neuronal differentiation, and neuronal survival of its targets throughout life (Garcia-Segura *et al.*, 2001; Wang *et al.*, 2002; McEwen, 2002; Behl, 2002). 17β-estradiol has been associated with a decreased risk, delayed onset and progression, or enhanced recovery from numerous traumatic or chronic neurological and mental diseases. However, recent studies suggest that estrogen replacement therapy may help to reduce the risk (Baldereschi *et al.*, 1998; Henderson *et al*, 1996; Kawas *et al.*, 1997; Tang *et al.*, 1996) and severity (Honjo *et al.*, 1995; Ohkura *et al.*, 1994; Ohkura *et al.,* 1995) of Alzheimer's-related dementia in postmenopausal women. In addition, studies have shown that estrogen replacement can enhance measures of verbal memory and associative learning in young and older women (Henderson *et al.*, 1996). These findings raise important questions about the potential of hormone replacement therapy to enhance cognitive performance and to reduce or prevent age-related cognitive decline in postmenopausal women. The neurobiological mechanisms that underlie these effects are currently unknown, but most likely reflect effects of estrogen on the survival, connectivity, and function of

specific neural systems.

1.5.2. Biosynthesis Pathway and Metabolism of Estrogen

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 *et al.*, 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 *et al.*, 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 *et al.*, 1969).

1.5.3. Mechanism of Action of Estrogen Neuroprotection

1.5.3.1. Genomic and Non-genomic Neuroprotective Effects of Estrogen

The general neuroprotective activities of 17β-estradiol are manifold. It is a steroid molecule that can bind to specific intracellular estrogen receptor receptors (ERs). In the mammalian brain, intracellular ERs are widespread and are found in the cerebral cortex, midbrain, hippocampus, brain stem, hypothalamus and pituitary gland. Activation of

ERs has long-term genomic effects such as regulation of nerve cell development and protection of the brain during aging (McEwen & Alves, 1999), which helps to reserve memory and other cognitive functions of hippocampus. The genomic neuroprotective activity of estrogen promotes the outgrowth of neurite, sprouting of neurons, synaptogensis, and the expression of neurotrophic factors such as nerve growth factor (NGF) and increases the synthesis of acetylcholine (McEwen & Alves, 1999; Toran-Allerand, 1996). There are two ERs, ERα and ERβ, have been identified. ERα, which mediates most of estrogen's transcriptional actions in the brain (White *et al.*, 1987) and is expresses in hippocampus. ERβ is abundantly expressed, which the neural role remains largely uncharacterized (Kuiper *et al.*, 1996; Tremblay *et al.*, 1997), but may be modulatory, inhibitory or even reciprocal with ERα (Lindberg *et al.*, 2003; Toran-Allerand *et al.*, 2004). ERα and ERβ are complementary but not redundant and are genetically and functionally distinct. In addition, estrogen affects other regions of the brain that express ERs including basal forebrain transmitter systems for acetylcholine, serotonin, dopamine and noradrenaline (McEwen & Alves, 1999). The mechanisms of the neurotrophic activities of estrogen suggest that estrogen and its receptors could prove to be important novel targets in the research for neuroprotective agents (McEwen & Alves, 1999).

However, not all of the neuromodulaory activities of estrogen are a result of nuclear receptor occupation, receptor activation and alteration of gene transcription (McEwen & Alves, 1999). In addition to the classical ERs, the neuroprotective functions can be at the membrane-binding sites (membrane receptors) by modulating neurotransmission (Wetzel *et al.*, 1998) or by modulating the excitability of neuronal membranes (Gu *et al.*, 1999), which is independent on ERs (non-genomic).

1.5.3.2. Antioxidant Effects of Estrogen

With respect to its chemical structure, estradiol is a steroid but also a monophenolic compound. 17β-estradiol possesses an aromatic A ring phenolic moiety at the C3, which serves as a radical scavenger (Behl, 1997) and a lipophilic carbohydrate moiety (Figure 1.11). 17β-estradiol is a natural antioxidant of membrane phospholipid peroxidation (Sugioka, 1987). The phenolic A ring of 17β-estradiol is a powerful inhibitor of lipid peroxidation (Moosmann & Behl, 1999; Nakano *et al.*, 1987; Sugioka *et al.*, 1987) and 17β-estradiol has been reported to be a more potent inhibitor of the propagation of lipid peroxidation in Mongolian gerbils brain tissue than vitamin E (Hall, 1991). Several *in vivo* studies show that 17β-estradiol has an ability to act as an antioxidant and reduce lipid peroxidative damage (Hall & Sutter, 1998) (figure 1.11).

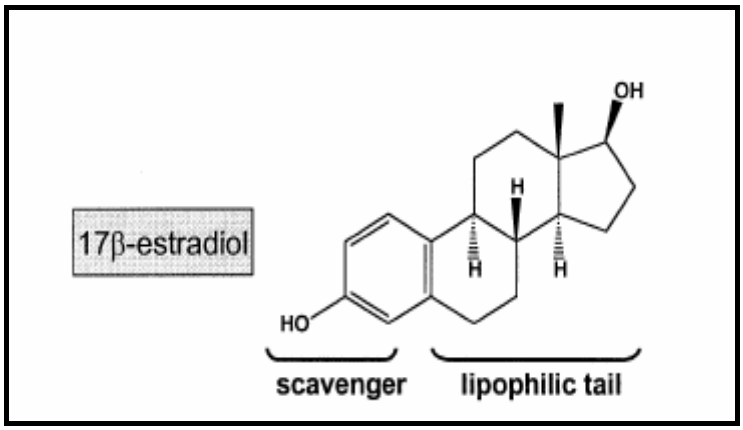


Figure 1.11. The monophenolic antioxidants structure of 17β-estradiol: the radical scavenging headgroup and the lipophilic tail (Behl, 2002).

17β-estradiol also exerts an indirect antioxidant action secondary to its ability to increase $NO^{\cdot}$. $NO^{\cdot}$ can act as an inhibitor of the lipid peroxidation chain reactions by scavenging lipid peroxyl radicals (Winkv *et al.*, 1995). Therefore increased $NO^{\cdot}$ levels will decrease lipid peroxidation damage (figure 1.12). Behl *et al.*,(1995) showed that oxidative cell death caused by Aβ, H_2O_2, and glutamate in mouse clonal hippocampal

cells and demonstrated that preincubation of the cells with 17β-estradiol prevents oxidative stress-induced cell damage and cell death (figure 1.12). However, the antioxidant activity of estrogen is not dependent on estrogen receptors and is therefore, non-genomic in nature.

However, the over-expression of the antioxidant enzyme Cu, Zn superoxide dismutase (Cu, Zn SOD) by estrogen is further evidence for *in vivo* antioxidant neuroprotective effect of estrogen (Hall & Sutter, 1998).

1.5.3.3. Neuroprotective Effects of Estrogen on β-Amyloid Production

Estrogen has been suggested to be beneficial in the prevention of AD (Henderson *et al.*, 1996; Tang *et al.*, 1996), is known to protect neurons from the cytotoxic effects of Aβ. Both high (Goodman *et al.*, 1996a) and physiological (Green *et al.*, 1996) doses of 17β-estradiol protect hippocampal neurons *in vitro* against Aβ-induced toxicity. An *in vivo* study (Shi *et al.*, 1998) shows that 17β-estradiol decreases production the Aβ precursor APP. Therefore, 17β-estradiol not only protects against Aβ toxicity, but decreases its production as well.

However, some reports (Mattson & Rydel, 1992; Behl, 1994) have demonstrated that Aβ toxicity is due to the formation of oxygen radicals and the subsequent initiation of membrane lipid peroxidation. It has been suggested that high doses of 17β-estradiol attenuate Aβ toxicity by reducing oxidative stress (Behl, 1995; Goodman *et al.*, 1996a) (figure 1.13). Thus, the neuroprotective effects of 17β-estradiol against Aβ in AD are due to antioxidant action.

1.5.3.4. Neuroprotective Effects of Estrogen on Glutamate-induced Excitotoxicity

Glutamate is the major excitatory amino-acid neurotransmitter in the brain and spinal cord (Wikinski & Acosta, 1995). At normal concentrations, glutamate is an essential signal molecule, but excessive glutamate release may have negative effects (Wikinski & Acosta, 1995; Hugon *et al.*, 1996a). The activation of glutamate receptors, e.g. NMDA receptors, causes a significant Ca^{2+} influx into neurons (Wikinski & Acosta, 1995; Scatton, 1993). If the Ca^{2+} influx is sufficiently large, this may result in cell death (Schousboe *et al.*, 1997). Glutamate receptors of the NMDA type are most densely concentrated in the cerebral cortex, hippocampus and cerebellum compared to other brain regions (Scatton *et al.*, 1993). Both the increased metabolism and excitotoxicity resulting from glutamate-induced stimulation of NMDA receptors may be reduced by estrogen (Weaver *et al.*, 1997). Several studies have shown that estrogen may protect against neuronal death due to excitotoxicity. 17β-estradiol attenuates the toxicity of glutamate exposure to hippocampal cell line culture (Behl, 1995; Goodman *et al.*, 1996a) and murine hippocampal HT-22 cells (Behl, 1995). Moreover, these data suggest that the neuroprotective effects against glutamate-mediated excitotoxicity are due to the steroidal chemical antioxidant action of 17β-estradiol rather than its ability to produce a genomic effect via the estrogen receptor that is critical for neuroprotection against excitotoxicity.

1.5.3.5. Neuroprotective Effects of Estrogen on MAP Kinase Pathways

Singer *et al.*, (1999) suggest that in addition to an antioxidant effect, estrogen protection against glutamate toxicity is mediated by activation of signalling pathways

by mitogen-activated protein kinase (MAPK) (Figure 1.12). MAPK plays an important role in both growth factor signalling and stress signalling (Seger & Kreb, 1995) and expression of MAPK is involved in blocking apoptosis (Sakata *et al.*, 1995).

1.5.3.6. Neuroprotective Effects of Estrogen on bcl-2 Expression

Bcl-2 is an anti-apoptotic protein (Hockenberry *et al.*, 1990) and is a survival factor that can block both necrotic and apoptotic cell death (Bredesen, 1995) and over-expression of bcl-2 protects neurons from glutamate toxicity (Zhong *et al.*, 1993; Singer *et al.*, 1998). Estrogen up-regulates bcl-2 expression in brain (Garcia-Segura *et al.*, 1998). Figure 1.12 shows the neuroprotective actions of estrogen on bcl-2. 17β-estradiol includes the prevention of activation of caspases, inhibition of free radical formation and increases in regulation of calcium sequestration (MacManus & Linnik, 1997). 17β-estradiol pre-treatment increased bcl-2 levels and enhanced survival of NT neurons *in vitro* after exposure to glutamate (Singer *et al.*, 1998). Therefore, bcl-2 induction may be a mediator of neuroprotection of estrogen.

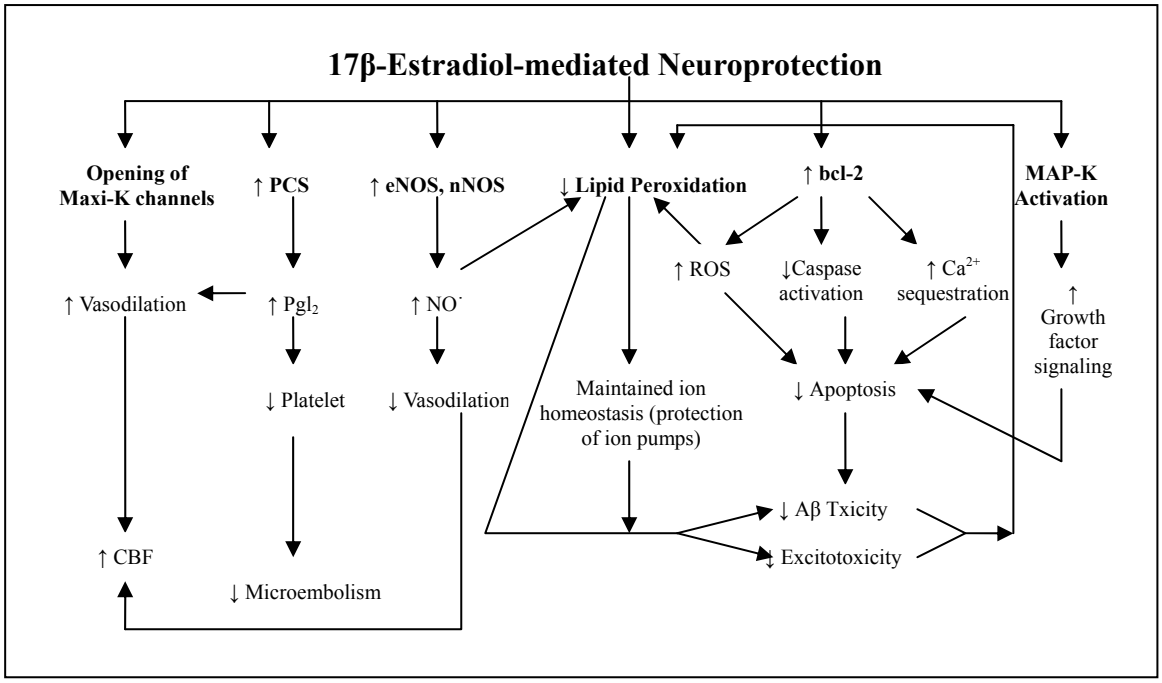


Figure 1.12. Mechanisms by which 17β-estradiol may exert its neuroprotective effects. PCS, prostacyclin synthetase (Roof & Hall, 2000).

1.5.4. Neuroprotective Effects of Estrogen and Alzheimer's Disease

AD afflicts more women than men, has an earlier onset in women, and progresses more rapidly in women. Many epidemiological studies have pointed to the protective role of estrogen in AD. One extensive clinical study assessing the protective role of estrogen in AD was conducted by Paganini-Hill and Henderson (1996). These authors found an inverse relationship between estrogen use and AD; the risk of AD decreased with increasing dose and duration of estrogen replacement therapy. Exogenous estrogen treatment appears to be the key, because there was no relationship between AD and age at menarche or age at the last menstrual period. However, due to the formation in adipose tissue, women who have high body weight, are presumably have high levels of estrogen, thus conferred some decreased risk for AD.

One of the earliest clinical trials assessing the potential impact of estrogen on AD was

a small pilot study of seven women who had been diagnosed with senile dementia of the Alzheimer's type (Fillit, 1986). Low-dose estrogen therapy modestly improved cognitive function and mood in some of the women. However, the study of Henderson *et al.,* (1994) also shows that estrogen not only protects against the onset of AD, but also helps to maintain the cognitive function of those women who already have AD. The cognitive function of women with AD who had received estrogen replacement therapy was well below non-demented control subjects, but better than the cognitive function of those AD patients not taking estrogen. A double-blind study of 12 women with mild to moderate AD, estrogen (0.05 mg/day of 17β-estradiol, delivered via a skin patch), improved attention and verbal memory within the first week of treatment, but the effects were temporary, and diminished once estrogen treatment was stopped (Asthana *et al.*, 1999). Estrogen increased activation of cortical regions involved in verbal memory tasks during storage and retrieval of verbal material, without altering verbal or nonverbal performance, in postmenopausal women given estrogen treatment (1.25 mg/day, oral dose, 21 days, double-blind) and assessed using functional magnetic resonance imaging.

Figure 1.13 shows the potential cellular mechanisms that underly estrogen's neuroprotective actions in AD. 17β-estradiol may affect Aβ deposition by the regulation of Aβ metabolism and ApoE expression (Jaffe *et al.*, 1994; Chang *et al.*, 1996; Xu *et al.*, 1998a, 1998b), which deposition of amyloid in the neuritic plaques are one of the neuropathological hallmarks of AD. 17β-estradiol increases cognitive function by enhancing cholinergic neurotransmission. Cholinergic neurons in the basal forebrain degenerate in AD patients (Whitehouse *et al.*, 1981) and 17β-estradiol can up-regulate the expression of choline acetyltransferase in AD in the rat brain (Luine *et al.*, 1985). Finally, 17β-estradiol may influence affective disorders

associated with AD by acting on serotonin receptor signaling (Halbreich *et al.*, 1995). 17β-estradiol decreases the G proteins that couple serotonin receptors to intracellular signaling molecules (Raap *et al.*, 2000) or via alterations in serotonin transporter expression (McQueen *et al.*, 1997).

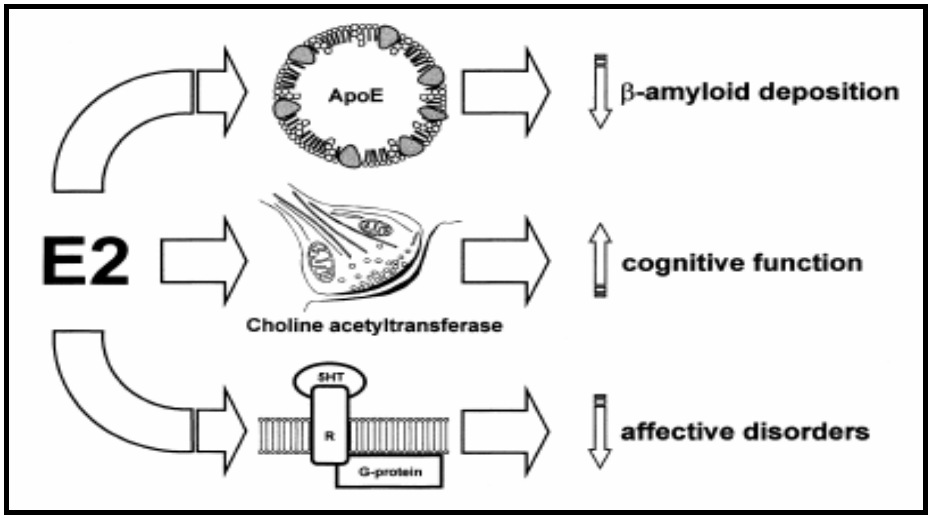


Figure 1.13. Mechanisms of actions of 17β-estradiol in Alzheimer's disease (Garcia-Segura *et al.*, 2001).

However, the etiology of AD is clearly multifactorial, but for many of the factors, estrogen may provide protective value, and estrogen receptor malfunction may increase risk of AD. Considerable support is therefore available for the use of estrogen replacement therapy to decrease vulnerability to AD, and in those individuals who are already diagnosed with AD to maintain cognitive function (Henderson, 1994; 2000; Mulnard *et al.*, 2000)

1.6. Progesterone

1.6.1. Introduction

While the majority of researchers have focused on estrogen as the source of neuroprotection observed in females, there is evidence that another female hormone,

progesterone, may also play a beneficial role in the injured brain. Progesterone is known to play an important role in the regulation of behavioural and neuroendocrine functions by modulating brain neurotransmission, such as the inhibitory γ-aminobutyric acid (GABA) (Bureau & Olsen, 1990; Kokate *et al.*, 1994) and EAA systems (Wu *et al.*, 1991). Over recent years, research has revealed that multiple actions of progesterone in the central and peripheral nervous system. Progesterone is an important signalling molecule in the nervous system, regulating neuron and glial functions by multiple mechanisms of action. However, in nerve cells, the actions of progesterone are exerted classically through cytosolic/nuclear receptors specific for the steroid (Pfaff & McEwen, 1983). Progesterone has potent anti-convulsant, anxiolytic and seizure suppression activity (Belelli *et al.*, 1989; Bitran *et al.*, 1991; Twyaman & Macdonald, 1992). Treatment of experimental traumatic brain injury with progesterone reduces functional deficits and secondary neuronal loss (Roof, 1994; Roof, 1997). Progesterone reduces cerebral oedema (Betz & Coester, 1990a) and facilitates cognitive recovery after acute global cerebral ischemia (Gonzalez-Vidal *et al.*, 1998).

The presence of receptors and sources of progesterone within the nervous system as well as its modulation of inhibitory and EAA suggest a broader role for progesterone than simply as a gestational hormone. Thus, progesterone may be involved in the process of brain damage after ischemic insult and the administration of progesterone may be beneficial to the neuronal tissue.

1.6.2. Biosynthesis Pathway and Metabolism of Progesterone

Progesterone is synthesized by the ovaries and placenta. The adrenal glands contribute significant amounts of circulating progesterone in humans of both genders.

Progesterone is a precursor of gluco- and mineralocorticosteroids in the adrenal glands. Progesterone is secreted into the bloodstream (Feder *et al.*, 1968; Holzbauer *et al.*, 1969). Because of the lipid solubility, progesterone crosses the blood brain barrier easily and it diffuses throughout nervous tissues. Progesterone can be synthesized by neurons and glial and some of the progesterone present in brain, spinal cord and peripheral nerves is derived from local synthesis.

Figure 1.14 shows the biosynthesis and metabolism of progesterone. The first step in the biosynthesis is the conversion of cholesterol to pregnenolone, the precursor of all steroid hormones. The cholesterol moves from intracellular stores to the inner mitochondrial membrane, where the cytochrome P450scc is located. The cytochrome P450scc catalyzes the conversion of cholesterol to pregnenolone. Pregnenolone then moves from the mitochondria to the microsomal compartment, where it is converted to progesterone by the 3β-hydroxy- steroid dehydrogenase (3βHSD). Progesterone can be converted to 5a-dihydroprogesterone by 5α-reductase and the 3α-hydroxysteroid oxidoreductase (3αHSD) catalyzes the conversion of 5α-dihydroprogesterone to 3α,5α,tetrahydroprogesterone (allopregnanolone) (Schumacher, 2001).

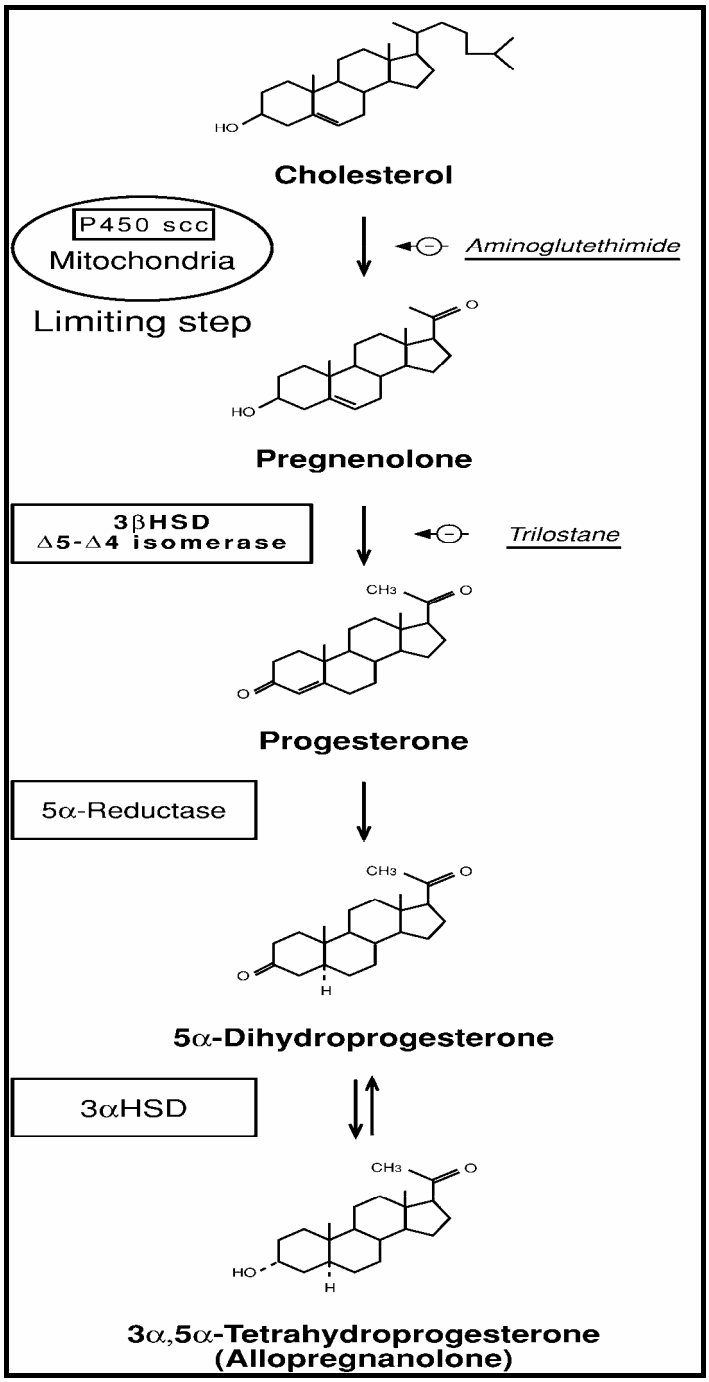


Figure 1.14. Biosynthesis and metabolism of progesterone (Schumacher *et al.* 2001).

1.6.3. Mechanism of Action of Progesterone Neuroprotection

The mechanism underlying neuroprotection against brain trauma and ischemia by progesterone has received less attention. The neuroprotective effects of progesterone have been reported to include improved blood-brain barrier integrity, limitation of free

radical induced lipid peroxidation and reduction of cerebral oedema (Betz & Coester, 1990a, b; Roof, 1994). Figure 1.15 summarizes the neuroprotective mechanisms of progesterone. Progesterone reduces membrane lipid peroxidation by promoting neuronal membrane stabilization (Roof *et al.*, 1994; Roof *et al.*, 1997) (figure 1.15) and acts as a free radical scavenger (Betz & Coester, 1990a). Progesterone, acts like other steroids, intercalates between the polyunsaturated fatty acid residues of the membrane phospholipids (Anderson, 1982). This causes cell membranes to be less fluid and protects them from ROS attack and calcium toxicity (Hall, 1993).

Progesterone binds to the intracellular progesterone receptor (PR) and activates the transcription of hormone-sensitive genes. High levels of PR mRNA and binding are found in brain regions involving in reproductive functions such as hypothalamus and the receptor is present in the brain, spinal cord and peripheral nerves (Hagihara *et al.*, 1992; Jung-Testas *et al.*, 1996; Lacor *et al.*, 1996; Lauber *et al.*, 1991; MacLusky & McEwen, 1980). Progesterone not only regulates nervous functions by activating gene transcription ('genomic effects'), they also modulate the activity of neurotransmitter receptors by acting directly on the neuronal membrane. Inhibition of EAA acid receptors or potentiation of GABA receptors can be neuroprotective. Excitotoxicity is reduced by progesterone's inhibition of excitatory amino acid receptors such as glutamate (Bergeron, 1996) (figure 1.15) and by its ability to depress spontaneous firing of neurons that could lead to post-injury seizures (Mohammad *et al.*, 1998). Physiological levels of progesterone enhance GABA-mediated inhibition of neuronal activity (Smith, 1994) and can alter the excitability of neurons by potentiation of GABA-evoked Cl^- currents. However, it has been shown recently that progesterone and its metabolites produce an antagonistic effect at the GABA receptor (Roof & Fritts, 1997).

Progesterone also attenuates EAA responsiveness (Smith, 1987; Smith, 1991). Progesterone depresses both kainite and NMDA responses of cerebellar neurons. Progesterone provides neuroprotection by NMDA antagonism, thus preventing neuronal death that caused by glutamate-induced excitoxicity (Hara *et al.*, 1993). Thus, progesterone may lower glutamate-induced excitotoxic cell death by potentiating inhibitory GABA receptor activation and by inhibiting excitatory EAA receptors (figure 1.15). In addition, progesterone also mediates anti-apoptotic actions through a progesterone-binding protein with $GABA_A$ receptor feature (Peluso *et al.*, 1998).

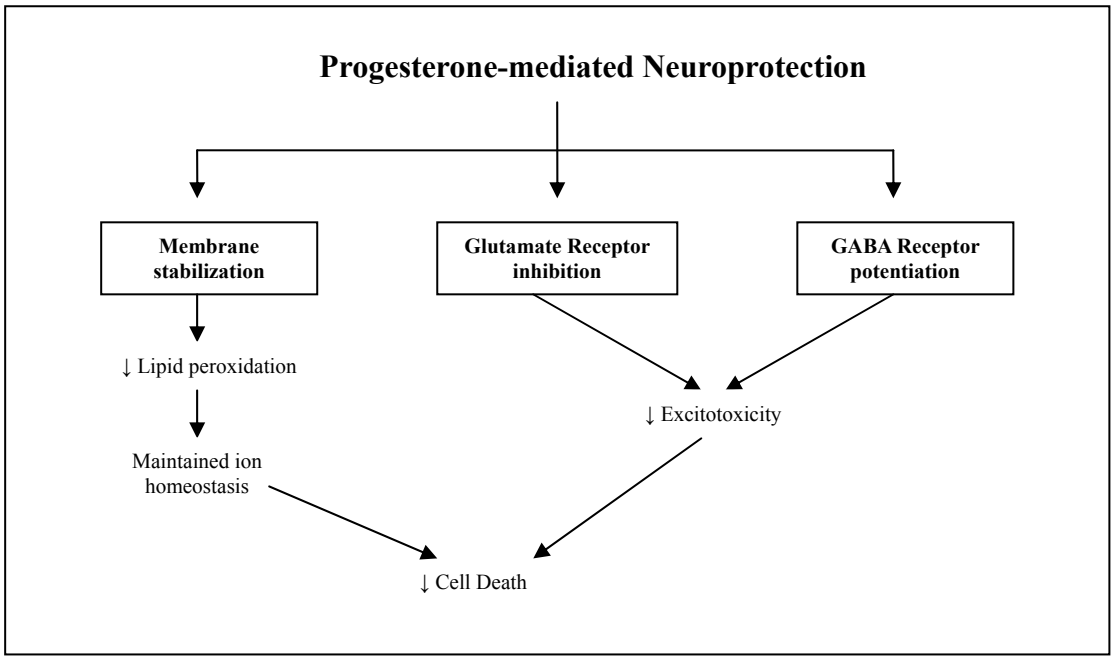


Figure 1.15. Mechanism by which progesterone may exert its neuroprotective effects (Roof & Hall, 2000).

1.7. Hormone Replacement Therapy and Alzheimer's Disease: The role of Homocysteine

Although estrogen and progesterone have neuroprotective properties in many animal models of neurodegeneration, the results from hormone replacement therapy (HRT,

estrogens and progestins) or estrogen-only replacement therapy (ERT), in women, are not yet conclusive. Most studies suggest that ERT increase memory and cognitive function in healthy women (Wise, 2003). ERT may also prevent or delay the onset of AD and improve cognition for women with AD (Asthana *et la.*, 2001; Paganini-Hill & Henderson, 1996), reduce the motor disability associated with PD (Dluzen & Horstink, 2003, Saunders-Pullman *et al.*, 2003) and decrease the risk of stroke in postmenopausal women (Paganini-Hill *et al.*, 2001). However, other studies indicate that ERT or HRT have no effect on PD (Saunders-Pullman *et al.*, 2003) and do not improve cognition of women with AD (Wise, 2003). Several explanations for discrepant results among different studies, such as differences in dose, formulation, route of administration, length of treatment, sample size, and age of the women receiving the treatment, have been proposed (Wise, 2003). However, the Women's Health Initiative (WHI) randomized trail studies show that long-term hormonal therapy started in women aging 65 or more years have beneficial effects in decrease the risk in AD and PD. WHI also shows that the highest efficacy of hormonal therapy is during the peri-menopause. During this period, the brain may be adapting local steroid synthesis to the new situation created by the loss of ovarian function; thus the steroid receptor machinery is not yet familiar and adjusted to the new physiological condition. In any case, the results of the WHI study emphasize the need for a better understanding on the effects of sex hormones in the brain.

However, reports from observational and clinical trials show that those women who are on HRT have lower plasma levels of Hcy (Barnabei *et al.*, 1999; Mijatovic *et al.*, 1998; van der Mooren *et al.*, 1994). Observational studies have found an increased risk of myocardial infarction and stroke associated with modestly elevated blood levels of Hcy (Aronow *et al.*, 2000; Bostom *et al.*, 1999). Hcy is a risk factor for AD,

dementia or poor cognitive function. Case control studies have found higher levels of Hcy in AD patients (Clark *et al.*, 1998; Nilsson *et al.*, 1996), and among AD patients, those with higher levels of Hcy have a greater extent of atrophy of the medial temporal lobe (Clark *et al.*, 1998). High levels of Hcy are associated with poorer cognitive function in population-based studies of community-dwelling elderly people and AD patients seen in clinics (Lehmann *et al.*, 1999; Riggs *et al.*, 1996). A recent report based on the Framingham study demonstrated that high levels of Hcy (14μmol/L or greater) are positively associated with the risk of developing AD and dementia (Seshadri *et al.*, 2002) and Hcy is associated with poorer cognitive performance only among postmenopausal women not taking HRT (Whitmer *et al.*, 2003). However, evidence indicates that Hcy plays a role in the progression of AD and neurological function in those without AD. The *in vivo* studies suggest that Hcy plays a neurotoxic role in cell injury (Althausen & Paschen, 2000). Hcy has been found to elicit DNA damage in rat hippocampal neurons (Kruman, 2000) and to potentiate Aβ and copper-mediated toxicity in mouse neuronal cultures (White *et al.*, 2001; Ho *et al.*, 2001). These findings demonstrate that in animal models, Hcy increases the vulnerability of hippocampal neurons to excitotoxic and oxidative injury. Thus, the neurophysiological mechanism for HRT and improved cognition may be a reduction of serum Hcy levels (Christodoulakos *et al.*, 2003).

1.8. Objectives

The study was undertaken to investigate the neuroprotective properties of estrogen and progesterone against damage induced by Hcy in the hippocampus. Hcy is known to be a neurotoxic and the concentration of Hcy in the brain increases with age. A high level of Hcy is associated with AD and loss of cognitive functions. Hcy is known to be an endogenous agonist at the NMDA receptor, causing neuronal damage due to massive calcium influx into the cell and induces apoptosis in hippocampal neurons. Accumulating evidence has suggested an action of estrogen and progesterone on excitatory amino acid receptors. Since the hippocampus is the primary region involved in memory formation and cognitive functions, and estrogen and progesterone have been reported to improve cognitive performance and memory, the hippocampus was chosen as the area of the brain to study.

The objective of the study was to investigate the protective effects of estrogen and progesterone against Hcy-induced oxidative stress and resultant neuronal damage and also to elucidate the cellular mechanisms whereby estrogen and progesterone may exert any protective effects in hippocampal neurons. In order to achieve these objectives, the following experiments were performed:

Chapter 2: Lipid peroxidation studies. This was done in order to show that both estrogen and progesterone reduce Hcy-induced lipid peroxidation in rat brains. Thus, the neuroprotective actions of both hormones are dependent on the antioxidant effects.

Chapter 3: Superoxide anion generation. In this chapter, NBT assay was employed. This was done to show both hormones have ability to reduce the superoxide anion neurodegeneration induced by Hcy in the rat hippocampus *in vivo.*

Chapter 4: Histology studies. In this chapter, Nissl staining was used for evidence of morphological changes in the rat hippocampus after treatment. This experiment was performed to show that both hormones have shown neuroprotection against Hcy-induced intrahippocampal lesions in the rat brain.

Chapter 5: Apoptosis. The TUNEL assay for programmed cell death was employed. This study indicated that both hormones were able to prevent against the Hcy-induced apoptotic cell death in both CA1 and CA3 regions of the rat hippocampus.

Chapter 6: Enzymatic antioxidants: Defense system. SOD and GSH are the most important antioxidant enzymes in the ROS defense system. Therefore, this chapter was to evaluate whether both hormones altered the effects of Hcy on both enzymatic antioxidant activities.

Chapter 7: Radioligand binding studies. NMDA receptor binding in isolated synaptic membranes was investigated. It was done to show that both hormones offered protection against Hcy-induced neurotoxicity via a NMDA receptor-dependent mechanism.

CHAPTER 2

LIPID PEROXIDATION STUDIES

2.1. INTRODUCTION

Biological membranes are vital for the existence of cellular integrity. The plasma membranes serve as barriers, protecting the cells from harmful compounds produced in the extracellular environment. These also function as compartmentalizing structures, which are essential for cellular function. Membranes are dynamic systems which are responsible for the production of ATP, and the binding of neurotransmitters which mediate transmission of nerve impulses (Bohinski *et al.*, 1987). Membranes also have the binding sites for regulatory molecules, such as hormones and function as permeable barriers for selective transport of substances in and out of the cells.

Lipid peroxidation, via the action of radical species, results in the attack of membrane lipids which cause destruction of the cell membrane, ultimately destroying the integrity of the cell (Southgate, 1999). The nervous system consists of substantial membranes and fatty acids (Halliwell & Gutteridge, 1989; Halliwell, 1992b). This implies an increased vulnerability of membrane lipid constituents in the nervous system to oxidative injury, either directly by cellular free radicals (Coyle & Puttfarcken, 1993; Olanow *et al.*, 1993; Bondy, 1995; Frölich & Riederer, 1995) or via other indirect or exogenic mechanisms (Nohl, 1993; Piotrowski *et al.* 1996). An increase in cellular load of free radicals results in an increase of lipid peroxidation and

loss of cellular compartmentalization (Gutteridge, 1988).

The presence of transition metals such as iron and any tissue injury results in the formation of highly reactive radical species such as the hydroxyl radical, capable of participating in the Fenton reaction. Thus, these events can lead to the initiation and propagation of lipid peroxidation.

2.2. THE COMPARATIVE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON HCY-INDUCED LIPID PEROXIDATION *IN VITRO*

2.2.1. INTRODUCTION

The brain is more susceptible to peroxidation because it is one of the most metabolically active organs in the body (McArdle *et al.*, 1986). Lipid peroxidation is able to cause extensive damage and play a major role in the deterioration of the brain and spinal cord that occurs after traumatic or ischemic injury.

The aim of the study was to determine whether 17β-estradiol and progesterone are able to protect against lipid peroxidation induced by the neurotoxin, homocysteine (Hcy). It is believed that Hcy exerts its effects through mechanisms involving free radical generation and as a result, oxidative damage (Mujumdar *et al.*, 2001). A comparative study was performed to determine whether 17β-estradiol and progesterone offer protection against Hcy-induced lipid peroxidation.

Male rats were selected to test the effect of exogenously administered estrogen and progesterone instead of the female rat in order to exclude complicating effects on ischemic cell damage of hormonal fluctuations during the estrus cycle. The assay used to measure lipid peroxidation is the Thiobarbituric acid (TBA) test. This assay involves the reaction of malondialdehyde (MDA) with TBA to yield a pink coloured complex, which can be measured colorimetrically at 532nm using a

spectrophotometer. MDA is the end product of lipid peroxidation from cell membranes, and is taken as a reliable indicator of oxidative stress (Halliwell & Gutteridge., 1990; Reiter *et al.*, 1995).

2.2.2. MATERIALS AND METHODS

2.2.2.1. Chemical and Reagents

All reagents used were of analytical grade. 17β-estradiol, progesterone (4-Pregnene-3,20-dione), DL-homocysteine, 2-thiobarbituric acid (98%) (TBA), 1,1,3,3-tetramethoxypropane (98%), butylated hyroxytoluene (BHT), were purchased from the Sigma Chemical Corporation (St Louis, MO, USA.). Tricholoracetic acid (TCA) and butanol were obtained from Saarchem (PYT) Ltd. (Krugersdorp, South Africa). All reagents were of the highest quality available.

2.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 250-300g were used. The animals were housed in a controlled environment with a 12:12 hour light: dark cycle, and were given access to standard laboratory chow and water *ad libitum*. Protocol for the experiments was approved by the Rhodes University animal ethics committee.

2.2.2.3. 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.

2.2.2.4. Preparation of Brain Homogenate

The brains were weighed and rapidly homogenised with 0.1M Phosphate buffer saline (PBS), pH 7.4, so as to give a final concentration of 10% w/v. This is necessary to prevent lysosomal damage of the tissue. The homogenate was centrifuged twice at 8000g for 10 minutes.

2.2.2.5. Lipid Peroxidation Assay

Lipid peroxidation was determined using the thiobarbituric acid (TBA) assay. This assay involves the reaction of malondialdehyde (MDA) equivalents with TBA to yield a pink complex. A modified method of Halliwell and Gutteridge (1990) was used in this set of experiments.

Homogenate (1ml) containing varying concentrations of DL-homocysteine (0, 0.25, 0.5, 1, 1.5 or 2mM) alone or in combination with varying concentrations of 17β-estradiol (0.05, 0.1, 0.2, 0.25, 0.5, 0.75 or 1mM) or progesterone (0.25, 0.5, 1mM) 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 methanol) and 1ml 25% TCA were added to the mixture. The samples were centrifuged at 2000g for 20 minutes at 4˚C to remove insoluble proteins. Following centrifugation, 2ml of 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 one 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 2000g for 10 minutes. An aliquot of 2ml of the top layer of the mixture was carefully removed with an autopipette and placed into separate tubes. This faction contained the extracted TBA-MDA complexes, which were then read at 532nm using a GBC UV/Vis 916 spectrophotometer. The MDA level was determined from a standard curve generated from 1,1,3,3-tetramethoxypropane. Final results were expressed as nmoles MDA/mg tissue.

2.2.2.6. 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 2.2.2.5. The absorbance was read at 532nm and plotted against the molar equivalent weight of MDA in the complex assayed. (Appendix 1).

2.2.2.7. 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 Hcy caused a significant increase in lipid peroxidation in a concentration-dependent manner in comparison to the control (figure 2.1).

Co-treatment of this homogenate with Hcy and increasing concentrations of 17β-estradiol or progesterone resulted in a significant reduction in lipid peroxidation (figure 2.2 and figure. 2.3 respectively). 17β- estradiol or progesterone at all concentrations tested, reduced the amount of MDA formed to the same level, i.e. ±0.04 nmoles/mg or ±0.06 nmoles/mg tissue respectively, at concentration of Hcy (1mM) tested. The results suggest that 17β- estradiol and progesterone protect against Hcy-induced lipid peroxidation in a manner that is independent of the concentration of 17β- estradiol or progesterone, at least within the concentration range tested.

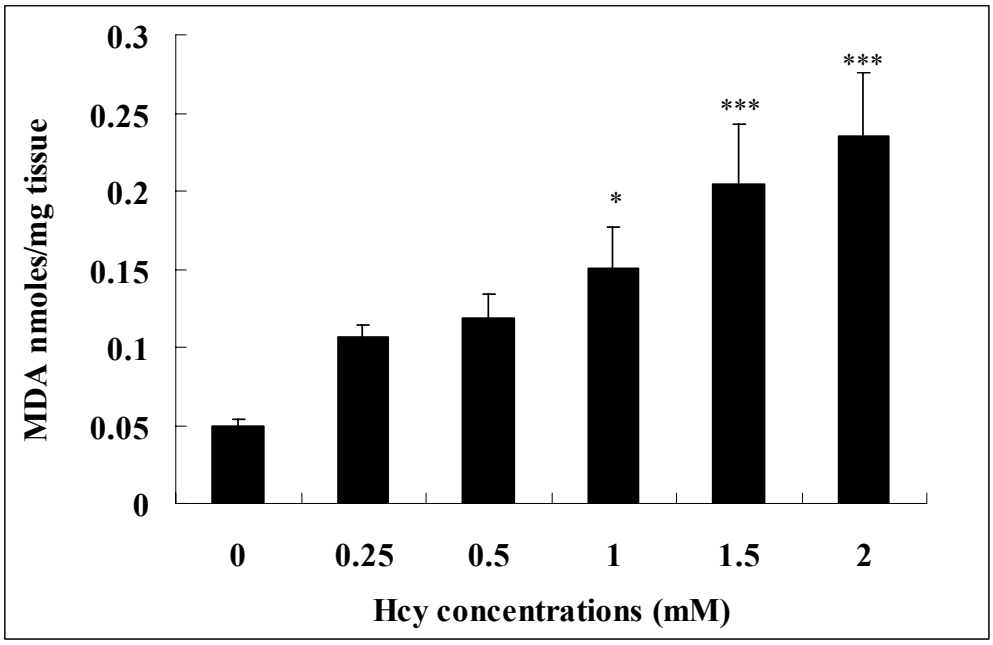


Figure. 2.1. Concentration-dependent effect of Hcy-induced lipid peroxidation in whole rat brain homogenate. Each bar represents the mean ±SEM; n=4. *($p<0.05$), *** ($p<0.001$) in comparison with control. Student-Newman-Keuls Multiple Range Test.

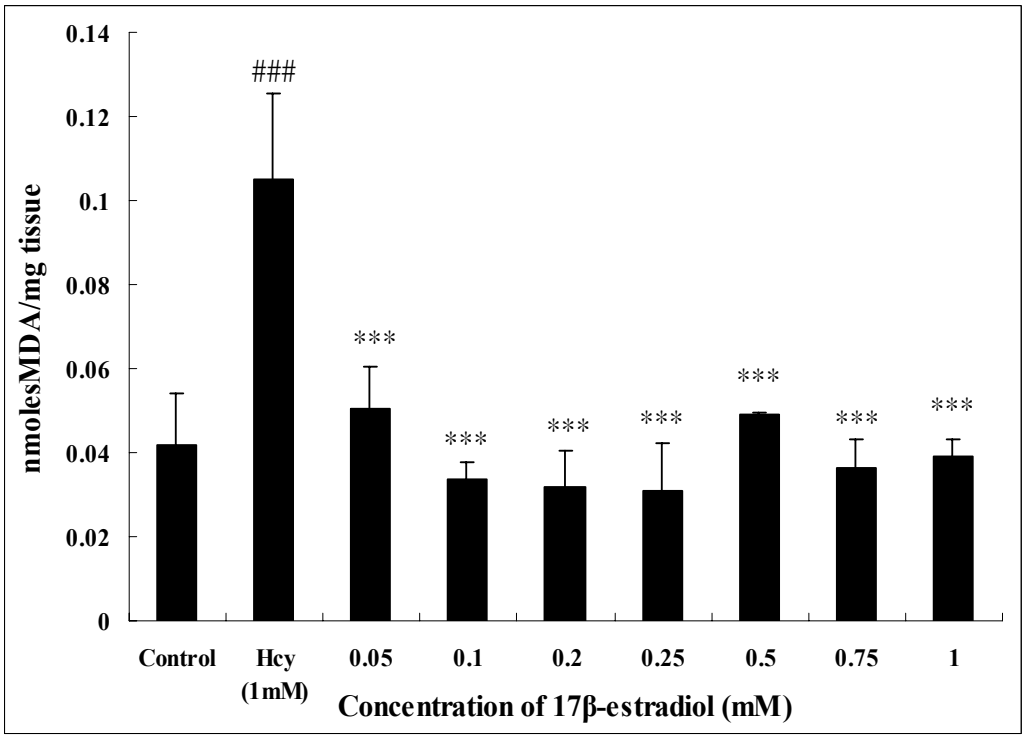


Figure. 2.2. The effect of 17β-estradiol on Hcy (1mM)-induced increase in MDA production in rat brain homogenate *in vitro*. Each bar represents the mean ±SEM; n=5. *($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) in comparison to Hcy; ### ($p<0.001$) in comparison to the control. Student Newman Keuls Multiple Range test.

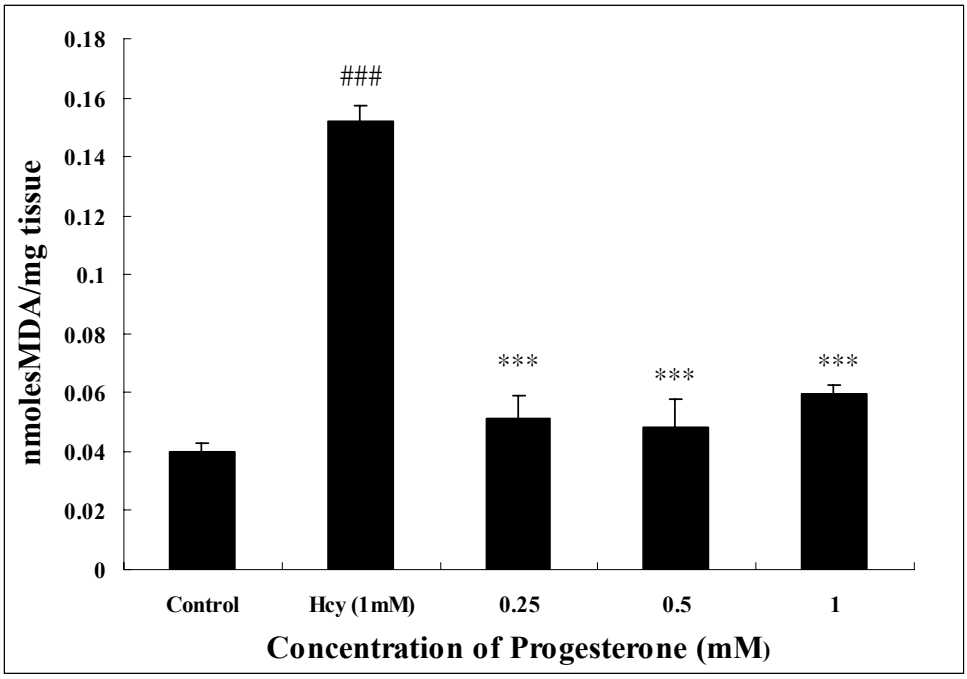


Fig. 2.3. The effect of progesterone on Hcy (1mM)-induced increase in MDA production in rat brain homogenate in vitro. Each bar represents the mean ±SEM; n=5. *($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) in comparison to Hcy; ### ($p<0.001$) in comparison to the control. Student Newman Keuls Multiple Range test.

2.2.4. DISCUSSION

The results of the study demonstrate that both 17β-estradiol and progesterone reduce Hcy-induced lipid peroxidation markedly *in vitro*. At concentration of 1mM Hcy, both 17β-estradiol and progesterone reduced the level of MDA formation in a concentration-independent manner. Furthermore, 17β-estradiol not only reduced the amount of MDA formed in the presence of Hcy but was also able to decrease MDA production to levels lower than those attained in samples without Hcy.

Previous studies show that Hcy induces lipid peroxidation at concentrations of 1mM and promotes iron-dependent oxidation of LDL (Alul *et al.*, 2003). Heinecke *et al.* (1993), Hirano *et al.,* (1994) and Lynch *et al.* (1997) reported that Hcy alone is incapable of oxidizing LDL; rather LDL oxidation is dependent upon the availability of free ferric (Fe^{3+}) iron. Oxidation of LDL by Fe^{3+} requires the presence of reduced thiols (Lynch *et al.*, 1997) to convert Fe^{3+} to Fe^{2+} (Lynch *et al.*, 1993 & 1995). Hcy is a thiol compound which is well-recognized biological reductant. Jocelyn *et al*., (1972) and Lynch *et al*., (1997) has shown that Fe^{3+} is reduced to Fe^{2+} when incubated with LDL in the presence of Hcy. Wood and Graham (1995) noted that the ability of Hcy to facilitate Fe^{3+} -dependent LDL oxidation was directly related to autooxidation. As Hcy autooxidation generates superoxide (Jocelyn, 1972), it is conceivable that the superoxide also contributes to Fe^{3+} reduction and thus, initiation of LDL oxidation (Lynch *et al.*, 1993; Lynch *et al.*, 1995).

Vedder *et al.,* (1999) showed that both 17β-estradiol and progesterone decrease Fe^{3+}-induced lipid peroxidation in rat brain and hippocampal HT22 cell homogenates. However, this author also showed that 17β-estradiol inhibits Fe^{3+}-induced lipid

peroxidation accompanied by an increased survival of primary neuronal culture cells, whereas progesterone reduces lipid peroxidation due to the membrane stabilizing effect (Roof *et al.*, 1997). One way in which both 17β-estradiol and progesterone could protect from Hcy-induced lipid peroxidation *in vitro* is by the antioxidant effects of these agents (Behl, 1995; Roof *et al.*, 1997) and the ability to bind Fe^{3+}, thereby preventing the conversion to Fe^{2+}.

In fact, high concentrations of 17β-estradiol (0.05-1nm) and progesterone (0.25-1nm) are used in this study. Roof and Hall, (2000) suggest that for both estrogen and progesterone, the neuroprotective actions involved can be dependent on the exogenous dose administered or the endogenous level of estrogen and progesterone employed in the case of pharmacological studies.

2.3. THE COMPARATIVE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON HCY-INDUCED LIPID PEROXIDATION *IN VIVO*

2.3.1. INTRODUCTION

The results obtained from section 2.2 shows that both 17β-estradiol and progesterone protect against lipid peroxidation in rat brain homogenates following exposure to Hcy. Baydas et al. (2003) showed that injection of Hcy directly into the rat brain causes a significant rise in MDA levels in the hippocampus. Therefore, it was decided to investigate whether both 17β-estradiol and progesterone could offer neuroprotection by determing lipid peroxidation before and after the intrahippocampal injection of Hcy *in vivo*.

Moreover, in postmenopausal women, the concentration of circulating estrogen and progesterone decrease dramatically. Several reports suggested that females may be more neuroprotected than male due to higher levels of circulating estrogen and progesterone (Groswasser *et al.*, 1998). Clinical trials have shown that estrogen replacement therapy or estrogen/progestin replacement therapy decreases Hcy level in postmenopausal women (Barnabei *et al.*, 1999). Hormone replacement therapy may protect the brains of these women against oxidative stress.

2.3.2. MATERIALS AND METHODS

2.3.2.1. Chemicals and 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. Dosing of the Animals

Animals were divided into four groups of five animals each in two treatment regimes. In treatment regime 1 (Table 1), the animals in group 3 and 4 received single daily doses of 100μg 17β- estradiol or 100μg progesterone in 100μl olive oil injected subcutaneously respectively, for 5 days prior to bilateral intrahippocampal Hcy (0.14μmol/μl) injection. Similarly, the animals in groups 1 and 2 received vehicle for 17β- estradiol and progesterone, viz. olive oil. On the 6th day, the animals in group 1 were injected with phosphate-buffered saline (PBS); groups 2 to 4 were injected with Hcy, directly into the hippocampal region, bilaterally. Hcy was dissolved in PBS at pH7.4. Following the bilateral intrahippocampal injections, the animals of groups 1 and 2 received subsequent daily doses of olive oil, each day for 5 days, while as before, the animals in group 3 and 4 received daily doses of 17β-estradiol or progesterone in olive oil for 5 days respectively.

In treatment regime 2 (Table 2), on the 1st day the animals in group 1 were injected with PBS; group 2 to 4 were injected with Hcy, directly into the hippocampal region bilaterally. The animals in group 3 and 4 received single daily doses of 100μg progesterone in 100μl olive oil injected subcutaneously respectively, for 7 days after

bilateral intrahippocampal Hcy (0.14μmol/μl) injection. Similarly, the animals in groups 1 and 2 received vehicle for 17β-estradiol and progesterone, viz. olive oil.

Table 2.1. Treatment regime 1 for each group of animals.

Treatment Group	Daily treatment for 5 days prior to sterotaxic surgery (subcutaneous)	Bilateral intrahippocampal injection	Daily treatment for 5 days after sterotaxic surgery (subcutaneous)
1 [Control]	100μl Olive oil	PBS	100μl Olive oil
2 [Hcy only]	100μl Olive oil	0.14μmol/μl Hcy in PBS	100μl Olive oil
3 [Est + Hcy]	100μg 17β-estradiol in 100μl Olive oil	0.14μmol/μl Hcy in PBS	100μg 17β-estradiol in 100μl Olive oil
4 [Prog + Hcy]	100μg Progesterone in 100μl Olive oil	0.14μmol/μl Hcy in PBS	100μg Progesterone in 100μl Olive oil

Table 2.2. Treatment regime 2 for each group of animals.

Treatment Group	Daily treatment for 7 days after stereotaxic surgery (subcutaneous)	Bilateral intrahippocampal injection
1 [Control]	100μl Olive oil	PBS
2 [Hcy only]	100μl Olive oil	0.14μmol/μl Hcy in PBS
3 [Est + Hcy]	100μg 17β-estradiol in 100μl Olive oil	0.14μmol/μl Hcy in PBS
4 [Prog + Hcy]	100μg Progesterone in 100μl Olive oil	0.14μmol/μl Hcy in PBS

2.3.2.4. Surgical Procedure

2.3.2.4.1. 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 as shown in figure 2.4. 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 respiration became too weak. A good indication of the depth of anaesthesia was monitored by the colour of the limbs and tail, which displaced a faint, almost pale pinkness. This was indicative of 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.

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

2.3.2.4.2. Bilateral Intrahippocampal Hcy Injection

Hcy (0.14µmol/µl) was injected intrahippocampally using stereotaxic surgery techniques. Female rats were anaesthetised with diethyl ether and placed into a stereotaxic apparatus (figure 2.4). The skull was orientated according to the König and Klippel stereotaxic atlas (1963). After a saggital cut in the skin of the skull, the bregma and lambda sutures 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. Hamilton syringe, with a cannula of diameter 0.3mm, was used to inject 0.14µmol/µl of Hcy in 8µl of PBS bilaterally, 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 cannula 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. The rats of group 1 were used as controls and subjected to the same surgical procedure. However, stereotaxic injections into the hippocampus were free of Hcy and comprised of PBS.

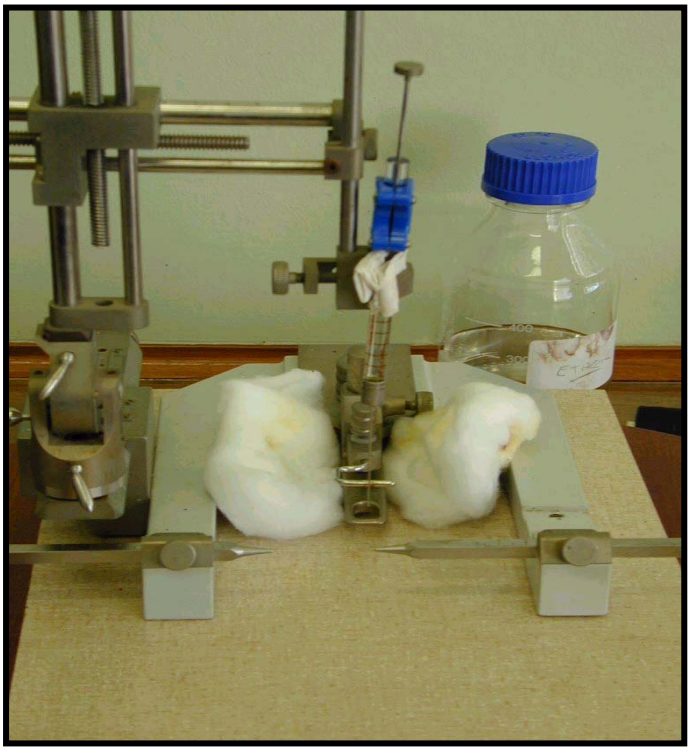


Figure 2.4. A view of the stereotaxic apparatus used for the bilateral intrahippocampal injection of Hcy (Stoelting, IL, USA).

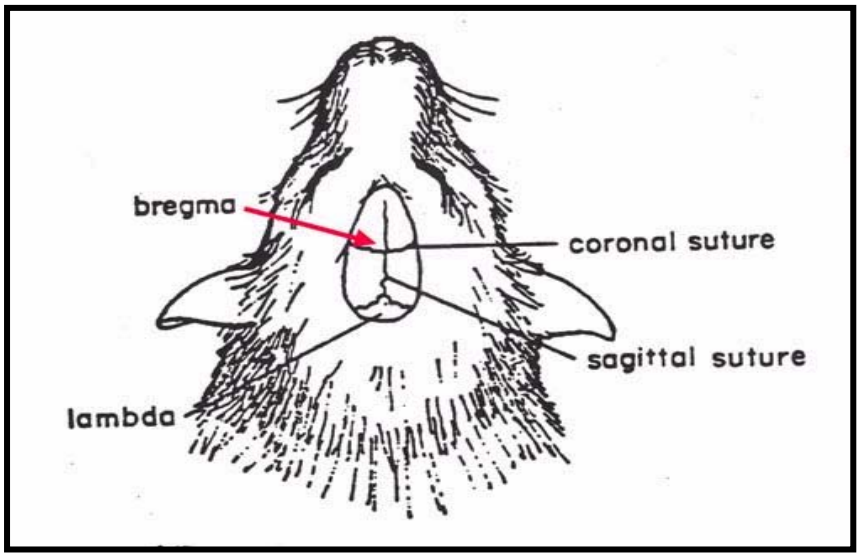


Figure 2.5 A view of the rat skull after the skin has been cut. The sutures shown are used as a reference point for the measurement of the coordinates for the intrahippocampal injection (Heron, 2001).

2.3.2.5. Homogenate Preparation

As described in section 2.2.2.4

2.3.2.6. 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, one hour incubation at 37˚C was excluded because no drugs were added to the homogenate. The homogenate (1ml) containing 0.5ml BHT and 1ml TCA was then 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 Hcy, the MDA formed as a result of Hcy-induced lipid peroxidation would have bound to such molecules (Gutteridge & Halliwell, 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 hydrolysis by oxidation of polyunsaturated fatty acids. However, pigments generated during hydrolysis interfere in the colorimetric assessment 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)

2.3.2.7. Statistical Analysis

As described in section 2.2.2.7.

2.3.3. RESULTS

The intrahippocampal injection of Hcy at 0.14μmol/μl *in vivo* caused a significant increase in lipid peroxidation (Figure 2.6) assessed by the formation of TBARS (TBA reacting substances during the assay). Pre-treatment and post-treatment of 17β-estradiol or progesterone (Figure 2.7), caused a significant reduction in Hcy (0.14μmol/μl)-induced TBARS formation with a p-value less than 0.01 and 0.001 respectively. Furthermore, 17β-estradiol and progesterone reduced the level of TBARS formed, which were below that of the control.

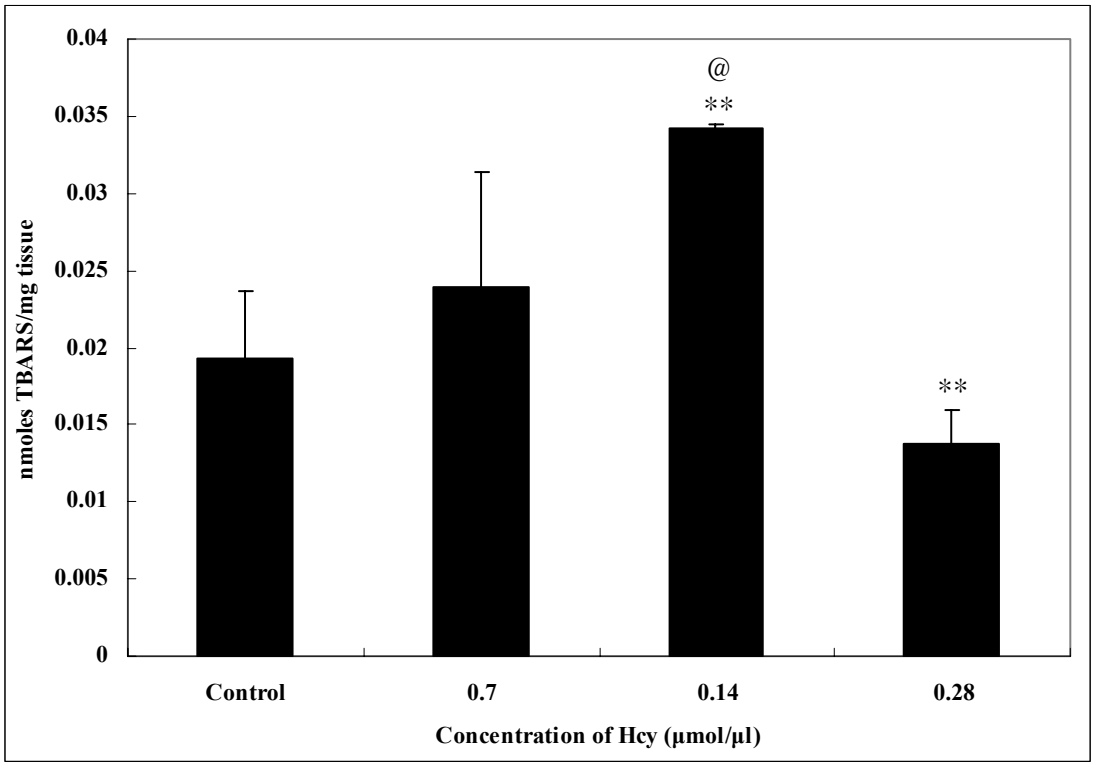


Figure 2.6. The effects of bilateral intrahippocampal injection 0.7, 0.14 and 0.28µmol/µl of Hcy on of TBARS formed. Each bar represents the mean ±SEM; n=5. *($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) in comparison to Hcy 0.7µmol/µl. @ ($p<0.01$) in comparison to control. Student Newman Keuls Multiple Range test.

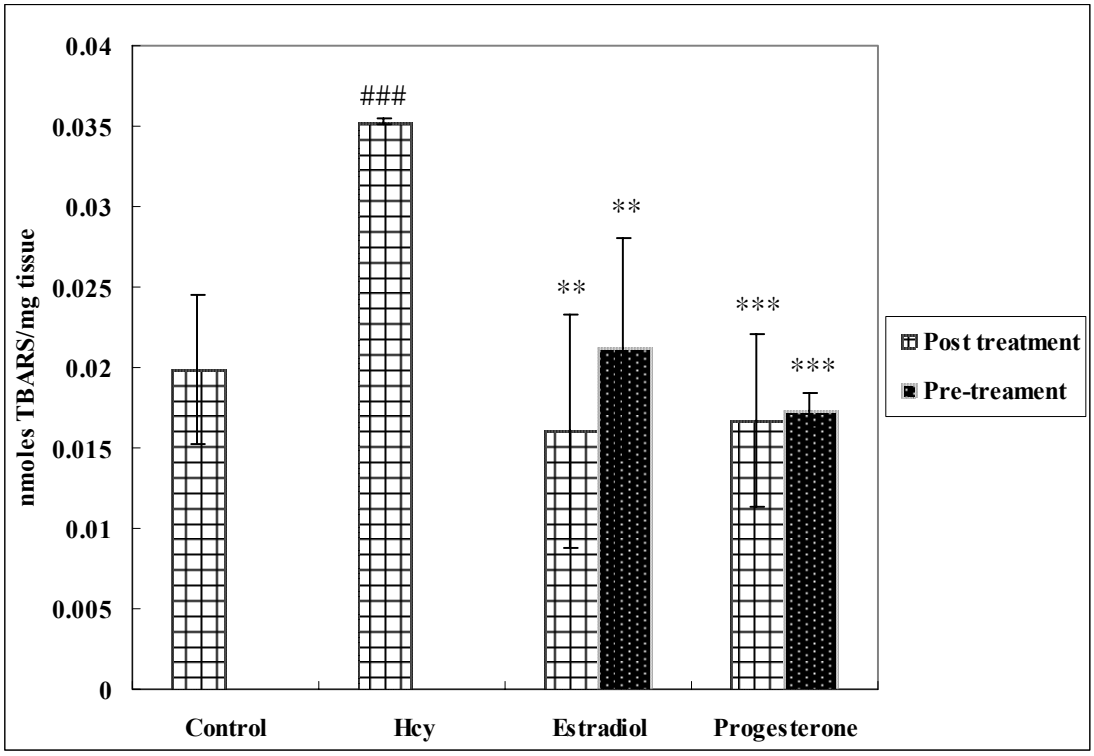


Figure 2.7. The effects of 17β-estradiol and progesterone on Hcy-induced lipid peroxidation *in vivo*. Values represent the mean ±SEM (n=5), (*p<0.05 for Hcy in comparison to the control; ***p<0.001 for estradiol/progesterone in comparison to Hcy)

2.3.4. DISCUSSION

The results obtained show that either 17β-estradiol or progesterone are able to protect against Hcy-induced lipid peroxidation *in vivo.* Hcy is an agonist at the glutamate site of the NMDA receptor and is therefore a potential excitotoxin. Overstimulation of NMDA receptors by Hcy results in excessive calcium influx and reactive oxygen generation that cause neuronal damage (Lipton *et al.*, 1997; Kim *et al.*, 1998; Kruman *et al.*, 2000). In addition to the antioxidant ability of both hormones, it is possible that both hormones also act on the NMDA receptor. This assumption is based on the results of the present study that both hormones (pre- and post-treatment) decrease the level of lipid peroxidation to below that of the control and thus show total protection against Hcy. Nevertheless, if both hormones only act as an antioxidant, cell damage due to Hcy would still arise and can only prevent further damage. However, this needs to be investigated further.

2.4. CONCLUSION

The results in this chapter demonstrate that Hcy-induced lipid peroxidation is markedly inhibited by both 17β-estradiol and progesterone *in vitro* and *in vivo*.

Hcy is a sulfur-containing amino acid which is formed during the metabolism of methionine (Nygard O *et al.*, 1999). It is an excitatory amino acid which markedly enhances the vulnerability of neuronal cells to oxidative injury *in vitro* and *in vivo* (Outinen *et al.*, 1998; Kruman *et al.*, 2000). Moreover, there is reported evidence implicating Hcy promotion of *in vivo* lipid oxidation (Young *et al.*, 1997; Roberts and Morrow, 2000).

Alul *et al.,* (2003) and Lynch *et al.,* (1997) reported that Hcy promotes iron-dependent oxidation of LDL and is dependent upon the availability of Fe^{3+}. These authors further reported that in the presence of Hcy, Fe^{3+} is reduced to Fe^{2+} when incubated with LDL. In chapter 3, it is also shown that intrahippocampal Hcy injection causes a significant induction of $O_2^{\bullet -}$ radicals, which initiates lipid peroxidation (Heinecke *et al.*, 1987).

The possible mechanisms by which 17β-estradiol offers protection against lipid peroxidation have been reported by Heron, (2000) and Ayres *et al.*, (1998). 17β-estradiol offers protection against lipid peroxidation *in vitro* due to its antioxidant activity or interaction with iron. It is also reported that 17β-estradiol inhibits LDL oxidation (Negre-Salvayre *et al.*, 1993). An interaction between 17β-estradiol and Fe^{3+} which is induced in the presence of Hcy would cause a decrease in Hcy-induced lipid peroxidation. Alternatively, progesterone has membrane stabilising effects and that could contribute to the reduction in damage due to lipid peroxidation (Roof, 1994).

From the *in vivo* results obtained (Fig. 2.7), both 17β-estradiol and progesterone also reduce the amount of TBARS production to levels lower than those attained in samples without Hcy. This demonstrates that 17β-estradiol is able to protect against Hcy-induced neurotoxicity completely. High concentrations of Hcy is a source of hydrogen peroxide, which estrogen, by enhancing glutathione content can act as a free radical scavenger by neutralizing hydrogen peroxide. It also reduces peroxides and maintains protein thiols in the reduced form (Dimitrova *et al.*, 2002a, b). Thus estradiol appears to offer protection against lipid peroxidation.

However, progesterone does not exert its neuroprotective effects via interaction with

hydrogen peroxide related cellular mechanisms (Vedder *et al.*, 2000). It has been shown that progesterone modulates γ-aminobutyric acid (GABA) and excitatory amino acid neurotransmitter systems and has neuroprotective properties (Lockhart *et al.*, 2002). It has been shown that progesterone decreases necrotic and apoptotic cell death in an *in vitro* system of N-methyl-D-aspartate (NMDA)- mediated neuronal injury and protects hippocampal cells against neuronal cell damage induced by amyloid β protein and glutamate (Lockhart *et al.*, 2002). Foy et al., (1999) reported that 17β-estradiol enhances NMDA receptor-mediated excitatory postsynaptic potentials. The results of this report show that 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 processes. As shown in Chapter 7, both 17β-estradiol and progesterone offer protection against Hcy-induced neurotoxicity via a NMDA receptor-dependent mechanism. Therefore, it is possible that the protective effect of 17β-estradiol and progesterone on Hcy-induced lipid peroxidation *in vivo* (Fig. 2.7) is due to the action of estradiol and progesterone on the NMDA receptor, since Hcy is an agonist at the glutamate site of the NMDA receptor and is therefore a potential excitotoxin (Lipton *et al.*, 1997). Thus estradiol and progesterone could possibly act as NMDA receptor antagonists *in vivo*.

In conclusion, the present study shows that 17β-estradiol offers protection against Hcy-induced lipid peroxidation *in vitro* via a free radical scavenging mechanism. Both estrogen and progesterone have been shown to suppress lipid peroxidation induced by amyloid β-peptide (Goodman *et al.*, 1996b; Lockhart *et al.*, 2002). It is well known that estrogen and progesterone are no longer synthesized in the female after menopause, while concentrations of Hcy increase with age (Hernanz *et al.*,

2000). These effects occurring at the same time, could contribute to neurodegeneration that occurs predominantly with an increase in age. Further studies are presently underway to investigate the exact mechanism responsible for estrogen and progesterone's protective activity against Hcy-induced lipid peroxidation *in vitro* and *in vivo*.

CHAPTER 3

SUPEROXIDE RADICAL GENERATION

3.1 INTRODUCTION

Reactive oxygen species (ROS) are produced in the course of normal metabolism and serve important physiological functions. However, because of their high reactivity, accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative degradation of critical molecules, such as DNA, proteins, and lipids (Hugon *et al.*, 1996b). Although cells possess an intricate network of defense mechanisms to neutralize excess ROS and reduce oxidative stress, some tissues, especially the brain, are much more vulnerable to oxidative stress because of their elevated consumption of oxygen and the consequent generation of large amounts of ROS. For the same reason, the mitochondrial DNA of brain cells is highly susceptible to structural alterations resulting in mitochondrial dysfunction. Several lines of evidence strongly suggest that these effects of ROS may be etiologically related to a number of neurodegenerative disorders.

Cells possess highly effective antioxidant defence mechanisms to protect neurons against free radical species (Braughler & Hall, 1989). Under normal conditions these defense mechanisms are quite capable of protecting neurons, but under conditions of excessive ROS production, these mechanisms may not be able to cope and

neurotoxicity may result (DiFiglia, 1990). These protective mechanisms can be classified into two main categories (Dawson & Dawson, 1996), the enzymatic defence mechanisms, which include superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the non-enzymatic cellular antioxidants, which include Vitamins A and E. ROS are generated by the addition of a single electron to O_2. The radical is usually generated as a result of electron "leakage" from the electron transport chain located in the mitochondria (McCord,1985), and by activation of certain enzymes.

Superoxide anions can be very reactive and can lead to neurodegeneration (Patel *et al.*, 1996). It has been proposed (Fahn *et al.*, 1992) that the excitatory amino acids are neurotoxic because these lead to increased production of. Overstimulation of excitatory amino acids receptors results in the influx of Ca^{2+}, Na^{+}, and K^{+} into neuron (Poeggeler *et al.*, 1993). As these ions cause a change in the osmotic pressure, the neuron attempts to pump the ions out of the cell. The transport of ions is an active process that requires energy. Mitochondria are forced to produce more ATP, and so there is a chance of greater electron leakage. One of the by-products of the above activity is the production of
$O_2^{\bullet -}$.

The aim of the present chapter is to investigate the ability of 17β-estradiol and progesterone to reduce Hcy-induced $O_2^{\bullet -}$ generation in the rat hippocampus *in vivo*.

3.2 THE COMPARATIVE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE ON HCY-INDUCED SUPEROXIDE RADICAL GENERATION IN THE RAT HIPPOCAMPUS

3.2.1 INTRODUCTION

Previous experiments (see Chapter 2) have demonstrated that Hcy-induced lipid peroxidation *in vitro* and *in vivo*. Hcy is a sulphur-containing amino acid and auto-oxidation of Hcy resulting in generation of ROS, such as superoxide anion radicals and hydroxyl radicals and thus initiates lipid peroxidation (Heinecke *et al.*, 1987).

Several studies have shown that auto-oxidation of Hcy generates superoxide, which contributes to Fe^{3+} reduction and cause initiation of LDL oxidation (Lynch *et al.,* 1993 & 1995). Lipton et al., 1997 also stated that Hcy interacts with NMDA receptors, acting as an agonist at the glutamate binding site. In addition, the results obtained from chapter 7 also show that Hcy acts on the glutamate binding sites. The activation of NMDA receptors causes accumulation of Ca^{2+}, thus causing excitotoxicity and oxidative stress (Hoffman *et al.*, 1990; Kim & Pae, 1996).

The nitro-blue tetrazolium (NBT) assay, which is generally accepted (Halliwell & Gutteridge, 1985) to be a reliable method for assaying for superoxide anions, was used. The assay involves the reduction of the NBT ion to the insoluble diformazan form, which can be extracted with glacial acetic acid.

3.2.2 MATERIALS AND METHODS

3.2.2.1 Chemicals and Reagents

All reagents used were of analytical grade. 17β-estradiol, progesterone (4-Pregnene-3,20-dione), DL-homocysteine and nitro-blue tetrazolium (NBT) and nitro-blue diformazan (NBD) were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Glacial acetic acid was purchased from Saarchem (PTY) Ltd. (Krugersdorp, South Africa). 0.1% of NBT reagent was prepared by dissolving the NBT in ethanol before making up the solution to the required volume with Milli-Q water. All reagents were of the highest quality available.

3.2.2.2 Animals

Adult male rats of the Wistar strain, weighing between 250-300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.2.

3.2.2.3 Dosing of the Animals

As described in section 2.3.2.3

3.2.2.4 Surgical Procedures

As described in section 2.3.2.4

3.2.2.5 Homogenate Preparation

As described in section 2.3.2.5.

3.2.2.6 Preparation of Standards

NBD was used as a standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in glacial acetic acid was prepared to a final volume of 1ml. A calibration curve (Appendix two) was generated by measuring the absorbance at 20μmoles/ml intervals. The absorbance was read at 560nm using a Shimadzu UV-160A UV-visible recording spectrophotometer.

3.2.2.7 Nitroblue Tetrazolium Assay (NBT)

A modified method of Halliwell and Gutteridge (1985) was used for this assay. The lipid source viz. rat hippocampal homogenate (0.5ml) was incubated with 0.4ml of a 0.1% NBT solution in an oscillating water bath for 60 minutes at 37˚C. Termination of the assay and extraction of reduced NBT was carried out by centrifugation of the suspension at 2000 x g for 10 minutes. The supernatant was decanted and the pellet was resuspended with 2ml glacial acetic acid. The relative absorbance of the glacial acetic acid fraction was measured at 560nm and converted to μmoles Diformazan using a standard curve generated from nitroblue diformazan (NBD) in appendix two. Final results are expressed as μmoles Diformazan/mg protein.

The protein content of each homogenized rat brain tissue was determined by carrying out protein assays as described in section 3.2.2.8. All results were analyzed to show

statistical significance as described in section 2.2.2.7.

3.2.2.8 Protein Determination

All protein determinations were performed using the method described by Lowry et al., (1951). A standard curve was generated using bovine serum albumin (BSA) as a standard at concentration intervals of 60μg/ml, described in appendix three.

3.2.2.9 Statistical Analysis

As described in section 2.2.2.7.

3.2.3. RESULTS

The intrahippocampal injection of Hcy at 0.14μmol/μl *in vivo* caused a significant increase in hippocampal $O_2^{\bullet -}$ production in comparison to the control value (Figure 3.1). Both groups treated with of 17β-estradiol or progesterone showed a significant reduction in Hcy-induced $O_2^{\bullet -}$ production. Furthermore, both hormones in both treatment groups are able to decrease the $O_2^{\bullet -}$ levels below that observed for the control treated rats.

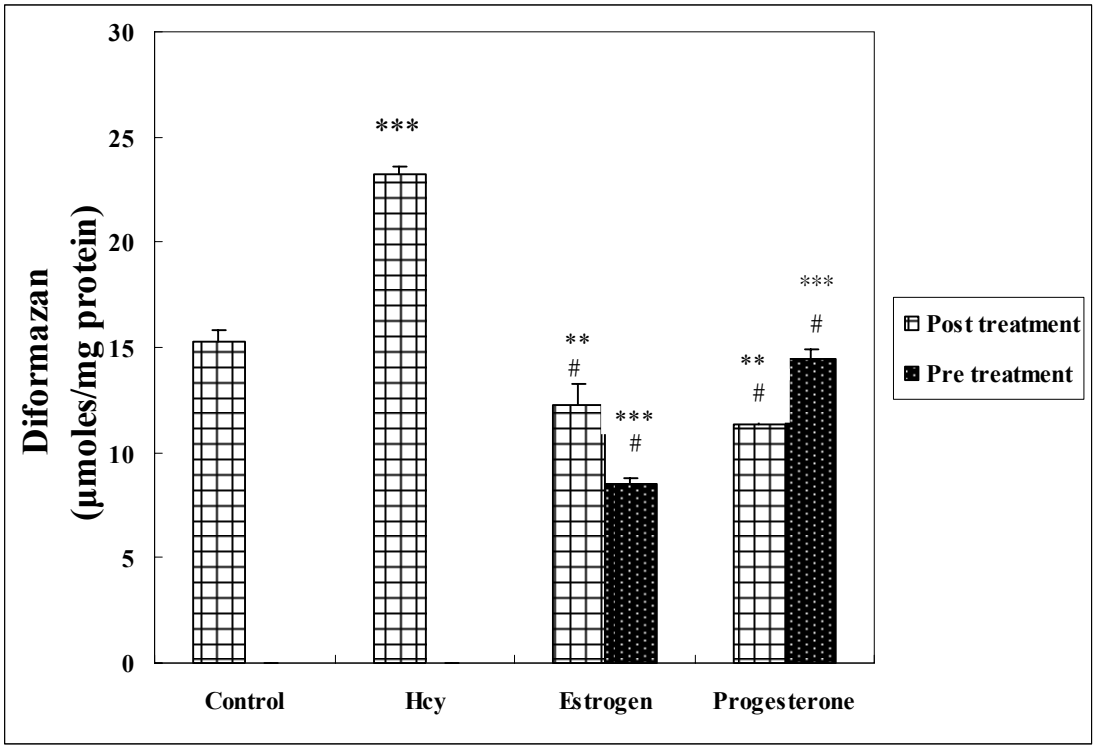


Figure 3.1. The effects of 17β-estradiol and progesterone on Hcy-induced $O_2^{\bullet -}$ generation in rat hippocampal homogenate *in vivo*. Values represent the mean ±SEM (n=4), (*** $p<0.001$ in comparison to the control; ** $p<0.01$ in comparison to the control; #$p<0.001$ for estradiol/progesterone in comparison to Hcy).

3.2.4. Discussion

The results obtained in this experiment show that the intrahippocampal Hcy injection causes a significant induction of $O_2^{\bullet -}$ generation in rat hippocampus. The increase in $O_2^{\bullet -}$ production could be caused by increased intracellular Ca^{2+} due to overstimulation of the glutamate receptors. $O_2^{\bullet -}$ is toxic to neurons (Fahn *et al.*, 1992) and could lead to neuronal death. Hcy is a sulphur-containing amino acid and Heinecke et al., (1987) have shown that Hcy causes generation of hydrogen peroxide, hydroxyl radicals and $O_2^{\bullet -}$ during the auto-oxidation reaction. In addition, Hcy causes Ca^{2+} influx via the stimulation of the NMDA receptor (Lipton *et al.*, 1997); a reduction in the number of binding sites of receptor (Chapter 7) and changes in the nature of the receptors in the

neurons (Chapter 7). Thus, Hcy is a potential neurotoxic agent due to its ability to increase $O_2^{\bullet -}$ generation in the brain.

However the Hcy-induced $O_2^{\bullet -}$ generation is reduced significantly by both regimens of either 17β-estradiol or progesterone. The neuroprotective effects of progesterone have been reported to include improved blood-brain barrier integrity and also free radical scavenging, which limit cell death by reducing lipid peroxidation (Betz *et al.*, 1990; Roof *et al.*, 1992; Shallert *et al.,* 1986; Yu, 1989). In addition, the results obtained in chapter 2 also show that progesterone has the ability to protect against Hcy-induced lipid peroxidation. Roof *et al.,* (1994) and Carlson *et al.,* (1990) showed that progesterone has membrane stabilising effects and antioxidant action. This causes the cell membrane to be less fluid and protects these from free oxygen radical attack and calcium toxicity.

Alternatively, 17β-estradiol 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, 1995). Hcy is a sulphur-containing amino acid and the auto-oxidation of this compound results in peroxynitrite anion. The superoxide anion interacts with nitric oxide to form peroxynitrite. It is also known that 17β-estradiol detoxifies peroxynitrite by increasing glutathione (GSH) levels (Pechanova *et al.*, 1999). Moreover, 17β-estradiol can modulate intracellular calcium levels through interactions with NMDA receptors (Joels *et al.*, 1995) and is able to up-regulate the number of binding sites (Chapter 7).

This experiment investigated the $O_2^{\bullet -}$ scavenging properties of 17β-estradiol and

progesterone induced by Hcy.

3.2.5. Conclusion

These results demonstrate that both 17β-estradiol and progesterone of both regimens are able to reduce Hcy-induced $O_2^{\bullet -}$ level in the hippocampus. Moreover, both hormones are able to reduce an Hcy-induced $O_2^{\bullet -}$ level, which is lower than the control treated group.

In conclusion the results of the present studies imply that both 17β-estradiol and progesterone are potent scavengers of $O_2^{\bullet -}$ and that these hormones have a definite role to play as antioxidants and in the protection of the brain against oxidative stress.

CHAPTER 4

HISTOLOGICAL STUDIES

4.1. INTRODUCTION

In chapter 2, it was shown that both 17β-estradiol and progesterone protect against Hcy-induced lipid peroxidation both *in vivo* and *in vitro*. However, these studies were only measurements of degraded lipid products, the TBA reacting substances. Although the decline in the formation of these degraded lipid products is an indication of decreased cell damage, it is important to examine the cells following Hcy administration.

Homocysteine induces neuronal cell death by activating *N*-methyl-D-aspartate (NMDA) receptor (Lipton *et al.*, 1997; Kim *et al.,* 1996; Ho *et al.*, 2002). Kruman, *et al*., 2000 reported that intrahippocampal injection of Hcy markedly increases the vulnerability of hippocampal neurons to neuronal cell death and oxidative injury in cell culture and *in vivo*.

The present study aims to examine the hippocampal neurons following an intrahippocampal injection of Hcy, and post- and pre-treatment with subcutaneous injection of 17β-estradiol or progesterone. Histological techniques were employed and the cells examined under a light microscope attached with a camera.

4.2. THE EFFECT OF 17β-ESTRADIOL AND PROGESTERONE AGAINST HCY-INDUCED DAMAGE TO HIPPOCAMPAL NEURONS

4.2.1. INTRODUCTION

Histology is the study of tissues and cells under a microscopea. It is also called microscopic anatomy, as opposed to gross anatomy which involves structures that can be observed with the naked eye. It also involves of the examination of preserved, sectioned and stained tissue (Hodgeson & Bernard, 1992).The Nissl stain, introduced by the German neurologist Franz Nissl in the late nineteenth century, and is commonly used to study neurons under the light microscope.

Neural tissues have a wide distribution throughout the body, innervating most viscera and peripheral tissues. Neurons are highly specialized cells that conduct nerve impulses and are easily excited to produce them. Typical neurons show a large cell body with a large central nucleus and many cytoplasmic extensions. The 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 or progesterone offers neuroprotection against Hcy-induced intrahippocampal lesions in the rat brain. After treatment, the brains of the rats were sectioned and the hippocampus of the rats was examined microscopically for evidence of any morphological changes

4.2.2. MATERIALS AND METHODS

4.2.2.1. Chemicals and Reagents

17β-estradiol, progesterone (4-Pregnene-3,20-dione), DL-homocysteine 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 male rats of the Wistar strain, weighing between 250-300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.2.

4.2.2.3. Dosing of Animals

As described in section 2.3.2.3.

4.2.2.4. Surgical Procedures

As described in section 2.3.2.4.

4.2.2.5. Histological Techniques

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

4.2.2.5.1. Fixing the brain

The animals were sacrificed and the brains removed as in section 2.2.2.3. 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 was 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.

4.2.2.5.5. 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.

4.2.2.5.6. Staining

The sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple, the nuclei purple, and leaves the background clear (Bauer, 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

4.2.2.5.7. 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.

4.2.2.5.8. Photo-microscopy

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

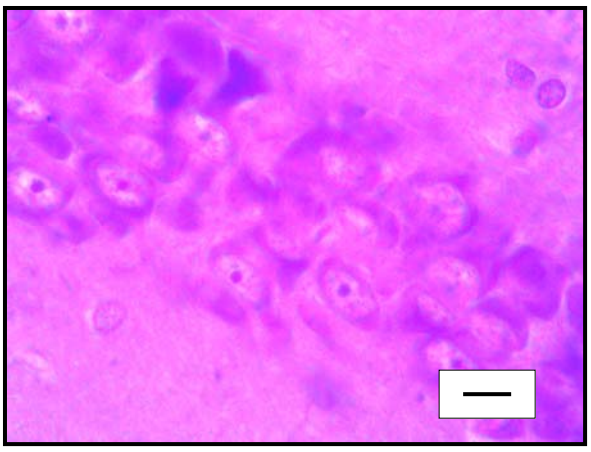
4.3 RESULTS

The results from figure 4.1 show that both 17β-estradiol and progesterone protect the neurons in both CA1 and CA3 regions of the hippocampus in Hcy-induced neurodegeneration. The hippocampal neurons in control group from both the CA1 (figure 4.1 (a)) and CA3 region (figure 4.1 (g)) appear to be undamaged. The neurons appear to have a pyramidal shape, continuous cell membrane lining and the cell nucleus is visibly observed.

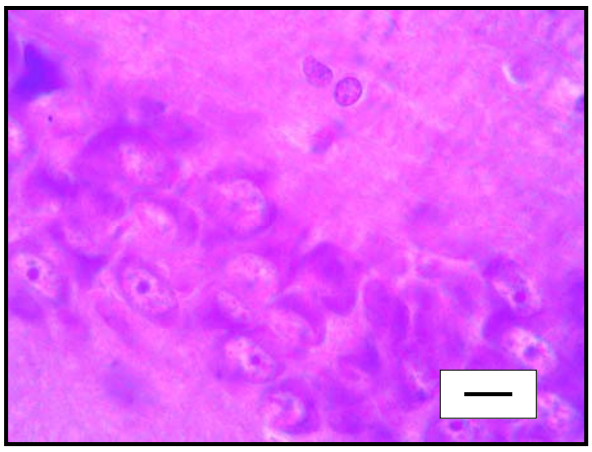
However, the neurons of animals treated with Hcy in the CA1 region (figure 4.1 (b)) and the CA3 region (figure 4.1 (h)) show signs of neuronal damage and degeneration as evident by the roundness and swelling. The cell nucleus is absent and the cell membrane is scattered. However, the neurons in CA1 (figure 4.1 (c)) and CA3 (figure 4.1 (i)) regions of the 17β-estradiol post-treated animals appear to be undamaged and show significant protection in comparison to the neurons of Hcy only treated animals. Similar results are obtained in the CA1 (figure 4.1 (d)) and CA3 (figure 4.1 (j)) regions of 17β-estradiol pre-treated animals.

Furthermore, figure 4.1 (e), (k), (f) and (l) demonstrate that the neurons are undamaged in CA1 and CA3 regions of post-treatment or pre-treatment of

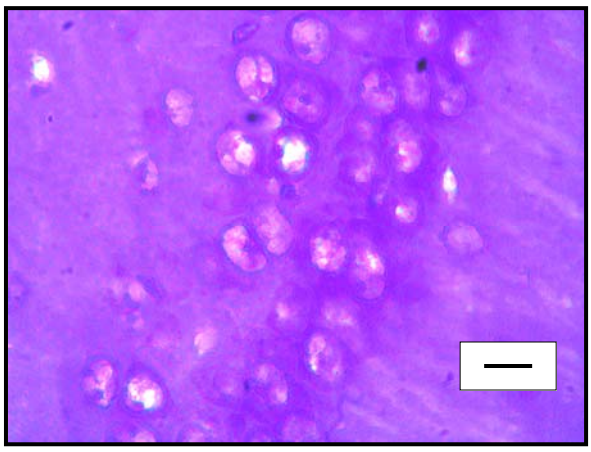
progesterone. It is also evident that the neurons of the both regimes of progesterone treated animals healthier than the neurons of the Hcy treated animals.



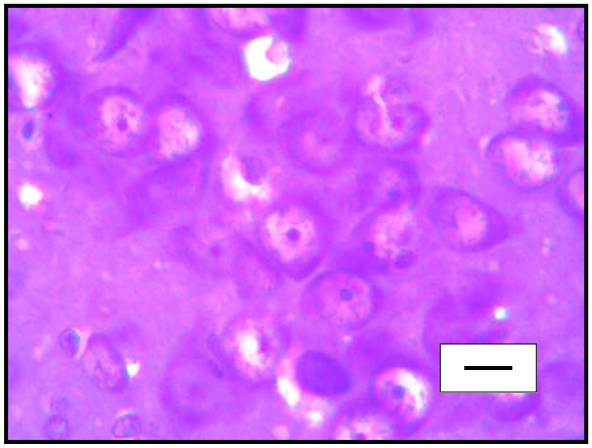
(a)



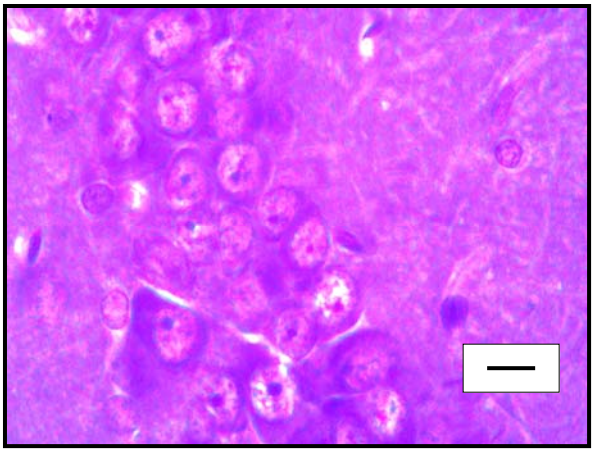
(g)



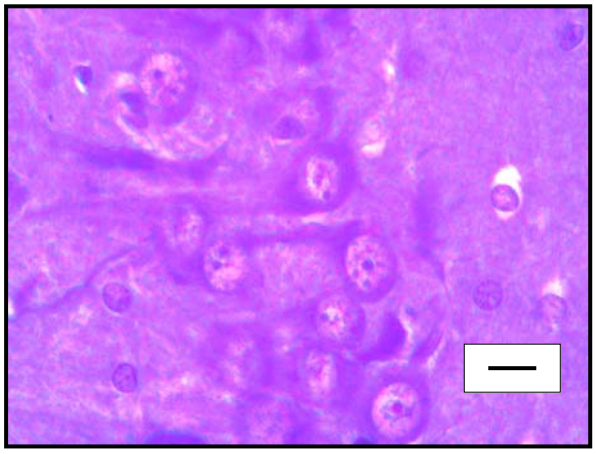
(b)



(h)



(c)



(i)

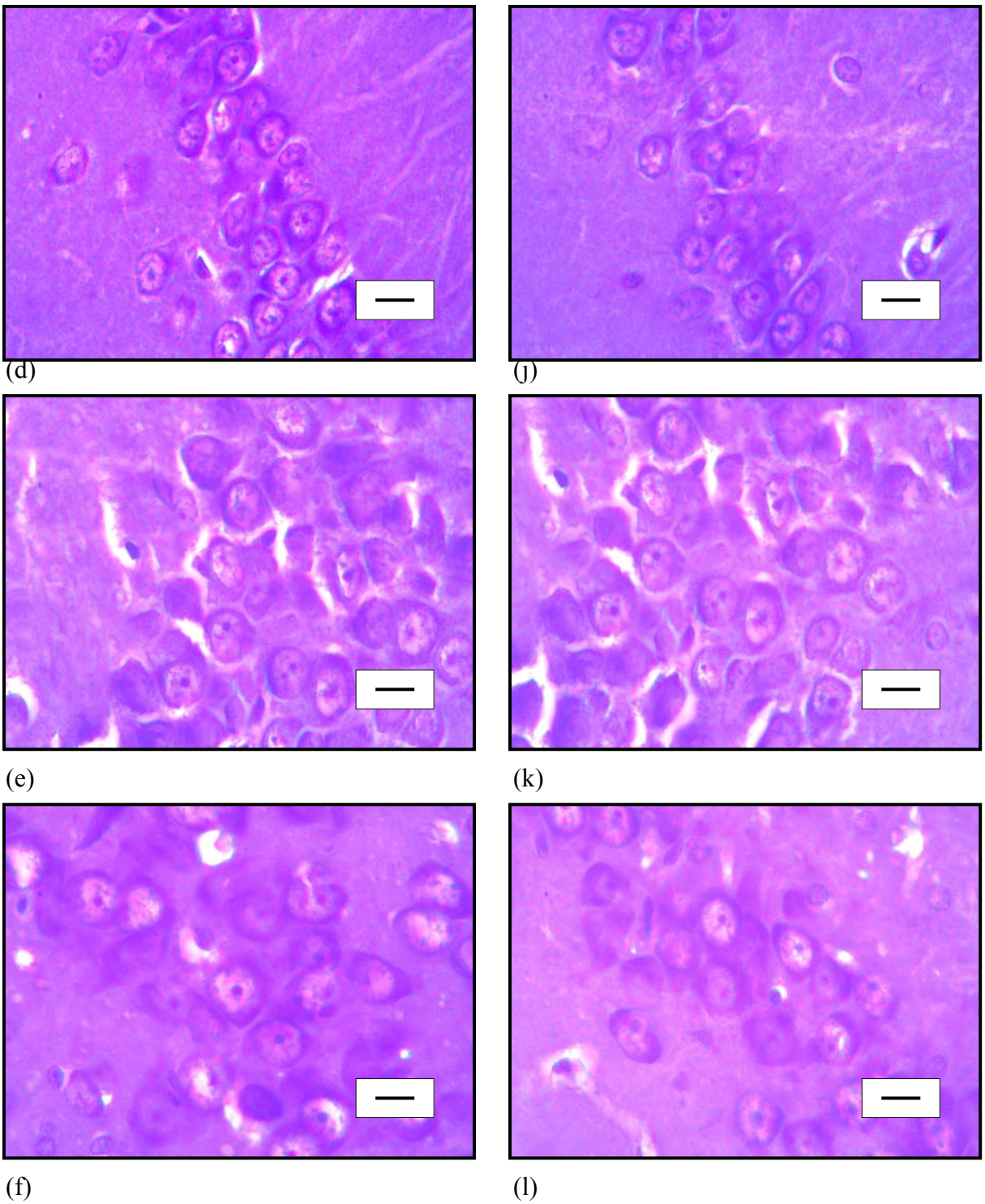


Figure 4.1. Hcy toxicity and the protective effects of 17β-estradiol or progesterone on rat hippocampal neurons. Micrographs (a-f) show cells in the CA1 region of the hippocampus from a control animal (a), an animal treated with Hcy (b), an animal treated with Hcy and 17β-estradiol (post-treatment) (c), an animal treated with Hcy and17β-estradiol (pre-treatment) (d), an animal treated with Hcy and progesterone (post-treatment) (e) and an animal treated with Hcy and progesterone (pre-treatment) (f). Micrographs (g-l) indicate cells in the CA3 region of the hippocampus from a

control animal (g), a Hcy treated animal (h), an animal treated with Hcy and 17β-estradiol (post-treatment) (i), animal treated with Hcy and 17β-estradiol (pre-treatment) (j), an animal treated with Hcy and progesterone (post-treatment) (k) and an animal treated with Hcy and progesterone (pre-treatment) (l). (Bar = 10μm)

4.4 DISCUSSION

These results (figure 4.1.) demonstrate that the structural damage of neurons that Hcy causes in CA1 and CA3 regions of the hippocampus. The results also demonstrate that 17β-estradiol or progesterone appears to protect against Hcy-induced neurotoxicity. The cell damage induced by Hcy is dependent on the NMDA receptor, since pretreatment with an antagonist prevents this damage. Overstimulation of NMDA receptors by Hcy results in Ca^{2+} to enter the neurons (Kim, 1999; Kruman *et al.*, 2000; Lipton *et al.*, 1997). The influx of these ions disturbs the ionic balance of the neurons, which result in swelling and lysis of neurons (Coyle *et al.*, 1993). This phenomenon is shown in figure 4.1. The influx of Ca^{2+} into the cell can also cause free radical production, thus causing neurotoxicity of neurons.

However, AMPA, a non-NMDA glutamate receptor, must be activated before activating NMDA receptors. This activation results in an influx of Na^{+} ions in the cell, causing a depolarization of the membrane. Depolarization of the membrane results in removal of the magnesium block, allowing the opening of the channel once Hcy has bound. Once the channel is opened, Ca^{2+} influx occurs in the neuron.

High concentrations of glutamate within the synapse result 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 & Daya, 1999). In addition, the activation of the NMDA receptor by Hcy results in an influx of Ca^{2+} ions. This together with the water uptake results in the swelling of the cells evident in the photomicrographs.

4.5 Conclusion

Hcy is an excitatory amino acid (EAA) and causes direct neurotoxicity by activating the NMDA subtype of glutamate receptor. Excessive stimulation of these receptors is known to mediate brain damage (Lipton *et al.*, 1997). Thus Hcy participates in the ensuing neurotoxic response in the brain.

Several studies have shown that 17β-estradiol protects against neuronal death due to excitotoxicity. Behl *et al.*, (1995) and Goodman et al., (1996) reported that preincubation with 17β-estradiol attenuates the toxicity of glutamate exposure to a hippocampal cell line culture, as measured by cell viability and lysis. In addition, progesterone also attenuates EAA responsiveness (Smith *et al.*, 1987; Smith 1991). These authors showed that progesterone reduces excitotoxic cell death by inhibiting excitatory EAA receptors.

From the results obtained in this chapter, it is evident that 17β-estradiol and progesterone attenuate Hcy insult in the rat hippocampus. This protection elicited by both hormones as well as that shown against the increase in lipid peroxidation, could be due to its antioxidant property, in this way preventing further damage induced by the free radicals formed during the Hcy damage. However, it is more likely that the

protection of both hormones against Hcy-induced neuronal damage involves the NMDA receptor.

CHAPTER 5

APOPTOSIS

5.1. INTRODUCTION

Apoptosis is an ancient Greek word used to describe the "falling off" of leaves from trees or petals from flowers, referring to the particular morphology of physiological cell death (Kerr *et al.*, 1972). It refers to the morphologic features of programmed cell death, which is characterized by cell shrinkage, nuclear condensation, nucleosomal DNA fragmentation (DNA ladder), membrane blebbing and fragmentation into membrane bound apoptotic bodies (Saikumar *et al.*, 1999).

Apoptosis has important biological roles in the development and homeostasis of cell populations, and in the pathogenesis and expression of disease processes. Excessive or insufficient apoptosis contributes to the pathogenesis of a wide variety of neurodegenerative diseases, such as Alzheimer's disease (AD). One hallmark of AD is the accumulation of β-amyloid (Aβ) (Haas *et al.*, 1992; Seubert *et al.*, 1992). Exposure of neuronal cells to aggregated Aβ induces multiple neurodegenerative events, including accumulation of cytosolic calcium, generation of reactive oxygen species and apoptosis; however, prevention of accumulation of calcium within the cytosol also prevents all of these other neurotoxic events (Ekinci *et al.* 1999, 2000). Moreover, DNA damage, which is a potent trigger of neuronal apoptosis, has been documented in studies of AD patients and animal models of AD (Mullaart *et al.*, 1990;

de la Monte *et al.*, 1998; Torp *et al.*, 1998).

A number of methods have been described to identify apoptotic cells (Afanasyev *et al.*, 1993; Bryson *et al.*, 1994). One of the methods is the TUNEL (TdT-mediated dUTP nick end labelling) method. It was designed as a histochemical technique to detect internucleosomal DNA fragmentation at the level of individual cells (Ben-Sasson *et al*., 1995). The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis and this allows discrimination of apoptosis from necrosis and from primary DNA strand breaks induced by cytostatic drugs or irradiation (Gorczyca *et al*., 1993).

Thus, in this chapter it was decided to investigate the ability of Hcy to promote apoptosis in the hippocampus and protection offered by the treatment with 17β-estradiol and progesterone using the *In Situ* Cell Death Detection Kit, POD. This kit is designed as a precise, fast, and simple, non-radioactive technique for the detection and quantification of apoptosis at single cell level in cells and tissues, based on labeling of DNA strand breaks (TUNEL technology) using light microscopy.

5.2. THE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE AGAINST HCY-INDUCED APOPTOSIS

5.2.1. INTRODUCTION

Hcy inhibits the expression of antioxidant enzymes as well as the synthesis of radical scavengers (e.g. glutathionine peroxidase, superoxide dismutase) which might potentiate the toxic effects of ROS (Huang *et al.,* 2001b). Homocysteine-induced ROS accumulation enhances the activation of NF-kappaB (Chern *et al.*, 2001). NF-kappaB is a redox-sensitive transcriptional factor which is crucial in the control of ROS-mediated apoptosis. Under these conditions, neuronal damage derives from excessive calcium influx and ROS leading to excitotoxicity. Moreover, primary inhibition of the mitochondrial respiratory chain indirectly induces NMDA receptor stimulation, which is termed secondary excitotoxicity (Greene & Greenamyre, 1995).

As shown in figure 5.1, the endoplasmic reticulum (ER) is an intracellular target of Hcy toxicity (Outinen *et al.*, 1998; Althausen & Paschen, 2000; Kokame *et al.*, 2000; Nonaka *et al.*, 2001; Zhang *et al.*, 2001; Mattson & Shea, 2003). ER stress is a condition in which misfolded proteins accumulate in the ER lumen. Homocysteine enhances the expression of both ER chaperone proteins (e.g. grp78, grp94, gadd153) (Outinen *et al.*, 1998; Althausen & Paschen, 2000; Nonaka *et al.*, 2001) and the novel homocysteine-induced unfolded non-chaperone Herp (Kokame *et al.*, 2000). The close relationship between the extent of cell injury and increase in ER stress response proteins may contribute to the homocysteine-induced apoptotic features.

Homocysteine induces apoptosis by mitochondrial dysfunction (Austin *et al.*, 1998; Mercie *et al.*, 2000; Bessede *et al.*, 2001). Calcium overload inside the cell damages mitochondria and causes neurotoxicity. Because of the collapse of the mitochondrial membrane potential, the production of ATP is compromised. This causes further accumulation of Ca^{2+}, since Ca^{2+} pumps need ATP to eliminate excess Ca^{2+} from of the cell (figure 5.1). However, Hcy is an endogenous ligand for the NMDA receptor (Zeise, 1988), and a portion of its neurotoxicity in cortical neurons is achieved by overstiumlation of NMDA receptors and resultant calcium influx (Lipton *et al.*, 1997; Kim, 1999; Kruman *et al.*, 2000) and leading to apoptosis (Lipton *et al.*, 1997). Furthermore, the consecutive leakage of cytochrome c from mitochondria as well as ROS (Huang, 2001b) activates the caspase-3 pathway. Caspases are a family of aspartate-specific cysteine proteases that are an important part of various apoptotic processes. The homocysteine-induced activation of the caspase-3 pathway (Kruman *et al.*, 2002) leads to DNA fragmentation which is a hallmark of apoptosis. This homocysteine-induced DNA damage (e.g. DNA strand breaks) in neurons further triggers a cell death cascade involving degradation or activation of poly-(ADP-ribose) polymerase (PARP) and induction of the tumor suppressor protein p53 (Kruman *et al.*, 2000). ROS accumulate in response to mitochondrial damage and in response to the activation of the arachidonic acid pathway by both homocysteine and Ca^{2+}. Autooxidation of homocysteine is known to generate ROS, whereby the prevention of homocysteine-induced toxicity by SOD, GPX and catalase suggest that hydrogen peroxide acts as a mediator of oxidative injury, leading to apoptosis (Austin *et al.*, 1998; Outinen *et al.*, 1998; D'Emilia & Lipton, 1999). Furthermore, Ekinci et al., (1999, 2000) suggests that calcium accumulation is an early and pivotal event in Aβ neurotoxicity. Hcy enhances the neurotoxicity of β-amyloid which is known to cause neurodegeneration (Ho *et al.*, 2001; Mattson *et al.* 1992a; Gray & Patel 1995). It has

also been shown that Hcy induces an increase in phospho-tau immunoreactivity and externalised phosphatidylserine which is indicative of apoptosis (Ho *et al.*, 2002).

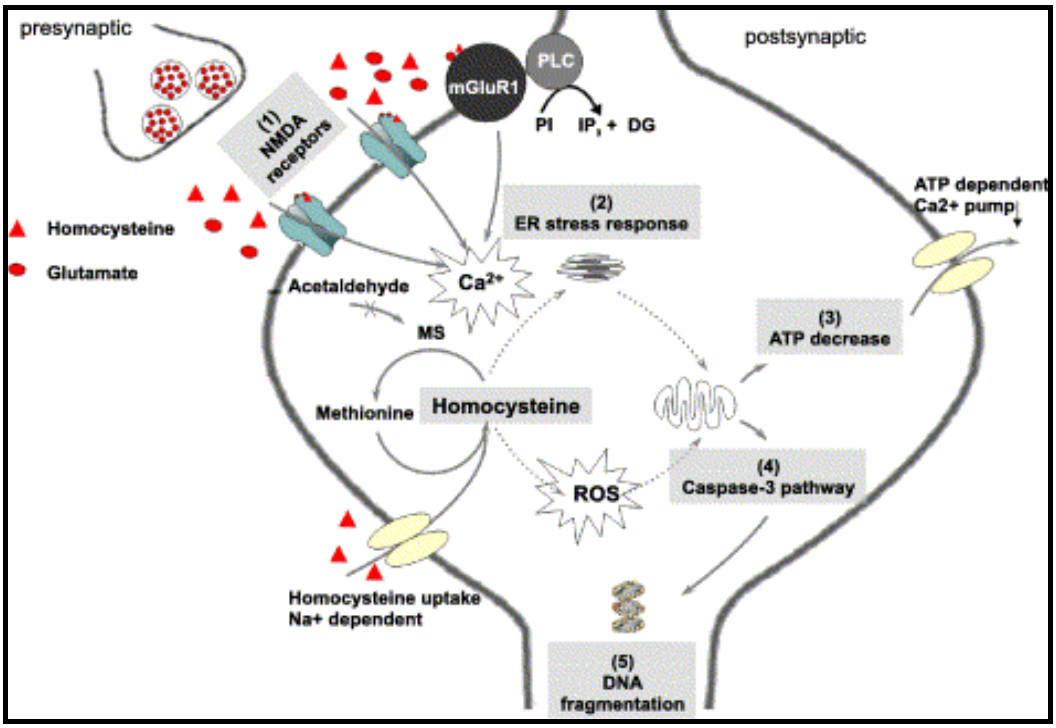


Figure 5.1. Extra- and intracellular neurotoxic mechanisms induced by homocysteine (Bleich *et al.*, 2004).

Since the ability of Hcy to induce damage in the CA1 and CA3 hippocampal neurons was shown in chapter four, it was decided to investigate whether Hcy results in apoptotic cell death as well. Thus, the aim of this chapter was to investigate whether damage of the hippocampus with Hcy results in apoptotic neuronal death and if co-treatment of rats with Hcy and 17β-estradiol or progesterone results in a reduction in positive apoptotic neurons, using the *in situ* cell death detection kit, POD.

5.2.2. MATERIAL AND METHODS

5.2.2.1. Chemicals and Reagents

17β-estradiol, progesterone (4-Pregnene-3,20-dione), DL-homocysteine were

purchased from Sigma St. Louis, MO, U.S.A. Aminopropyltriethoxysilane (APES) was purchased from NT lab Fluka. Paraffin wax was obtained from Lasec (South Africa). *In situ* cell death detection kit, POD, proteinase K (nuclease free), DNase 1, grade 1 (positive control), and DAB substrate were purchased from Roche Diagnostics, (Nonnenwald, Penzberg). Formaldehyde, glacial acetic acid, absolute ethanol, xylene, and chloroform were purchased from Saarchem, Gauteng, SA while the aqueous mountant, SHUR/MOUNT™ was purchased from Triangle Biomedical Sciences Inc, Durham, USA. All other chemicals were of the highest quality available and were purchased from commercial distributors.

5.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 250-300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.2.

5.2.2.3. Dosing of Animals

As described in section 2.3.2.3.

5.2.2.4. Surgical Procedure

As described in section 2.3.2.4.

5.2.2.5. Histological Techniques for Apoptosis Detection

The histological techniques were followed according to the method described by the *in situ* cell death detection kit, POD instruction manual (www.roche-applied-science.com/pack-insert/1684817a.pdf) and Maharaj, 2003.

5.2.2.5.1. Fixing the brain

The animals were sacrificed and the brains removed as in section 2.2.2.3. Immediately after death, animal tissues begin to break down as a result of autolysis and bacterial attack. Fixation functions to chemically stabilize proteins, and thus preserve structures (Southgate, 1999). The role of the fixative is to maintain the morphology of the tissues as close to *in vivo* morphology as possible and to prevent post-sampling necrosis. A recommended fixative used for the study of brain tissue is Davidson's solution. The ratio of fixative to tissue volume should be at least 10:1 to ensure good fixation.

There is no universal fixative and choice should be made taking into account later use of fixed material as well as practical aspects of fixative use (price, component availability, etc). Davidson's solution is an excellent choice for preserving the structure of the tissues (Lighter, 1996). In addition, tissue sections fixed with Davidson's solution can be stained later by different histochemical methods, as well as *in-situ* hybridization with DNA proteins. Davidson's alcohol formalin acetic acid fixative consists of 220mL of formalin (100%), 115mL of glacial acetic acid, 330mL of ethanol, and 335mL of water. This mixture is stored at room temperature prior to use (Lighter, 1996).

Brains were rapidly fixed in the Davidson's fixative mixture for 48 hours. After fixation, a slice of brain, 2mm thick was prepared to exclude the location of the injection track, which was normally apparent from the residual dimpling of the cortical surface produced by the needle penetration. Exclusion of the tissue directly below the site of the injection ensured that all damage was due to neurotoxin or drug and not due to the physical damage caused by the cannula needle. The 2mm block of brain tissues were then stored in 70% ethanol.

The fixation process was terminated by dehydrating the brain tissue. For apoptosis detection, traditional processing and embedding techniques cannot be used as described in chapter four, section 4.2.2.5.1. Thus, in order to reduce residual damage and improve the fluorescence of the tissue, reagents that could affect the fluorescence were eliminated from the procedure. Moisture was extracted from the tissue fragments by bathing them successively in a graded series of mixtures of ethanol, and this step was followed by the clearing and defatting process that involves the removal of ethanol by immersing the tissue in chloroform. The tissue was then submerged in molten paraffin wax (MP 57-58˚C) at 60˚C for one hour, which facilitated the removal of chloroform and while infiltrating the tissue without encountering water. At the end of this 1 hr period the brain tissue was placed under vacuum to remove any air that was trapped in the wax for 15min. Thereafter, the brain tissues were placed in new wax twice for a period of 1hr each. Finally the brain tissue was embedded in molten wax and this stage provides the hardness and support that the tissue requires for sectioning. The method used here is a modification of the method described by Geiger *et al*., (1997) and is described in Table 5.1. The tissues can be stored in paraffin indefinitely without visible influence on the quality of TUNEL reactions (Geiger *et al*., 1997).

Table 5.1. Fixation and Processing of Tissues for Paraffin Embedding.

Step	Processing Agent	Time (Hours)
Fixation	Davidson's Fixative solution	48 hrs
Dehydration	50% Absolute Ethanol	1 x 2 hrs
	70% Absolute Ethanol	1 x 2 hrs
	80% Absolute Ethanol	1 x 2 hrs
	90% Absolute Ethanol	1 x 2 hrs
	96% Absolute Ethanol	2 x 2 hrs
	100% Absolute Ethanol	3 x 2 hrs
Clearing	Absolute Ethanol: Chloroform (1:1)	1x 2 hrs
	Chloroform	1 x 2 hrs
	Xylene: Chloroform (1:1) at 60°C	1 x 1 hr
Wax Immersion	Melted Paraffin Wax (MP 57-58°C) at 60°C	1x 1 hr
	Vacuum at 60°C	15min
	Melted wax	2 x 1hrs
Embedding	In molten Wax	Overnight

5.2.2.5.2. Sectioning

Paraffin sections were cut by standard methods as described in chapter twelve, section 4.2.2.5.2. The sections of paraffin-embedded tissue was cut 5µm thick and placed on the APES coated slides. The treatment procedure for the slides is described below.

5.2.2.5.3. Treatment of Slides

When mounting paraffin sections on slides, it is important to use an appropriate adhesive to avoid loss of sections during the subsequent washing procedures. Sections can be mounted on Superfrost slides or on glass slides that have been coated (subbed)

with either aminopropyl triethoxysilane (APES) or poly-L-lysine. APES has been shown to be superior to poly-L-lysine in preventing tissue detachment from the glass (Ben-Sasson, 1995) and thus, APES was use to treat the glass slides prior to use. The slides were subbed at least 2 days prior to the application of the paraffin sections.

The method described by Herrington and McGee (1992) was used for treating the slides. Briefly, the slides were placed in a rack and cleaned by immersion for 30 min in 2% Decon 90 made in warm (60°C) distilled water. This was followed by rinsing in distilled water, and then in acetone and finally air dying. The slides were then immersed into 2% APES made in acetone for 30 min. Finally the slides were rinsed with acetone, washed in distilled water and air dried at 37°C. The slides were then stored in a dry place for 2 days prior to use.

5.2.2.5.4. Transferring Sections to Slides

One or two sections at a time were removed from the water bath and placed onto glass microscope slides using a thin paint brush. The paraffin slides were stored at room temperature until use to enable the section to adhere to the slide.

5.2.2.5.5. Deparaffinising Sections

Deparaffination should be as complete as possible, since remaining paraffin adversely affects the TUNEL reaction. The sections were heated 60°C for 20 min and then hydrated through several baths of xylene and a graded series of ethanol at concentrations ranging from 100 to 70% with an immersion time of 3 min per bath as shown in table 5.2. Thereafter, the sections were rinsed in PBS (pH 7.4) for 30 sec.

Care was taken to ensure that the slides did not dry out during the deparaffinising.

Table 5.2. Procedure for dewaxing and rehydrating brain sections.

Step	Processing Agent	Time
1	Heat at 60°C	20 min
2	Xylene	2 x 5 min each
3	100% Ethanol	2 x 3 min each
4	90% Ethanol	1 x 3 min
6	80% Ethanol	1 x 3 min
7	PBS	30 sec

5.2.2.5.6. In situ Cell Death Detection Kit, POD

For the TUNEL reaction, tissue sections were processed according to the procedure described below.

5.2.2.5.6.1. Deproteinisation with Proteinase K

After the PBS wash, the tissue sections were partially deproteinised by incubation with Proteinase K. Proteinase K treatment digests cross-linked proteins and thereby increases cell permeability and access to the nucleic acid targets i.e. DNA. Proteinase K is preferred because it does not require predigestion to reduce residual nucleases (Willson & Higgins, 1990). The concentration, incubation time, and temperature of proteinase K are extremely important and have to be optimized for each type of tissue as high concentrations can cause tissue damage and increase nonspecific staining (Tornusciolo, 1995). The slides were incubated in 20µg/ml proteinase K which was made up in 10mM Tris-HCl buffer, pH 8, for 15min at 37°C in a humidified chamber. Care was taken to ensure that the sections did not dry out.

Since the final immunohistochemical stain is peroxidase-independent (Geiger *et al.*, 1997), no inhibitions of endogenous peroxidases that can produce high levels of background staining and interfere with the interpretation of the results, was performed because H_2O_2 weakens TdT activity (Migheli *et al.*, 1995) and induces DNA breaks (Wijsman *et al.*, 1993). Thus, incubation step was terminated by washing the slides four times in PBS for 3min each. The experimental slides were kept in PBS while the positive control slide was removed for DNase treatment as described below.

5.2.2.5.6.2. Positive DNase Controls

There is a substantial amount of variation in positive staining when using the TUNEL method; therefore, at least two DNase control slides should be included with each experimental run. The type, size and fixation of the tissue are contribution factors to the variation in staining. Since DNA fragmentation is characteristic of apoptosis, application of DNase to control slides is ideal. DNase is an endonuclease that introduces breaks by hydrolyzing double-stranded or single-stranded DNA, preferentially at sites adjacent to pyrimidine nucleotides (Sambrook *et al.*, 1989); therefore, pretreatment with DNase results in intensive labelling of all nuclei. If the DNase controls do not stain, staining on the experimental slides may be artifact and not positive staining.

The concentration of DNase used was 3000U/ml prepared in 50mM Tris-HCl, pH 7.4 containing 1mg/ml BSA. After finger flicking for 10sec, sufficient DNase mixture was applied to the desired DNase control slides in order to cover the entire section and incubated for 10min at 25˚C. This mixture should not be made until needed, since thawing of the DNase I cause its inactivation. After DNase 1 pretreatment, the

positive control slides were washed thoroughly with PBS, since residual DNase activity can introduce high background.

5.2.2.5.6.3. Labelling Protocol

In the TUNEL method, TdT is used to incorporate biotinylated deoxyuridine at the sites of DNA breaks. Both single-stranded DNA and 3' overhangs of double-stranded DNA are good substrates for TdT. The TdT is generally inefficient at catalyzing the transfer of biotinylated dUTP to blunt or recessive ends (Deng & Wu, 1983). The *in situ* cell death detection kit, POD (Roche) contains three vials; vial 1 is the enzyme solution which contains the TdT from calf thymus (EC 2.7.7.31) in storage buffer; vial 2 which is the label solution contains the nucleotide mixture in reaction buffer. However, vial 3 is the converter-POD which is an anti-fluorescein antibody, conjugated with horseradish peroxidase (POD).

To prepare the reaction mixture 100µl label solution is removed from vial 2 and kept away for the 2 negative controls. The total volume of the enzyme solution (vial 1) is added to the remaining 450µl of label solution (vial 2) to obtain 500µl of TUNEL reaction mixture. The mixture is mixed well to equilibrate the components. To maximize efficiency the TUNEL reaction mixture is prepared during the 10min DNase treatment step and kept on ice until use. In addition, the TUNEL reaction mixture is sensitive to light therefore it was prepared in the dark. 50µl/section of TUNEL reaction mixture containing the enzyme and digoxigneninlabeled dUTP was added to both the experimental and DNase control slides while, 50µl of the labelling solution was added to each of the negative controls. All the slides were covered with a zip-lock bag, in order to prevent the slides from drying out and this also imposes an

even layering of the reaction mixture over the whole tissue section. All the slides were then incubated in a humidified chamber for 60min at 37°C in the dark. TdT is temperature-sensitive; temperatures above 40°C inactivate the enzyme (Geiger *et al.*, 1997) therefore the temperature was constantly monitored in the humidified chamber to ensure that a temperature of 37±2°C was maintained throughout the incubation period.

The reaction was then terminated by immersing the slides in PBS. The slides were washed three times in PBS for 5mins each.

5.2.2.5.6.4. Signal Conversion

Following the labeling with TUNEL reaction mixture results in fluorescein, POD antibody and DAB substrate was added. This allows for the detection of apoptotic cell death under light microscopy. The reaction of fluorescein-labeled DNA strand breaks with POD and DAB substrate is illustrated in figure 5.2.

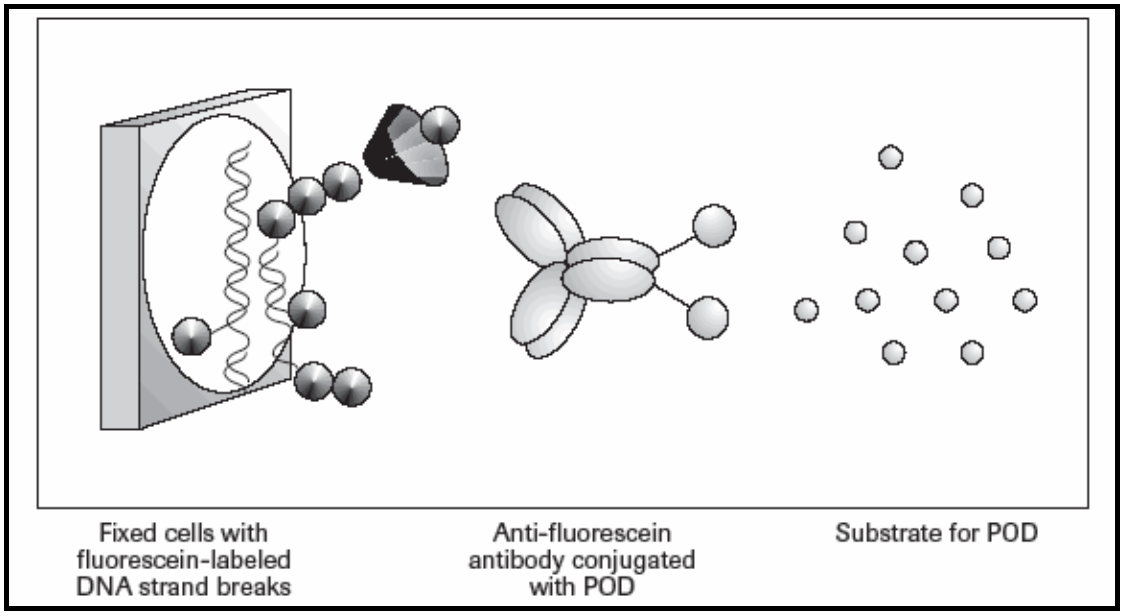


Figure 5.2. Illustration of the reaction mechanism of *In Situ* Cell Death Detection Kit, POD. The reaction allows for the viewing of apoptotic cells under light microscopy (www.roche-applied-science/pack-insert/1684817a.pdf).

After washing the slides with PBS, 50µl of converter-POD (vial 3) was added to each tissue sample. To ensure homogenous spread of converter POD throughout the tissue and to avoid evaporative loss the samples were covered with parafilm. The slides was incubated in a humidified chamber for 30 min at 37±2°C. The slides were washed three times in PBS.

The DAB substrate, metal enhanced utilizes cobalt chloride and nickel chloride in a special formulation to produce a dark brown/black precipitate in the presence of horseradish peroxidase (POD). A 10% of DAB substrate was prepared by utilizing peroxide buffer. 100µl DAB substrate was then added to each tissue sample and was covered with parafilm during incubation. The slides were incubated for 10 min at 15-25°C. Once again, the slides were rinse three times with PBS.

5.2.2.5.6.5. Mounting of Slides

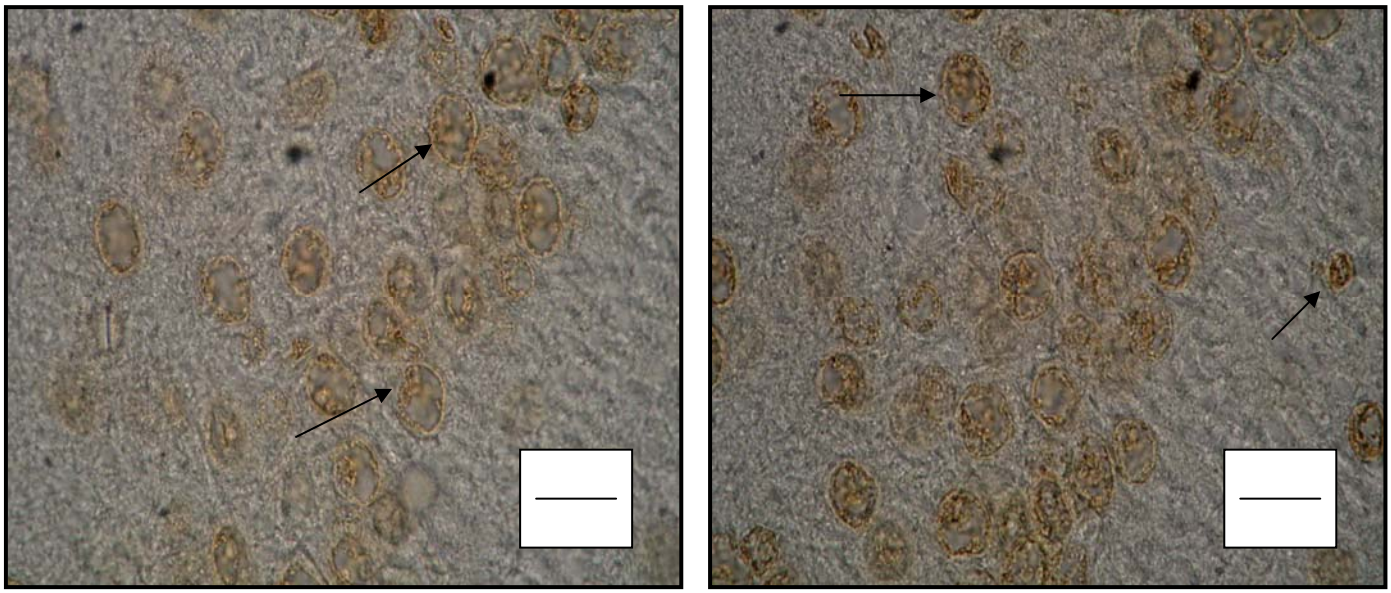
In order to prevent the drying out of the sections, the sections need to be mounted. However, while the slides were still wet enough SHUR/MOUNT^{TM} was added to them. SHUR/MOUNT^{TM} is an aqueous mountant that also assists in preserving the fluorescence of the tissue. Tissue sections were then covered with coverslips and allowed to dry in the dark.

5.2.2.5.6.6. Photo-microscopy

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

5.3. RESULTS

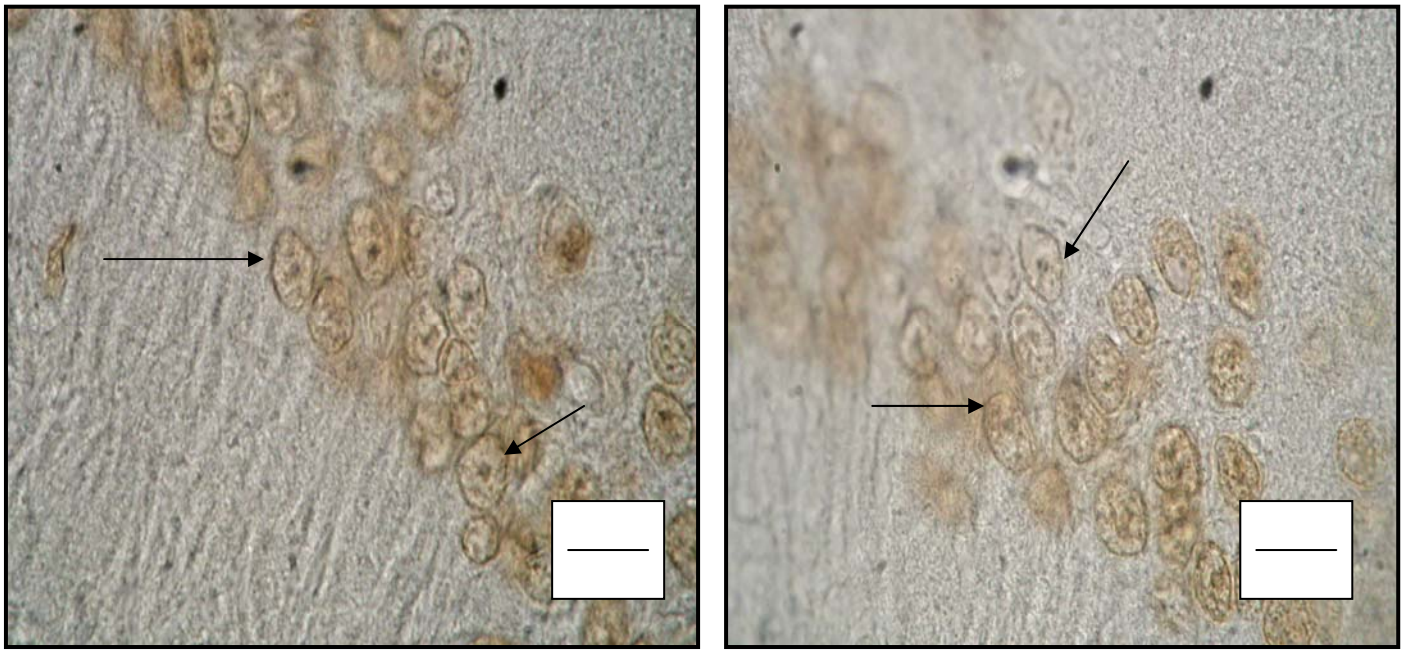
In figure 5.3 (a) and (b), the positive apoptotic cells of CA1 and CA3 regions are indicated with arrows. The positive control slides treated with DNase 1 are used as a guideline to ensure the experimental slides is not artifact. In the positive apoptotic control cells, the neuronal cells appeared scattered and the nuclei are stained in brown colour. The DAB substrate stains the apoptotic positive cells brown in colour. However, some of the nuclei are absence in the neuronal cells which is an indication of nuclear fragmentation.



(a) (b)

Figure 5.3. Detection of apoptotic cells (stained brown in colour) in the CA 1 and CA3 regions of the hippocampus. (a) CA1 region of positive control tissue, (b) CA3 region of the positive control tissue (Bar = 10μm). Arrows showing positive apoptotic cell death neurons.

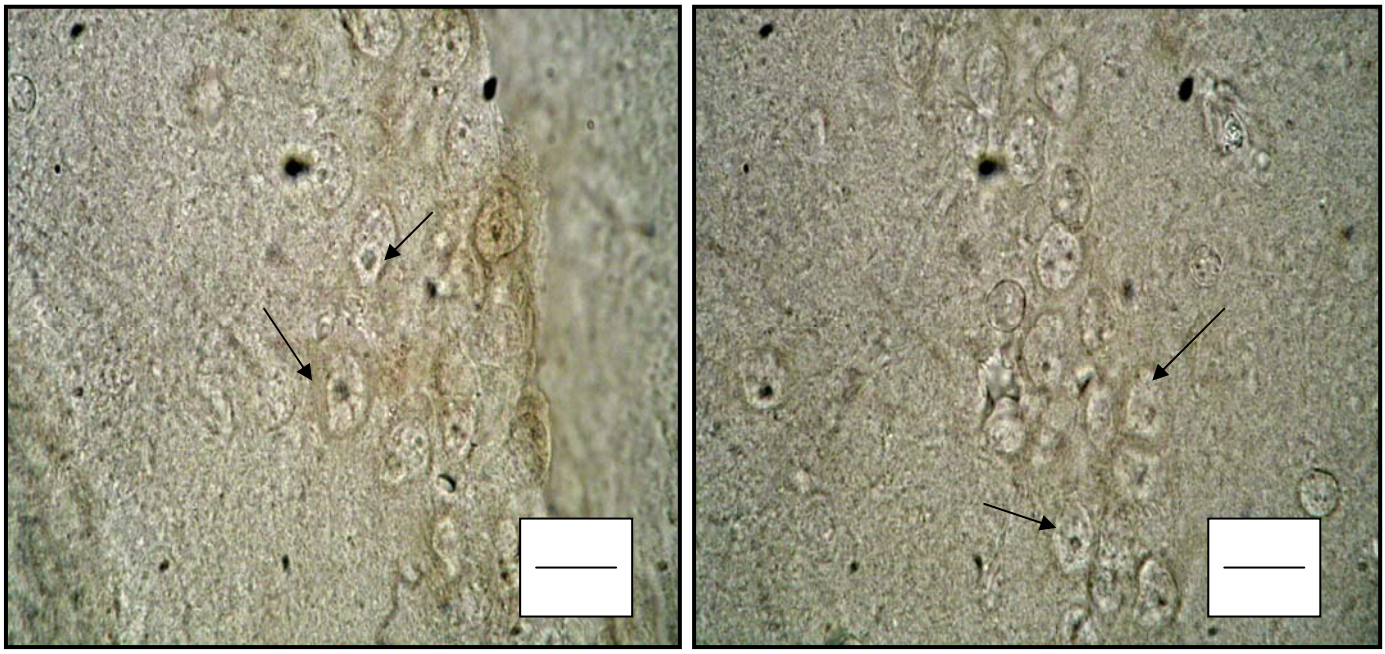
Figure 5.4 (a) and (b) show the negative control cells of the CA1 and CA3 regions of hippocampus respectively. The cells appeared clear, the nuclei can be seen clearly and lacking the brown staining. Thus there is no indication of apoptotic cell death occurring in the neurons. The neuronal cells appear healthy and undamaged.



(a) (b)

Figure 5.4. Hippocampal neuronal cells from negative control treated rat brain section. (a) CA1 region of negative control tissue, (b) CA3 region of negative control tissue (Bar = 10μm). Arrows show healthy neurons lacking the brown stained nuclei which are investigative of apoptosis.

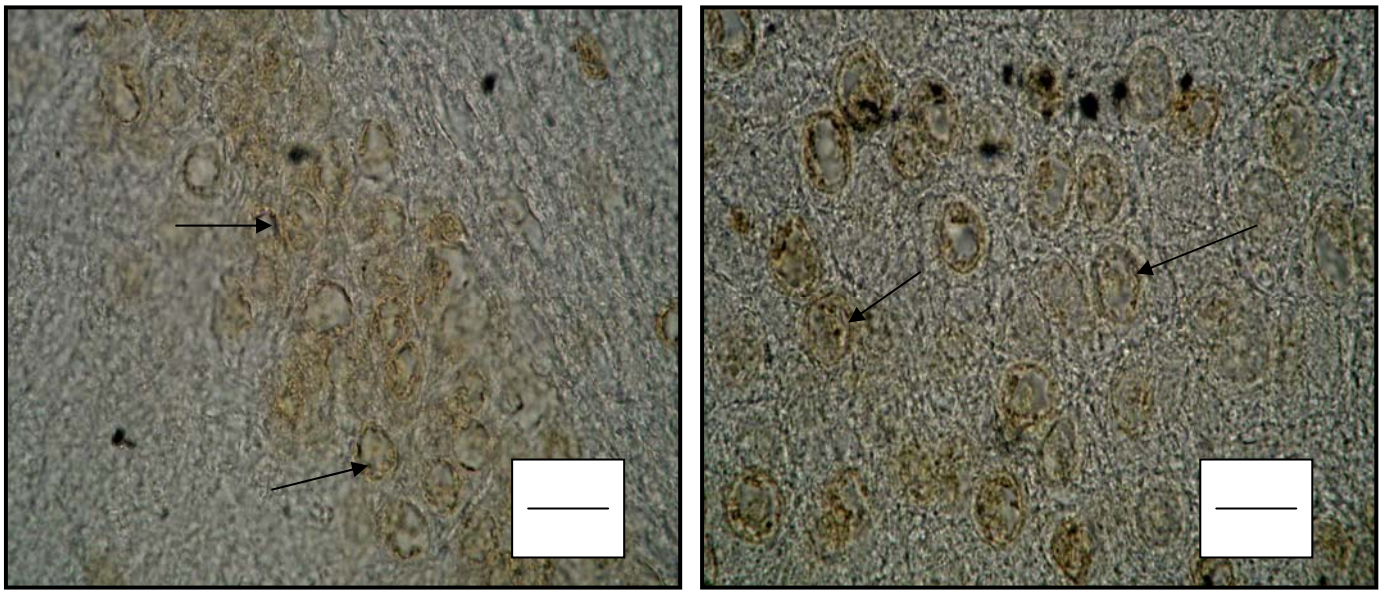
Figure 5.5 (a) and (b) show the CA1 and CA3 of the control treated groups rat brains. The neurons are clearly visible with continuous cell membrane. The pyramidal shape neuronal cells are observed with the absence of the brown staining nuclei. These indicate that there is no apoptotic cell death occurred. The neurons of the control group section resemble those from the negative control section as evident in figure 5.4 (a) and (b).



(a) (b)

Figure 5.5. Hippocampal neuronal cells from control treated group rat brains. (a) CA1 region of control treated group, (b) CA3 region of control treated group. (Bar = 10μm). Arrows show neurons lacking the brown stained nuclei.

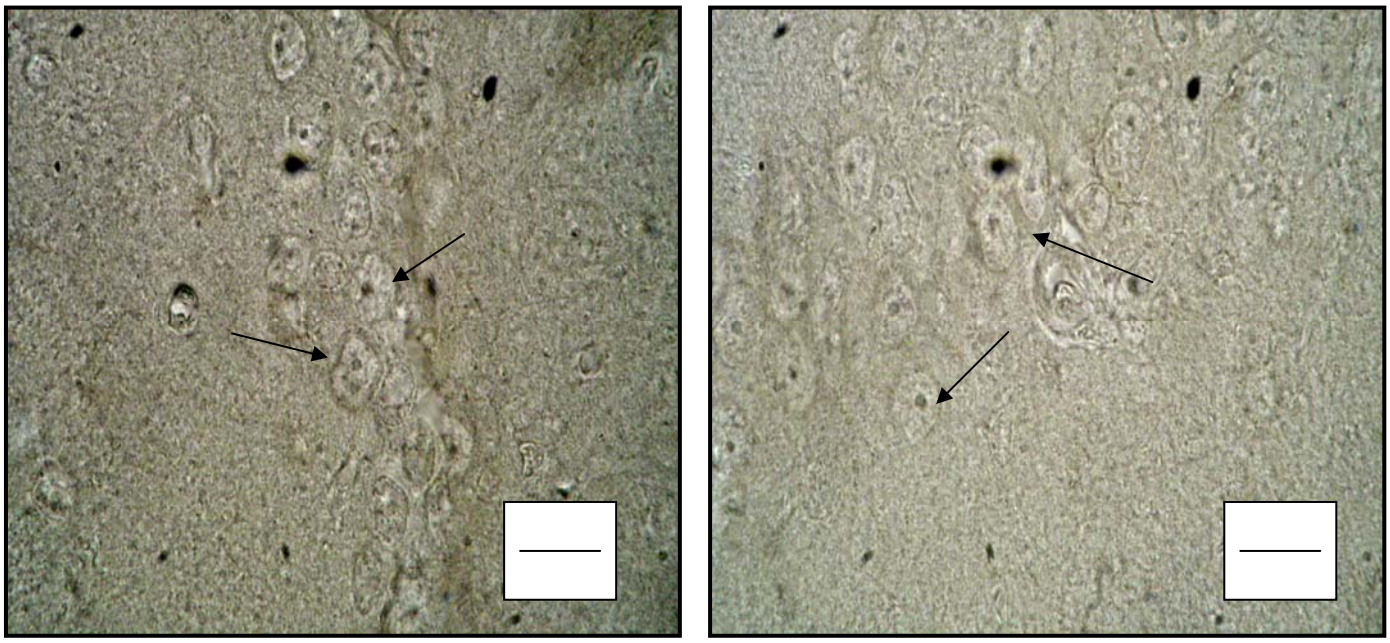
Figure 5.6 (a) and (b) show the apoptotic cells in the Hcy treated rats as indicated with arrows. The neuronal cells in both CA1 and CA3 regions are stained in brown colour intensely. The nucleus is absent in these cells indicating that nuclear fragmentation have occurred. These neurons are lack of uniformity in shape; this is result from blebbing of the cell membrane.



(a) (b)

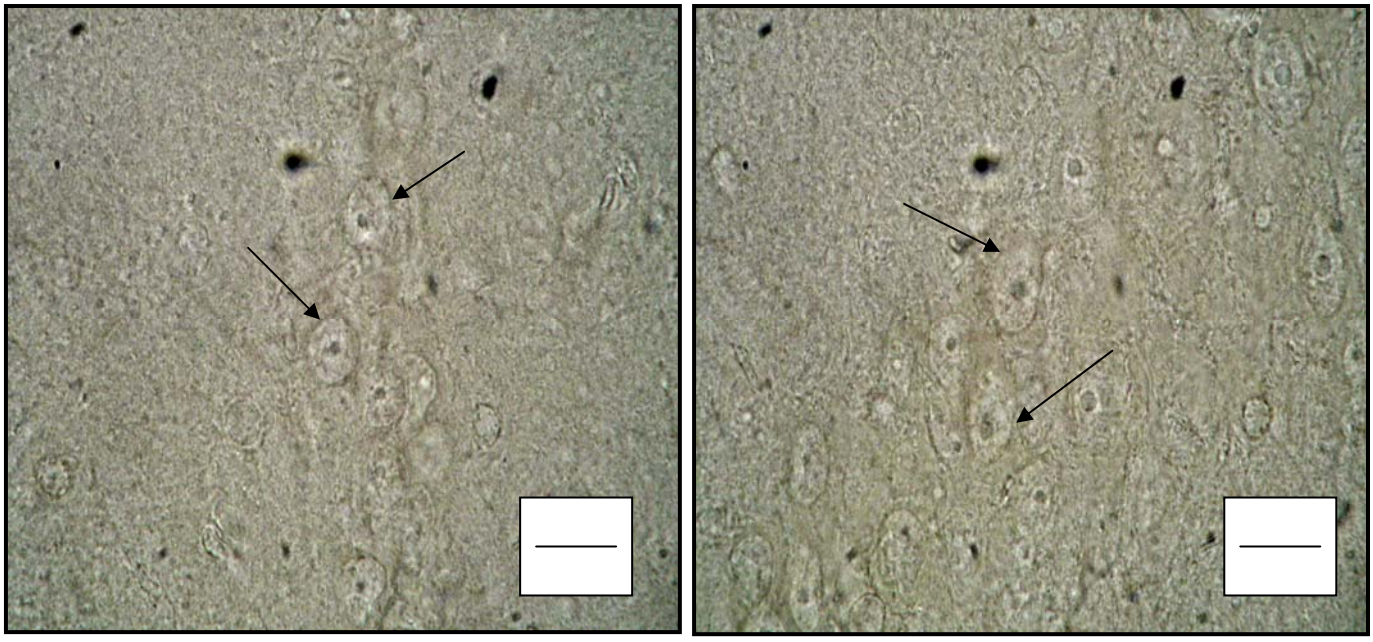
Figure 5.6. Hippocampal neuronal cells of Hcy treated rat brains. (a) CA1 region of Hcy treated rat group, (b) CA3 region of Hcy treated rat group. (Bar = 10μm). Arrows show brown stained apoptotic cells.

The pre- and post- treatments of either 17β-estradiol or progesterone rat brains are able to prevent against the Hcy-induced apoptotic cell death in CA1 and CA3 as evident in figure 5.7, 5.8, 5.9 and 5.10. Lack of brown staining of neuronal cells is an indication of non-apoptotic cells. The neurons of both treatments appear lack of intense brown staining that can be observed in the Hcy treated group (figure 5.6). However, the hippocampal neuronal cells of both regimes of 17β-stradiol or progesterone treated groups have no significant difference among the hippocampal neurons of the control treated rats. The neuronal cells appear as healthy as those in the control treated rats. In figures 5.7-5.10, the neurons posses visible nuclei and continuous cell membranes that are absent in the neurons of the Hcy treated group (figure 5.6). These results (figure 5.7 – 5.10) indicating that both regimens of 17β-estradiol or progesterone are able to protect against Hcy-induced apoptotic cell death.



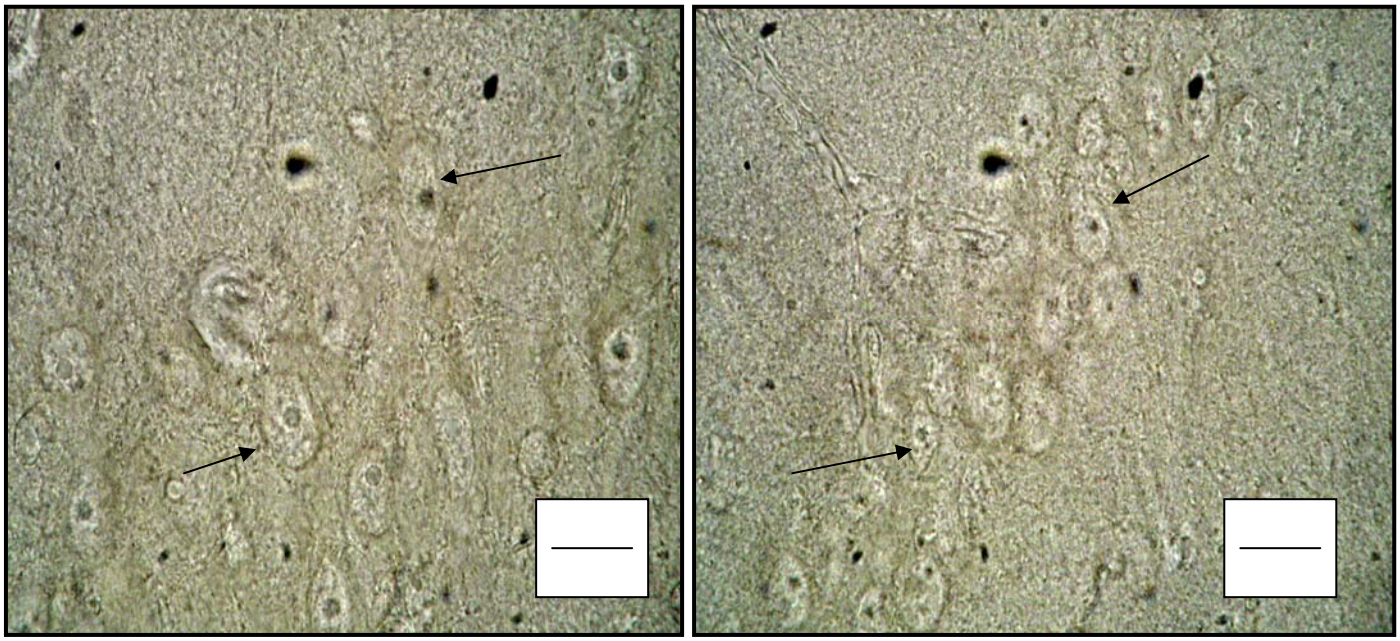
(a) (b)

Figure 5.7. Hippocampal neuronal cells of animals treated with Hcy and 17β-estradiol (Post-treatment). (a) CA1 region of treated rat group, (b) CA3 region treated rat group. (Bar = 10μm). Arrows show neurons lack of apoptotic cell death. (Bar= 10μm).



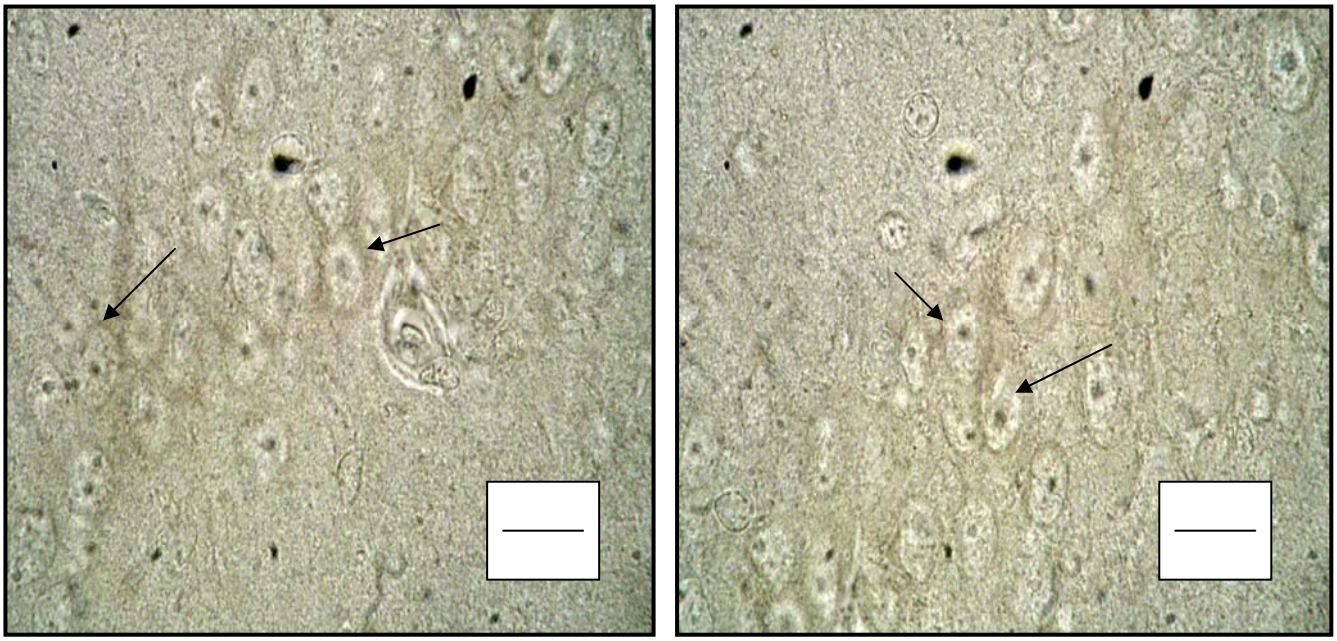
(a) (b)

Figure 5.8. Hippocampal neuronal cells of animals treated with Hcy and 17β-estradiol (Pre-treatment). (a) CA1 region of treated rat group, (b) CA3 region treated rat group. (Bar = 10μm). Arrows show neurons lack of apoptotic cell death. (Bar= 10μm).



(a) (b)

Figure 5.9. Hippocampal neuronal cells of animals treated with Hcy and progesterone (Post-treatment). (a) CA1 region of treated rat group, (b) CA3 region treated rat group. (Bar = 10µm). Arrows show neurons lack of apoptotic cell death. (Bar= 10µm).



(a) (b)

Figure 5.10. Hippocampal neuronal cells of animals treated with Hcy and progesterone (Pre-treatment). (a) CA1 region of treated rat group, (b) CA3 region treated rat group. (Bar = 10µm). Arrows show neurons lack of apoptotic cell death. (Bar= 10µm).

5.4. DISCUSSION

The TUNEL kit used in this study is specific and sensitive. This method allows for the detection of apoptotic cell death in the CA1 and CA3 regions of the rat hippocampus, under light microscopy, induced by Hcy. The DNase 1 treated positive control sections (figure 5.3, (a) and (b)), provides evidence that the experiment is carried out properly and correctly. The neurons are stained in brown colour intensively, which is one of the characteristic of apoptotic cell death. Therefore, the positive control is used as a guideline for the identification of apoptotic cells in the treatment sections.

Several studies showed that elevated Hcy levels increase the vulnerability of cultured hippocampal neurons to Aβ-induced death and to promote neuronal degeneration in mutant mice (Kruman *et al.*, 2000, 2002). Hcy is also a neurotoxic amino acid that potentiated the effects of Aβ on cytosolic calcium and apoptosis in differentiated SH-SY-5Y human neuroblastoma cells (Ho, 2001). A similar potentiation of Aβ-induced neurodegeneration has previously been proposed for glutamate (Mattson *et al.*, 1992a; Gray & Patel, 1995). The synergistic influences of Hcy and Aβ on calcium influx are likely to derive from stimulation of distinct calcium channels. Lipton et al., (1997) showed that the initial influx of calcium following exposure to Hcy occurs via NMDA channels; resultant calcium influx via NMDA channels fosters increased ROS (Lipton, 1997).

Moreover, Kruman *et al.*, (2000) has reported that Hcy can induce neuronal apoptosis and can increase neuronal vulnerability to excitotoxicity by a mechanism involving DNA damage which triggers the cell death cascade, poly-(ADP-ribose) polymerase (PARP) and induction of p53. However, Cantoni *et al.*, (1986) stated that one of the

potential mechanisms by which Hcy foster increases DNA damage is by inhibition of cellular levels of S-adenosylmethionine (SAM) and thus promote apoptosis (Endresen *et al.*, 1994, 1996).

The results illustrated in figure 5.6 (a) and (b) demonstrate the dark brown staining of CA1 and CA3 regions of hippocampal neuronal cells indicating that Hcy does induce apoptotic cell death and is in agreement with the reports mentions. It is clearly evident from figure 5.6 (a) and (b) that the apoptotic cells differ greatly in structure and shape when compared to the control treatment group (figure 5.5). The hippocampal cells shown in figure 5.5 do not exhibit apoptotic cell death characteristics such as cell shrinkage, bledding of cell membrane and nuclear fragmentation. The dark brown staining of neuronal cells in Hcy treated group indicate the formation of apoptotic bodies, which was absent in the control treated group.

The results illustrated in figure 5.7 and 5.8 show that both treatment regimens of 17β-estradiol significantly attenuate Hcy-induced apoptotic cell death and this is clearly evident by the lack of brown staining neuronal cells. Prevention of apoptosis by antioxidants indicates that oxidative stress plays an essential role in the combined neurotoxic effects of Hcy and Aβ. 17β-estradiol has been reported to attenuate the Aβ-induced neuronal death in rat hippocampal cultures via its antioxidant activity (Behl, 1995; Goodman, 1996; Gridley, 1997, 1998; Green, 1998; Keller, 1997). Green *et al.*, 1998 has shown that with physiological estrogen concentrations of 0.2-2nM can protect against Aβ-induced toxicity in murine neuronal HT-22 cell. Behl *et al.*, (1994) determined that hydrogen peroxide (H_2O_2) mediates Aβ toxicity, since Aβ causes the intracellular accumulation of H_2O_2. It has been suggested that high doses of 17β-estradiol attenuate Aβ toxicity by reducing the oxidative stress caused by it (Behl,

1995; Goodman *et al.*, 1996; Gridley *et al.*, 1997).

In addition, one of the anti-apoptotic mechanisms of 17β-estradiol is that it up-regulates bcl-2 expression in the brain (Garcia-Segura *et al.*, 1998; Wang & Phang, 1995). Bcl-2 is an anti-apoptotic protein, which is a survival factor that can block both necrotic and apoptotic cell death (Bredesen, 1995) and promotes cell survival in a variety of tissues including brain. 17β-estradiol increases bcl-2 expression in cultured hippocampal neurons with a reduction in measures of Aβ-induced apoptosis (Pike, 1999). It includes the prevention of the activation of capases, inhibition of free radical formation and regulation of calcium sequestration (MacManus & Linnik, 1997). However, two *in vivo* studies further support the hypothesis that bcl-2 is a mediator of neuroprotection of 17β-estradiol. Several reports show that 17β-estradiol protects against neuronal death due to excitotoxicity. Chern *et al.*, (2001) shows that Hcy-induced ROS accumulation enhances the activation of NF-kappaB and a recent report by Lu *et al.*, (2004) shows that 17β-estradiol can inactivate this factor in liver from inflammatory cell injury, thus preventing apoptosis.

Moreover, 17β-estradiol has been shown to inhibit NMDA-induced excitotoxicity, by reducing Ca^{2+} influx (Weaver Jr., 1997). Sur *et al.*, (2003) using the light microscope using TUNEL method, showed that 17β-estradiol reduce intracellular levels of Ca^{2+} influx in C6 glial cells and thus is neuroprotective against Aβ. The result of this study provides visual evidence of the potent neuroprotective effect of 17β-estradiol on Hcy-induced apoptotic neuronal cell death.

Alternatively, the results seen in figure 5.9 and 5.10 also demonstrate the anti-apoptotic effects of progesterone in Hcy-induced apoptotic neuronal cell death in CA1 and CA3

regions of rat hippocampus. The cells of both treatment regimens of progesterone illustrate cellular characteristics that were comparable to those of the control group (figure 5.4). Progesterone is a free radical scavenger and thus reduces oxidative damage (Roof *et al.*, 1997; Olson *et al.*, 1988). Progesterone mediates anti-apoptotic actions in rat granulose cells through a progesterone-binding protein with gamma aminobutyric acid (GABA)A receptor-like features (Peluso *et al.*, 1998). Progesterone exerts GABAergic effects in the brain and inhibits neuronal excitability (Smith, 1991) and thus decreases the cerebral ischemic damage (Gonzalez-Vidal *et al.*, 1998).

Progesterone (10μM) also decreases apoptotic cell death in an *in vitro* system of NMDA-mediated cytotoxic cell death in NT2 cell line culture by using TUNEL method (Lockhart *et al.*, 2002). Concas et al., (1998) also shows that progesterone protects against the glutamate-induced-increases in intracellular Ca^{2+} in rat hippocampal slices. In addition, progesterone protects hippocampal cells against neuronal cell damage induced by Aβ and glutamate (Gursoy *et al.*, 2001). These study further support that progesterone would be of therapeutic benefit in the Hcy-induced apoptosis.

5.5. CONCLUSION

Homocysteine is a neurotoxic amino acid that accumulates in several neurological disorders including Alzheimer's disease (Clark *et al.*, 1998; Gottfries *et al.*, 1998; Miller *et al.*, 1999). Recent studies have shown that Hcy can be directly toxic to cultured neurons; the mechanism may involve the activation of NMDA receptors (Lipton *et al.*, 1997) or apoptosis triggered by DNA damage (Kruman *et al.*, 2000).

In vitro studies have shown that neuronal cell death induced by toxic factors in AD, β-amyloid, is protected by estradiol both at higher concentrations (100nM -20μM)

(Keller *et al.*, 1997; Green *et al.*, 1998; Svensson & Nordberg, 1999) or lower physiological or therapeutic concentrations (0.2-2nM) (Green *et al.*, 1996; Gridley *et al.*, 1997, 1998) and progesterone (Gursoy *et al.*, 2001). Excessive generation of $O_2^{\bullet-}$ has been linked to programmed cell death and to the diminution of the life span of organisms (Greenland *et al.*, 1995; Orr & Sohal, 1994). The neuroprotective effects of both hormones are related to free radical scavenging. The results are confirmed by studies conducted in chapter 2 and 3, where both hormones reduced Hcy-induced $O_2^{\bullet-}$ generation and lipid peroxidation, respectively. However, this study provides visual evidence that the potent antioxidant properties of both hormones are converted to a neuroprotective role in the presence of potent neurotoxins, Hcy. The preservation of cellular integrity in both CA1 and CA3 regions of the hippocampus, by both hormones, implies that these agents could act as antioxidants and reduce excitotoxicity. Therefore, the further neuroprotective effects of both hormones, in Hcy treated rats, were investigated in chapter 6 and 7, respectively.

CHAPTER 6

ENZYMATIC ANTIOXIDANTS: DEFENCE SYSTEM

6.1 INTRODUCTION

Free radicals are molecules, which have an unpaired electron in the outer orbit (Gilbert, 2000). These are generally unstable and very reactive. Oxygen and nitrogen free radicals convert to other non-radical reactive species, such as hydrogen peroxide and peroxynitrite (Evans *et al.*, 2001). However, excessive production of free radicals can potentially damage different macromolecules such as proteins, nucleic acids and lipids, thus leading to cellular degeneration (Cohen & d'Arcy Doherty, 1987). Brain cells have a highly active oxidative metabolism with elevated levels of ATP consumption, yet they contain only low to moderate enzymatic antioxidant defence molecules such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities (Cooper, 1997; Clarke *et al.*, 1999). Consequently, in preventing neurodegeneration, it is essential to preserve the antioxidant molecules such as SOD and GPx in the brain.

Brain antioxidant defences function properly during most of human life. However, a number of neurodegenerative processes, which involve reactive oxygen (ROS) and nitrogen species, become evident with age (Sayre *et al.*, 2000). Brain cells possess a complete set of antioxidant defenses, including enzymatic defense molecules such as

SOD and GPx, which can react with ROS and neutralize them before inflict damage in the brain.

Hcy can increase oxidative stress by inhibiting expression (Nonaka *et al.*, 2001) or function (Yamamoto *et al.*, 2000) of key antioxidant enzymes such as SOD and GPx, which might potentiate the toxic effects of ROS (Huang *et al.*, 2001). Upchurch *et al.*, (1997) found that Hcy reduced GPx mRNA concentration and enzyme activity in bovine aortic endothelial cells.

Therefore, the aim of this chapter was to investigate the effects of Hcy on SOD and GPx activities in rat hippocampus, *in vivo*, as well as to evaluate whether pre- and post-treatments of 17β-estradiol or progesterone altered the effects of Hcy on both enzymatic antioxidant activities.

6.1.1 Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx)

Enhanced ROS production leads to oxidative stress and ultimately to cellular damage. Due to their extreme reactivity, it is difficult to detect and quantify ROS. Therefore, involvements of ROS in pathological conditions have generally inferred from measurement of indirect markers of oxidative stress, such as changes in the activities of antioxidative enzymes. SOD and GPX are among the most important members of the antioxidant defense system.

Copper-zinc superoxide dismutase (Cu/Zn-SOD), the cytosolic isoform of SOD, is responsible for scavenging superoxide ions ($O_2^{\bullet -}$), which is the major ROS in the cell. GPx, on the other hand, is responsible for scavenging hydrogen peroxide (H_2O_2) (Ramanathan *et al.*, 2002). A steady state level of $O_2^{\bullet -}$ and H_2O_2 is always present in cells due to normal metabolism. Therefore, changes in activities of SOD and GPx

would reflect changes in levels of ROS, such as $O_2^{\bullet-}$ and H_2O_2 (Nohl and Hegner, 1978). It has been shown that under oxidative stress conditions, the overproduction of ROS is able to inhibit SOD activity (Yunoki *et al.*, 1998) and Davies et al., (1987) has shown that SOD exposed to hydroxyl radicals is an excellent substrate. Decreased SOD activity has been observed under aged rats (Gupta *et al.*, 1991) and in Alzheimer's brains (Chen *et al.*, 1994; Richardson, 1993).

6.2 THE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE IN HCY-INDUCED MODIFICATION OF SOD FUNCTION IN RAT HIPPOCAMPUS *IN VIVO.*

6.2.1 INTRODUCTION

Superoxide is an anionic radical formed by the reduction of molecular oxygen through the acceptance of a single electron. Dismutation of superoxide yields hydrogen peroxide, which produced by enzymes such as L-amino acid oxidase, glycolate oxidase, and xanthine oxidase (Sies, 1991). The superoxide anion does not cross cell membranes and, by itself, is not very reactive towards cell constituents. Hydrogen peroxide is also a comparatively inactive molecule, but unlike superoxide, it can easily cross cell membranes. This property, combined with the membrane permeability of hydrogen peroxide, gives superoxide and hydrogen peroxide the ability to affect the integrity of distant molecules within the cell (Cochrane *et al.*, 1991a; Halliwell & Gutteridge, 1989).

Enzyme antioxidants act on specific ROS after these are formed and degrade them to less harmful products. Hydrogen peroxide is first converted from superoxide radical by SOD which is not a free radical by itself, but is a precursor of the highly reactive hydroxyl radical. Detoxification of hydrogen peroxide is then carried out by GPx, which reduces hydrogen peroxide to water in the presence of GSH. Therefore, the dismutation of the superoxide anion by SOD serves as one of the antioxidant defense mechanism in the brain.

The auto-oxidation of Hcy results in the generation superoxide anion, thus leading to increased production of hydrogen peroxide (Heinecke et *al.*, 1987). This effect can be inhibited by SOD, a scavenger of superoxide anions (Alvarez-Maqueda *et al.*, 2004).

However, the activities of these enzymatic antioxidants have been shown to be regulated by hormones (Pereira *et al.*, 1998). Azevedo *et al*., (2001) shows that the total SOD activity is 83% higher in female rats than in male rats. In chapter 2 and chapter 3, both 17β-estradiol and progesterone of both treatment regimes show potent antioxidant activity, which reduce lipid peroxidation and superoxide anion generation in rat hippocampus.

Therefore, the aim of this study is to investigate the effect of Hcy on SOD activity in rat hippocampus, *in vivo,* as well as to assess whether post- and pre-treatment with 17β-estradiol and progesterone alter the effect of this neurotoxin on SOD activity.

6.2.2 MATERIALS AND METHODS

6.2.2.1 Chemicals and Reagents

All chemicals and reagents were of the highest quality available and purchased from commercial distributors. Pyragallol was purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A. EDTA was purchased from Merck, Darmstadt, Germany.

6.2.2.2 Dosing of Animals

As described in chapter 2, section 2.3.2.3.

6.2.2.3 Surgical Procedures

As described in chapter 2, section 2.3.2.4.

6.2.2.4 Preparation of Cytosolic/Particulate fractions

The hippocampi were homogenized in potassium phosphate buffer (pH 7.8, 0.1M) using a glass Teflon homogenizer. The homogenate was then centrifuged at 100 000 x g for 60 min at 4ºC. The supernatant obtained corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were resuspended in the buffer, freeze thawed three times, and centrifuged at 100 000 x g for 60 min at 4ºC. This supernatant corresponds to the particulate fraction containing Mn-SOD. The cytosolic and particulate fractions were mixed together in order to obtain the total enzyme fraction.

6.2.2.5 Superoxide Dismutase Assay

The method of Marklund and Marklund (1974) was employed to assay the SOD activity, with minor modifications. Pyragallol in the presence of 10mM EDTA auto-oxidizes rapidly in alkaline solution (50mM Tris-HCl; pH 8.2). The reaction mixture consisted of: supernatant (approximately 500µg protein), 300µl of 2mM pyragallol, 300µl of 10mM EDTA and 62.5mM Tris-HCl was used to make up to 3ml. Pyragallol auto-oxidation was monitored over a period of 3 minutes with and without the enzyme protein. The absorbance was read at 420nm using a SHIMADZU UV-Absorbance Spectrophotometer. The auto-oxidation of pyragallol was linear with the activity of the enzyme present. Fifty percent inhibition/ (mg protein$\cdot$min^{-1}) was taken as one unit of enzyme activity.

6.2.2.6 Statistical Analysis

As described in chapter 2, section 2.2.2.7.

6.2.3 RESULTS

The intrahippocampal injection of Hcy at 0.14μmol/μl *in vivo* caused a significant down-regulation in SOD activity (figure 6.1) when compared to the control. Both pre- and post-treatment of 17β-estradiol or progesterone, caused a significant increase in SOD activity when compared with the Hcy-treated group. Furthermore, 17β-estradiol and progesterone up-regulate the SOD activity and was higher than that of the control group.

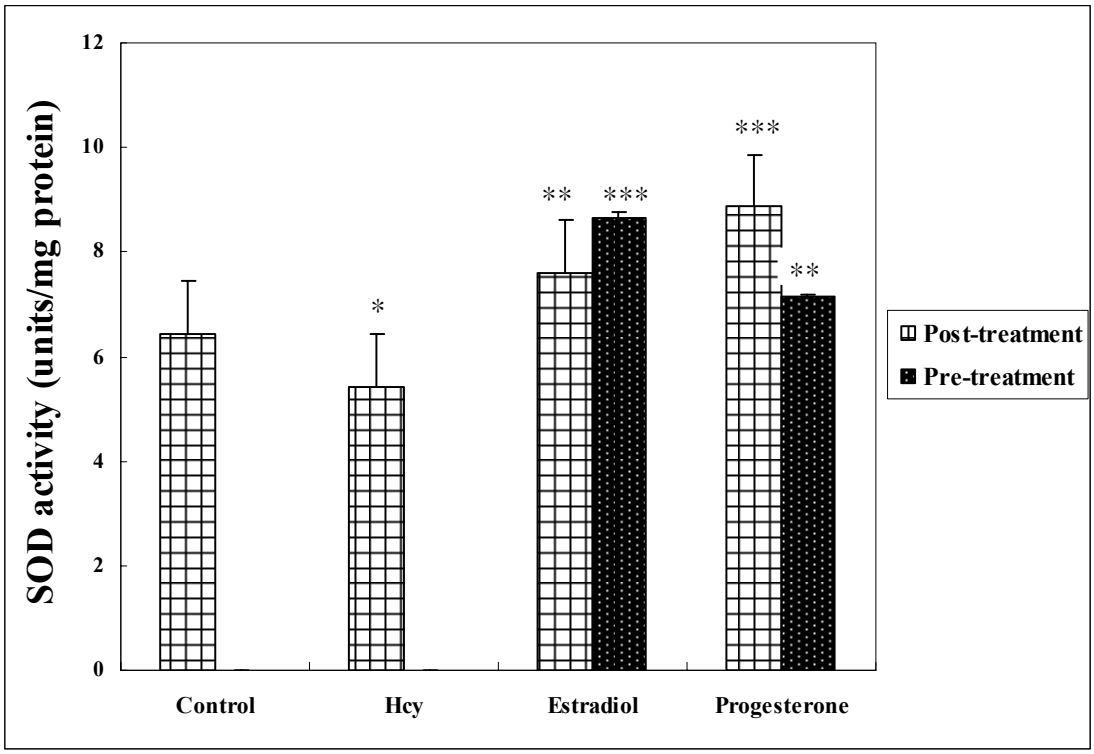


Figure 6.1. The effect of the administration of post- and pre-treatment of 17β-estradiol or progesterone on SOD activity following intrahippocampal administration of Hcy in rat mitochondrial homogenate. Each bar represents the mean ±SEM, n=5. *(p<0.01) Hcy vs. control; *** (p<0.001) Pre-treatment of 17β-estradiol vs. Hcy and post-treatment of progesterone vs. Hcy; ** (p<0.05) post-treatment of

17β-estradiol vs. Hcy and pre-treatment of progesterone vs. Hcy. Student Newman-Keuls-Multiple Range test.

6.2.4 DISCUSSION

Oxidative stress is an imbalance between oxidant and antioxidant activity, which occurs during a variety of pathologies (Bast *et al.*, 1991). SOD is the first enzyme identified to have a free radical as its substrate (McCord, 1969), provides a first line defense against oxidative stress by catalyzing the dismutation of $O_2^{\bullet -}$ into H_2O_2 and O_2.

Hcy is believed to exert its effects through mechanisms involving $O_2^{\bullet -}$ generation (chapter 3) and as a result, oxidative damage (Mujumdar *et al.*, 2001). Several reports show that Hcy and H_2O_2 act synergistically to promote mitochondrial damage (Austin *et al.*, 1998) or cause cytochrome *c* release from mitochondria, which is a key component involved in mitochondrial-mediated cell death (Toborek *et al.*, 1995). However, Outinen et al., (1999) demonstrated that Hcy inhibits gene expression of SOD in human umbilical vein endothelial cells and compromised hepatic Cu-Zn SOD activities (Huang *et al.*, 2001). The results from figure 6.1 show that Hcy significantly down-regulates the SOD activity, as compared to the control treated group.

Pre- and post treatment of 17β-estradiol significant enhances the SOD activity in the hippocampus (figure 6.1). Hall and Sutter *et al.*, (1998) compared Cu-Zn SOD in male and female transgenic mice and provide evidence for 17β-estradiol's *in vivo* antioxidant neuroprotective effect. The results shown in figure 6.1 also indicate that 17β-estradiol interferes with the Hcy neurotoxicity.

Pre- and post treatment of progesterone show significant enhanced SOD activity in the hippocampus (figure 6.1). Progesterone is a free radical scavenger (Roof *et al.*, 1992; 1994) and is able reduce lipid peroxidation (Roof *et al.*, 1992; chapter 2). The up-regulated SOD activity, in comparison to Hcy treated group, could therefore be due to the $O_2^{\bullet -}$ scavenging properties of progesterone (chapter 3).

Therefore, the results presented in this experiment provide more understanding of the free radical scavenging and antioxidant mechanisms of both hormones involving Hcy induced neurotoxicity.

6.3 THE EFFECTS OF 17β-ESTRADIOL AND PROGESTERONE IN HCY-INDUCED MODIFICATION OF GLUTATHIONE PEROXIDASE ACTIVITY IN RAT HIPPOCAMPUS *IN VIVO*

6.3.1 INTRODUCTION

In particular, changes in glutathione (GSH) levels occur in AD. Elevated GSH levels in hippocampus and midbrain were reported in AD (Adams *et al.*, 1991). GSH protects cells against oxidative and free radical damage and provides cells with their reducing power (Meister, 1988; 1994b). The functions of GSH include maintenance of thiol groups in proteins as well as in synthesis and repair of DNA (Meister, 1988; 1994b).

H_2O_2 is produced during neurotransmitter turnover in brain mitochondria and is detoxified through reduction to water. If H_2O_2 is not reduced, it can lead to formation of reactive hydroxyl radicals and cause formation of lipid hydroperoxides, which can damage the mitochondrial membrane and thus, inhibit brain mitochondrial function (Zoccarato *et al.*, 1988). However, GPx is the enzyme that involved in the reduction of H_2O_2 to water. The reduction of H_2O_2 through GPx in the mitochondria, results in the formation of GSSG, which is reduced back to GSH (Meister, 1988; 1994b). GPx therefore is an antioxidant enzyme that scavenges H_2O_2.

Hcy is a well known thiol, which induces ROS through its auto-oxidation to H_2O_2 (Loscalzo, 1996) and initiates lipid peroxidation via inhibition of GPx activity in intracerebroventricularly administered Hcy rats (Baydas *et al.*, 2003).

The aim of this study is to investigate the effects of Hcy in GPx activity in rat hippocampus as well as to establish whether pre- and post-treatment of 17β-estradiol and progesterone would alter the effects that Hcy may have.

6.3.2 MATERIALS AND METHODS

6.3.2.1 Chemicals and Reagents

All chemicals and reagents were of the highest quality available and were purchased from commercial distributors. NADPH, reduced glutathione, glutathione reductase (Sigma type III) and *tert*-butyl-hydroperoxide (*t*-butyl-HPx) were purchased from Sigma Chemical Corporation, St. Louis, MO, U.S.A.

6.3.2.2 Dosing of animals

As described in chapter 2, section 2.3.2.3.

6.3.2.3 Surgical Procedures

As described in chapter 2, section 2.3.2.4.

6.3.2.4 Glutathione Peroxidase Activity

GPx activity was measured by the method described by Sinet *et al*. (1975) using *t*-butyl-HPx as a substrate. The hippocampi were homogenized in PBS 0.05M (pH 7.4) in buffer solution (1mg/5µl) using a Teflon homogenizer. Hippocampal protein (30µg)

of was added to 500 µl of PBS containing 10^{-3} M reduced glutathione, 2 units of yeast glutathione reductase (Sigma type III) and 2 x 10^{-4} NADPH and incubated for 10 minutes at 37 ºC. The reaction was initiated by the addition of *t*-butyl-HPx to a final concentration of 10^{-3} M, under constant agitation. One enzyme unit was defined as 1µM NADPH/mU per mg protein. The results were analyzed using a one-way analysis of variance (ANOVA). If the F values were significant, the Student's Newmans-Keuls Multiple Range test was used to compare the treated and control groups. The level of significance was accepted at $p<0.05$ (Zar, 1974).

6.3.2.5 Statistical analysis

As described in chapter 2, section 2.2.2.7.

6.3.3 RESULTS

There is a significant down-regulation in GPx activity after the intrahippocampal administration of Hcy as compared to the control treated group (figure 6.2).

The results from figure 6.2 also show that pre- and post-treatments of either 17β-estradiol or progesterone cause a significant increase in GPx activity as compared to the Hcy treated group.

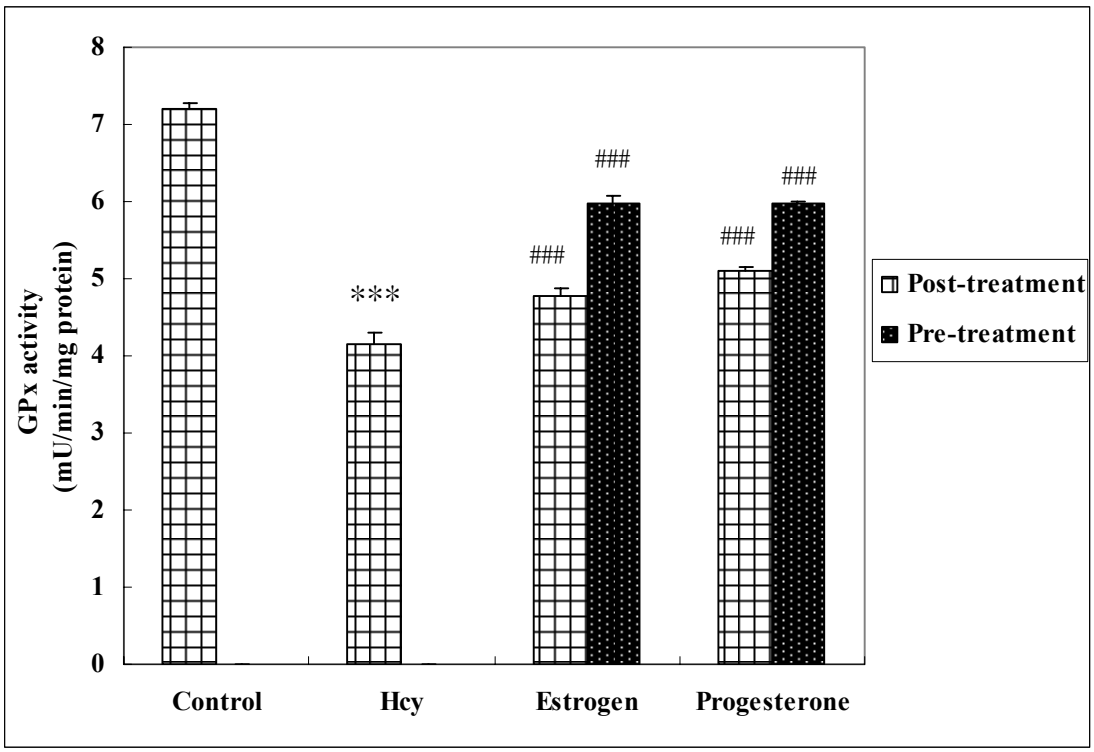


Figure 6.2. The effect of the administration of post- and pre-treatment of 17β-estradiol or progesterone on GPx activity following intrahippocampal administration of Hcy in rat hippocampal homogenate. Each bar represents the mean ±SEM, n=5. *** (p<0.001) Control vs. Hcy; ### (p<0.001) post- and pre-treatment of 17β-estradiol vs. Hcy and post- and pre-treatment of progesterone vs. Hcy. Student Newman-Keuls-Multiple Range test.

6.3.4 DISCUSSION

The results obtained from this study demonstrate that intrahippocampal injection of Hcy results in a significant reduction of GPx activity when compared to the control group (figure 6.2). This demonstrates that Hcy, down-regulates GPx activity and thus potentiates the generation of reactive oxygen species. Baydas et al., (2003) show that intracerebroventricular Hcy (143µg/kg) injection significantlys down-regulates GPx activity in hippocampus and cerebellum. However Baydas *et al*., (2003) also shows that Hcy can initiate lipid peroxidation via the inhibition of GPx activity, which correlates with the results obtained in chapter 2, section 2.3.3.

The pre- and post-treatment of 17β-estradiol and progesterone up-regulate the GPx activity, in comparison to the Hcy-treated group (figure 6.2) could result from an inhibition of lipid peroxidation (chapter 2) and free radical scavenging properties (chapter 3). Azevedo et al., (2001) showed that both 17β-estradiol and progesterone increase GPx activity in male and female rat macrophages. There is evidence that estrogen decreases ROS and free radical production both *in vitro* and *in vivo*, and suppresses estrogen synthesis in postmenopausal women and is responsible for enhanced oxidative stress (Ayres *et al.*, 1998; Gura, 1995; Trevisan *et al.*, 2001). This evokes concern regarding adverse effects on antioxidant defense in premenopausal females (Tiidus, 2000). Nevertheless, ovarian hormones control the involvement of endogenous antioxidant enzymes in the protective effect against oxidative stress in nervous tissue. A number of studies imply that there exists a correlation between the antioxidant status and hormonal levels (Inal *et al.*, 1997; Rapoport *et al.*, 1998; Cardozo-Pelaez *et al.*, 2000). A recently report by Baydas et al., (2001) showed that Hcy inhibits GPx activity in hippocampus and thus initiates lipid peroxidation. Further evidence to support the antioxidant effects of both hormones was the reduced MDA levels, a lipid peroxidation by-product observed in chapter 2.

Furthermore, Ayres et al., (1998) and Behl et al., (1995) show that, estrogen-mediated antioxidant activity may prevent the formation of radicals, following exposure to H_2O_2. Chapter 3 demonstrates that both hormones are potent scavengers of $O_2^{-\bullet}$. Thus, it provides another mechanism whereby these hormones could prevent the formation of H_2O_2.

6.3.5 CONCLUSION

Enhanced ROS production leads to oxidative stress and in due course to cellular damage and is a causative factor of a variety of neurodegenerative disease including AD is associated with a decreased antioxidant capacity of the brain (Benzi & Moretti, 1995). Because of their extreme reactivity, it is difficult to detect and quantify ROS. Therefore, measurements of indirect markers of oxidative stress, such as changes in the activities of antioxidative enzymes, which involve ROS in pathological conditions is used. SOD and GPx are among the most important members of the antioxidant defense system. The ability of pre- and post treatment of both hormones to modify the antioxidant defense mechanism of SOD and GPx, in the presence of Hcy provide further evidence that the neuroprotective mechanisms of 17β-estradiol and progesterone can be multifactor. These results however, correlate and associate with the results obtained in chapter 3, which indicate that these agents are free radical scavengers.

CHAPTER 7

RADIOLIGAND BINDING STUDIES

7.1. INTRODUCTION

NMDA sensitive L-[H^3]-glutamate binding sites located in the CA1 region of the hippocampus constitute the major glutamatergic system of the neurotransmitter system in the hippocampus (Monaghan & Cotman, 1986). Overstimulation of NMDA receptors, plays a key role in excitotoxic calcium influx to neurons (Mayer & Westbrook, 1987; Michaels & Rothman, 1990), resulting in excitotoxic neuronal injury (Gosh & Griffiths, 1994).

The receptor binding assay is an important tool in many disciplines in the biological sciences and that is one procedure that has improved the development of neuroscience research (Bylund & Yamamura, 1990). This provides data regarding quantitation, pharmacological specificity, modulation and distribution of NMDA receptors.

Hcy is an endogenous ligand (Zeise *et al.*, 1988) and an agonist at the glutamate site (Kim, 1999) of the NMDA receptor and part of its neurotoxicity is caused by overstiumlation of NMDA receptors and thus a resultant Ca^{2+} influx (Lipton *et al.*, 1997; Kruman *et al.*, 2000). Hcy elicits excitation of cerebellar neurons (Lee *et al.*, 1988; Kruman *et al.*, 2000). These authors show that Hcy enhances glutamate excitotoxicity and is as least as excitotoxic as glutamate.

In this experiment the effect of Hcy and 17β-estradiol or progesterone with Hcy on NMDA receptors was investigated, in order to determine whether these hormones could offer protection against Hcy-induced neurotoxicity.

7.2. THE EFFECTS OF 17β-ESTRADIOL, PROGESTERONE, AND INTRAHIPPOCAMPAL HCY ADMINISTRATION ON GLUTAMATE BINDING *IN VIVO* AND *IN VITRO*

7.2.1. INTRODUCTION

Excessive stimulation of NMDA receptors is known to mediate brain damage (Simon, 1984; Lipton, 1994). 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).

In the previous experiment in chapter 4, it was determined that both treatment regimens of 17β-estradiol and progesterone appeared to offer protection against Hcy-induced neurotoxicity in the hippocampus. The neurons in CA1 region of the hippocampus did not appear to swell, nor undergo neurodegeneration in CA3 region. However, the previous studies (chapter 4) were not able to quantify the protection offered by both hormones.

Moreover, in chapter 6, the results show that the role of 17β-estradiol and progesterone in protect against Hcy-induced neurotoxicity due to their abilities to upregulate the enzymatic antioxidant activities. Therefore, the mechanism by which 17β-estradiol and progesterone are neuroprotective may extend beyond that of an antioxidant. The present study aims to investigate the effect of 17β-estradiol and progesterone on glutamate binding to the NMDA receptor in comparison to Hcy.

7.2.2. MATERIALS AND METHODS

7.2.2.1. Chemicals and Reagents

17β-estradiol, progesterone (4-Pregnene-3,20-dione), DL-homocysteine and N-methyl-D-aspartate (NMDA) were purchased from Sigma (St. Louis, USA). [H^3]Glutamate (43.0 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.

7.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 250-300g were used for the experiment, and were housed and maintained under the conditions described in section 2.2.2.2.

7.2.2.3. Dosing of Animals

As described in section 2.3.2.3.

7.2.2.4. Surgical Procedures

As described in section 2.3.2.4.

7.2.2.5. 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). The pellets were then resuspended in 20ml of 0.32M sucrose and rapidly frozen in liquid nitrogen and stored at -70˚C until use.

7.2.2.6. 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. Therefore, this study was considered to be an *in vivo* investigation.

On the day of the binding study, the frozen synaptic membranes were thawed, diluted up to 40 volumes with buffer and centrifuged at 50 000 x g at 4˚C for 20 minutes. The pellets were resuspended and treated with 0.08% Triton X-100 for 10 minutes. This was followed by centrifugation at 50 000 x g at 4˚C for 20 minutes. The pellets were resuspended in 50mM Tris acetate buffer. Protein concentration was determined as in 3.2.2.8.

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 fiber (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 pmoles glutamate bound.

7.2.2.7. 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 as in 7.2.2.5. 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 or progesterone dissolved in ethanol or Hcy 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. An aliquot of 400µl of membrane suspension was added to all the tubes. Results were expressed as percent glutamate bound in the presence of 17β-estradiol, progesterone or Hcy.

7.2.3. RESULTS

7.2.3.1. Saturation Binding Studies – calculation 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:

$$f = \text{specific activity}^{-1} \times \text{volume}^{-1} \times \text{efficiency}^{-1} \times Ci/\ 2.2 \times 10^{12} dpm$$

Efficiency refers to the efficiency of the scintillation counter (65%), volume is the incubation volume i.e. 0.5ml and the final number is the definition of a curie in terms of dpm (disintegrations per minute). The unit of this factor is pmoles/cpm (Bylund & Yamamura, 1991). The K_D and B_{max} values were obtained from Graphpad Prism 4®.

Saturation binding plots were generated for control (figure 7.1), Hcy (figure 7.3), both regimens of 17β-estradiol (figure 7.5 and 7.7) and progesterone (figure 7.9 and 7.11). The data was converted to Scatchard plots so as to determine the B_{max} for each of the treated groups (see figure 7.2, 7.4, 7.6, 7.8, 7.10 and 7.12).

The B_{max} values obtained in each case are shown in Table 7.1. Hcy caused a significant down-regulation in the number of glutamate binding sites when compared to control groups. Moreover, animals which received a combination of Hcy and 17β-estradiol or progesterone of both treatment regimens showed a similar number of

glutamate binding sites compared to control animals. Therefore both regimens of either hormone appear to be able to up-regulate the number of glutamate binding sites compared to the control and Hcy only treated group.

Simultaneously, the glutamate receptors of 17β-estradiol or progesterone treated groups in combination with Hcy injections had very similar K_D values compared to the control group. The receptors of the Hcy only treated group had a much higher glutamate binding affinity than either control or drug treated groups, with a K_D value 10 fold lower than the control.

Table 7.1. Summary of B_{max} values obtained from glutamate receptor binding studies.

Group	**K_D (nM)**	**B_{max} (pmoles/mg protein)**
Control (PBS)	2.57	245.8±57.3
Hcy	0.28	81.45±4.3
Hcy + 17β-estradiol (post-treatment)	1.70	227.30±54.6
Hcy + 17β-estradiol (pre-treatment)	2.31	184.64±42.2
Hcy + progesterone (post-treatment)	1.51	195.75±24.9
Hcy + progesterone (pre-treatment)	2.68	226.62±66.9

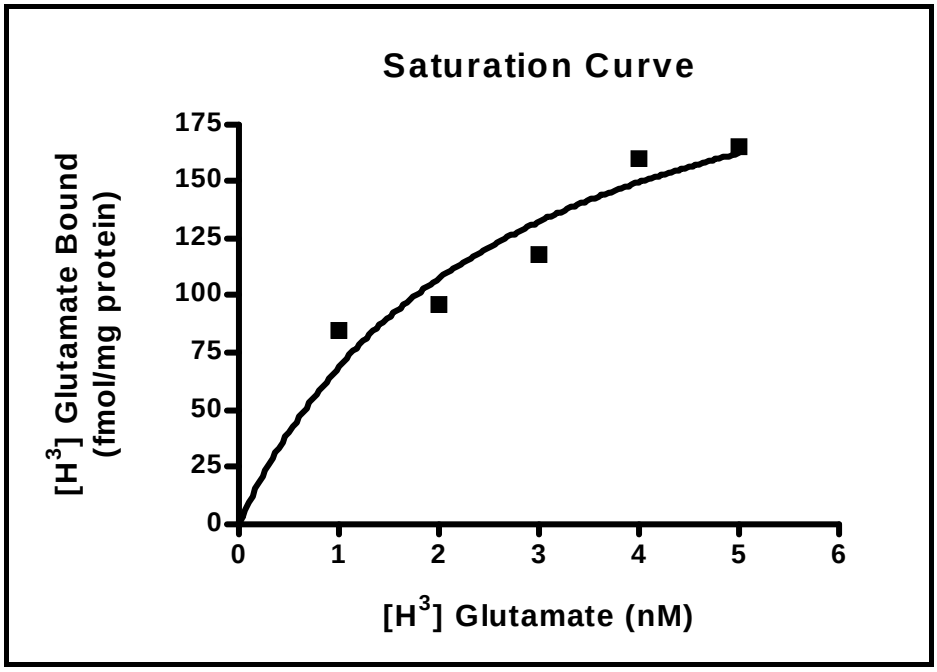


Figure 7.1. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from control rats. Three animals from each group were used in saturation binding experiments.

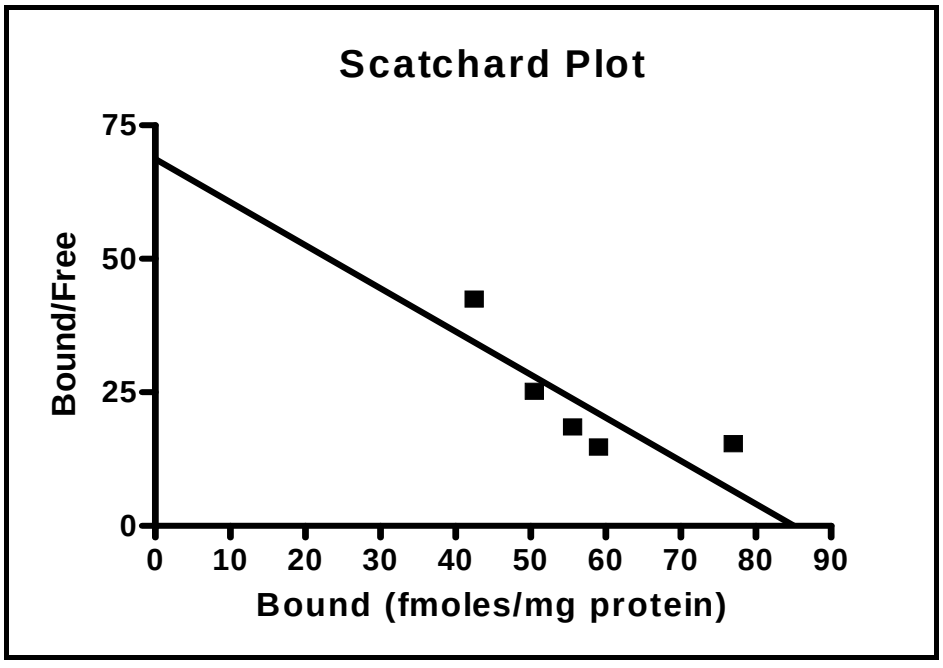


Figure 7.2. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from control rats.

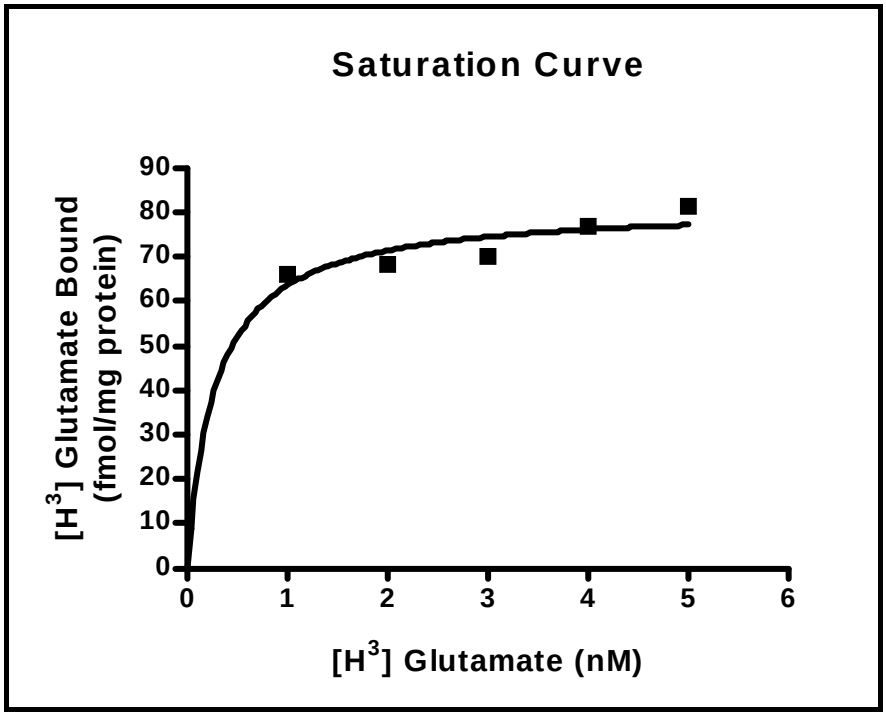


Figure 7.3. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from Hcy only treated rats. Three animals from each group were used in saturation binding experiments.

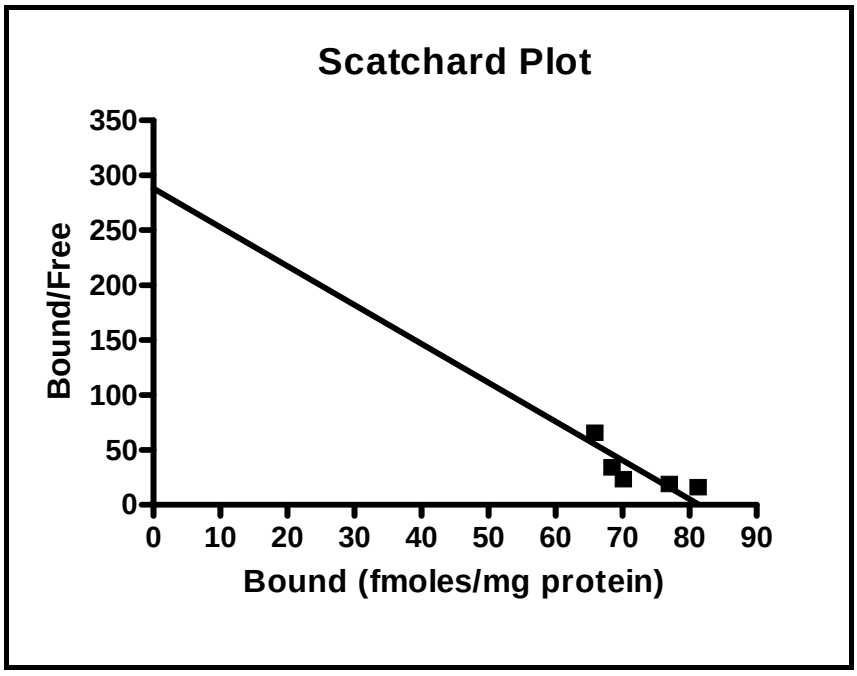


Figure 7.4. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from Hcy only treated rats.

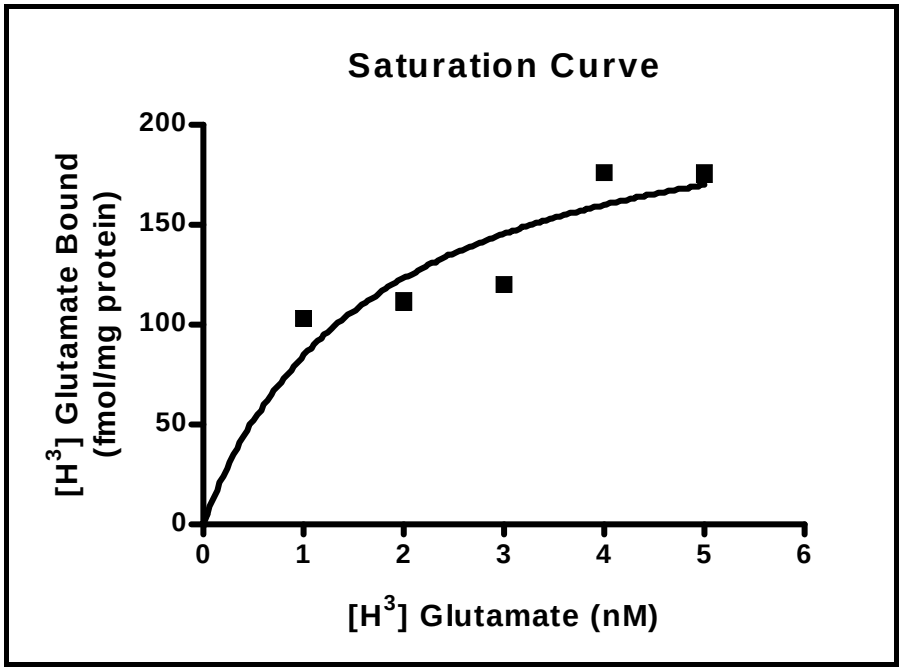


Figure 7.5. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from post-treatment of 17β-estradiol and Hcy treated rats. Three animals from each group were used in saturation binding experiments.

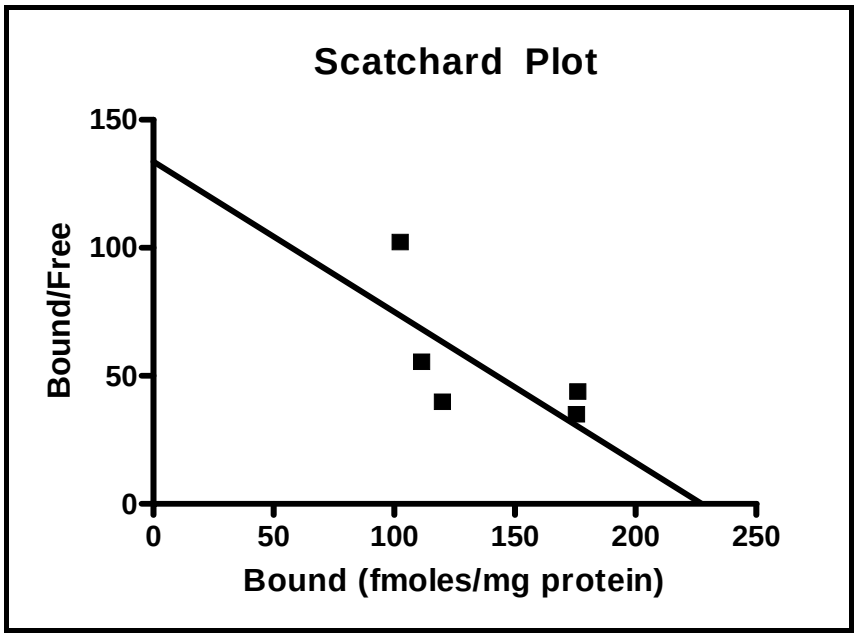


Figure 7.6. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from post-treatment of 17β-estradiol and Hcy treated rats.

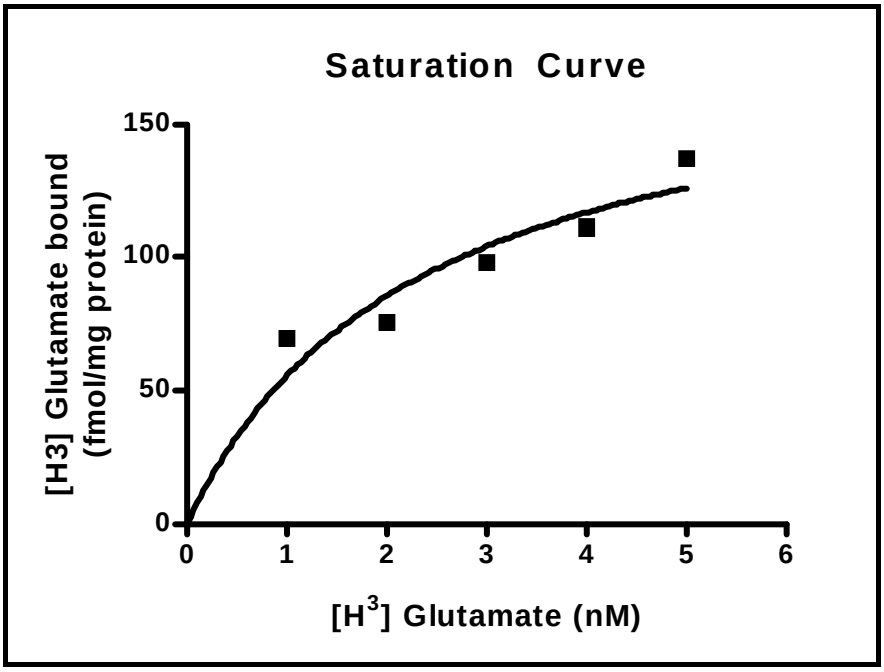


Figure 7.7. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from pre-treatment of 17β-estradiol and Hcy treated rats. Three animals from each group were used in saturation binding experiments.

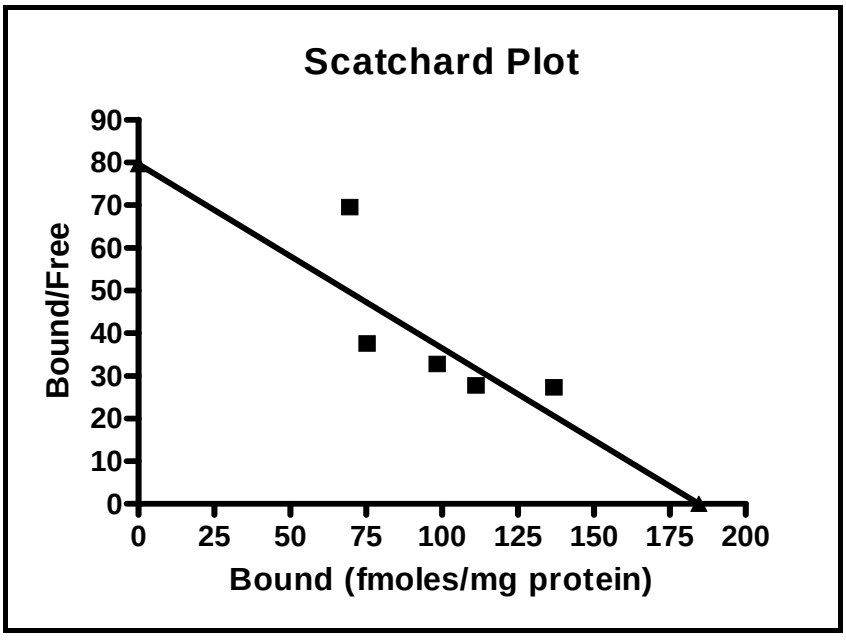


Figure 7.8. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from pre-treatment of 17β-estradiol and Hcy treated rats.

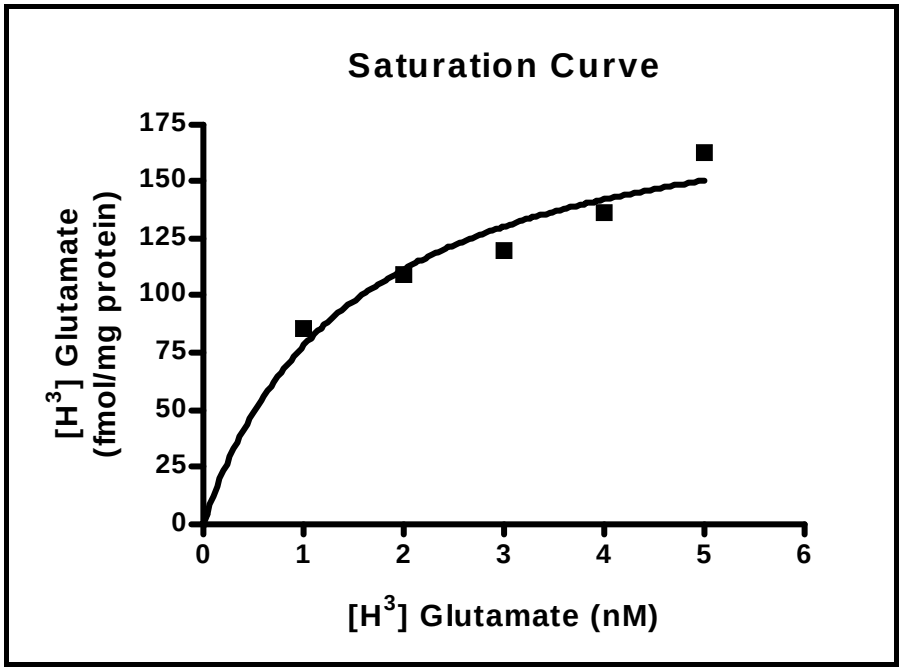


Figure 7.9. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from pre-treatment of progesterone and Hcy treated rats. Three animals from each group were used in saturation binding experiments.

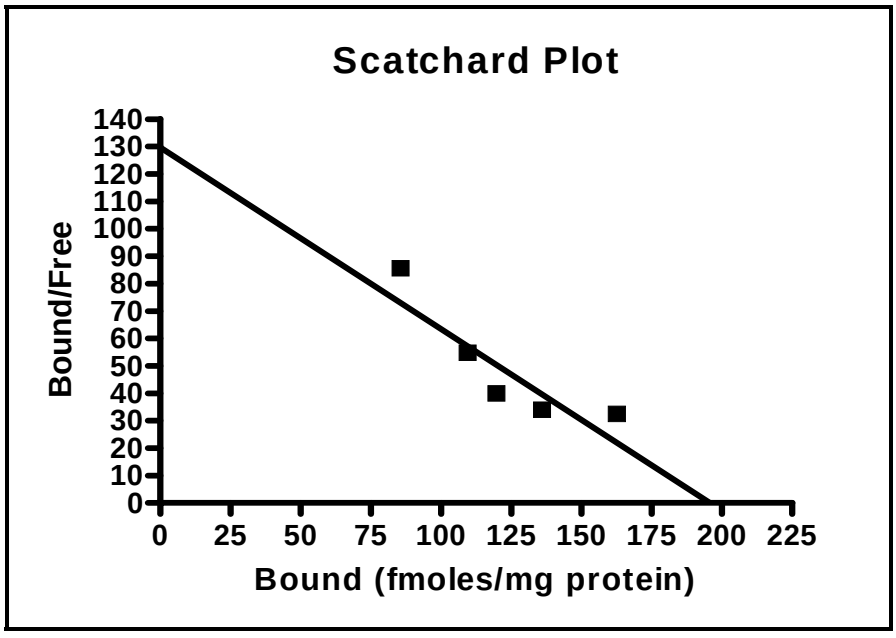


Figure 7.10. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from pre-treatment of 17β-estradiol and Hcy treated rats.

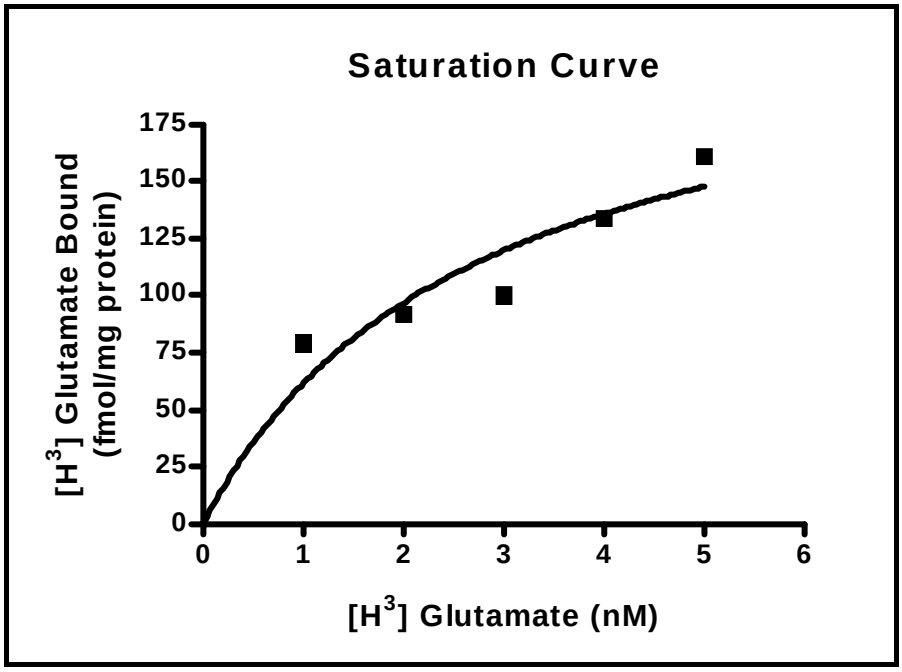


Figure 7.11. Saturation binding plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from post-treatment of progesterone and Hcy treated rats. Three animals from each group were used in saturation binding experiments.

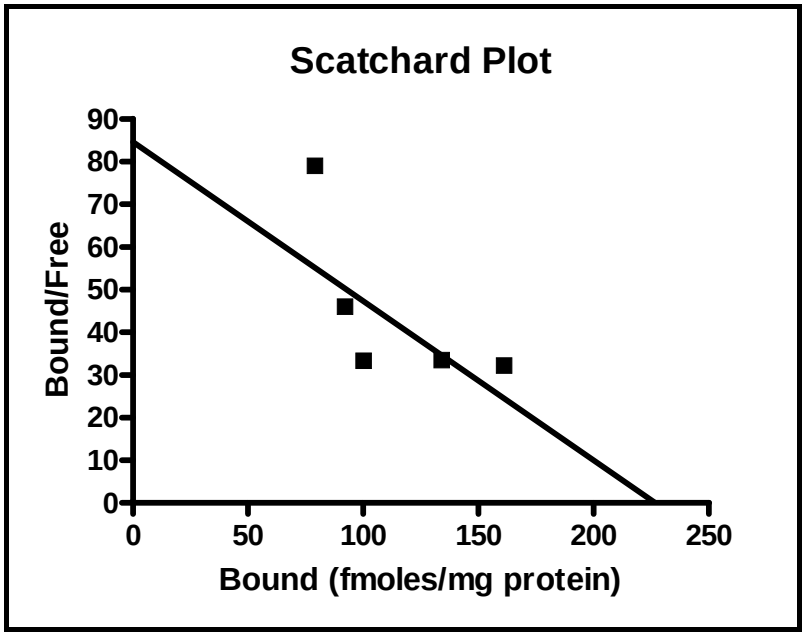


Figure 7.12. Scatchard plot of [H^3]-Glutamate binding to hippocampal synaptic membranes from pre-treatment of progesterone and Hcy treated rats.

7.2.3.2. Receptor-Displacement Studies – calculations and data analysis

The affinity of the unlabelled ligand for the receptor can be determined directly by measuring its ability to compete with, and thus inhibit, the binding of a radioactive ligand to its receptor. A competition 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 diluents tube (tubes 1 in this case) and subtracting the mean of the non-specific values (NSB; tube 8). For tubes 2 through 7 P were calculated by subtracting the non-specific binding from the amount bound (B), dividing by B_0 and multiplying by 100:

$$P = B\text{-}NSB / B_0 \times 100$$

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

$$Logit = ln[P/(100\text{-}P)]$$

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).

Glutamate receptor-displacement binding studies show that both treatment regimes of 17β-estradiol or progesterone are more effective in reducing the amount of glutamate

bound compared to Hcy. 17β-estradiol and progesterone exhibit a greater inhibition of glutamate binding as opposed to Hcy as evidenced by the sharper decrease in percent glutamate bound at low concentrations of 17β-estradiol and progesterone (figure 7.13). However, the IC_{50} values obtained are lower to that obtained for Hcy at the same concentrations, calculated from the plots in figure 7.13. Furthermore, the K_i for both regimes of 17β-estradiol or progesterone in comparison to that for Hcy is generally the same, which indicates equal inhibition of glutamate binding by 17β-estradiol or progesterone (Table 5.2.).

Table 7.2. Summary of the data obtained for inhibition binding studies. [H^3]-Glutamate binding was observed in the presence of 17β-estradiol, progesterone or Hcy.

Tested Ligand	**IC_{50} (nM)**	**K_i (nM)**
Hcy	1.36	5.03
17β- estradiol	0.22	3.16
Progesterone	0.34	2.97

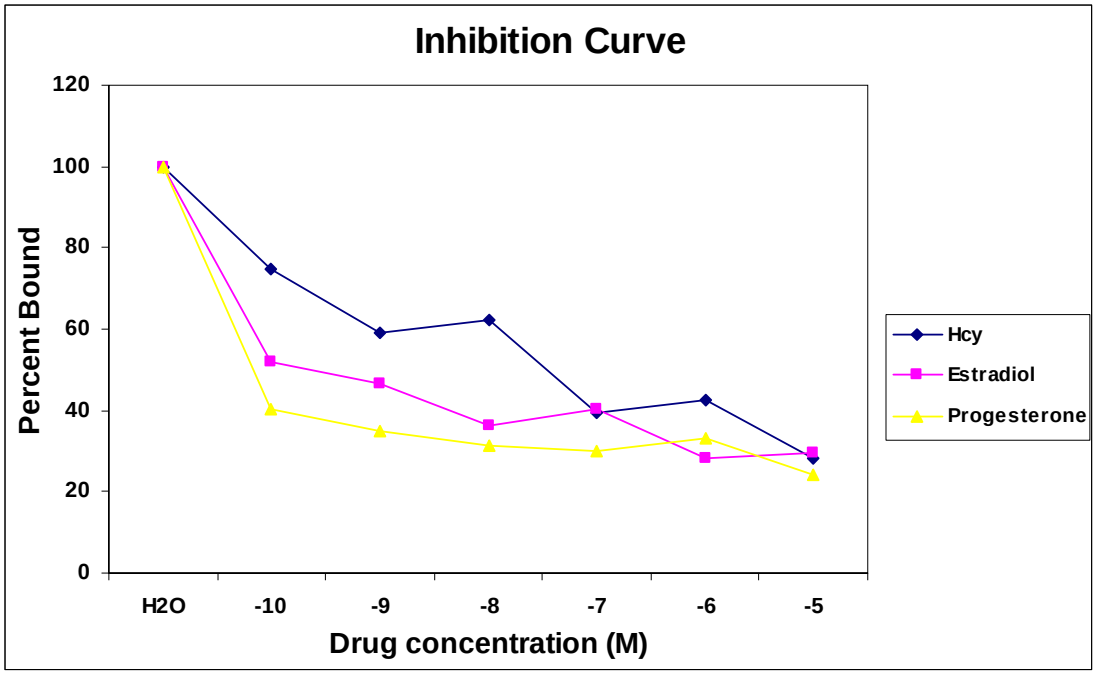
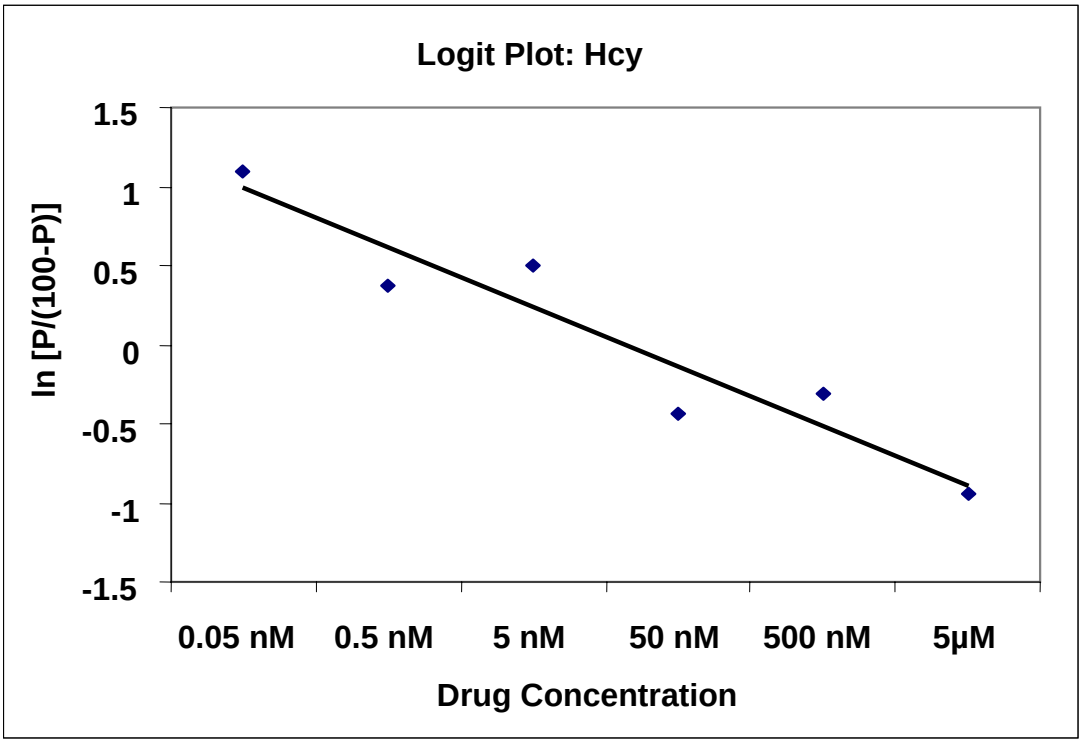
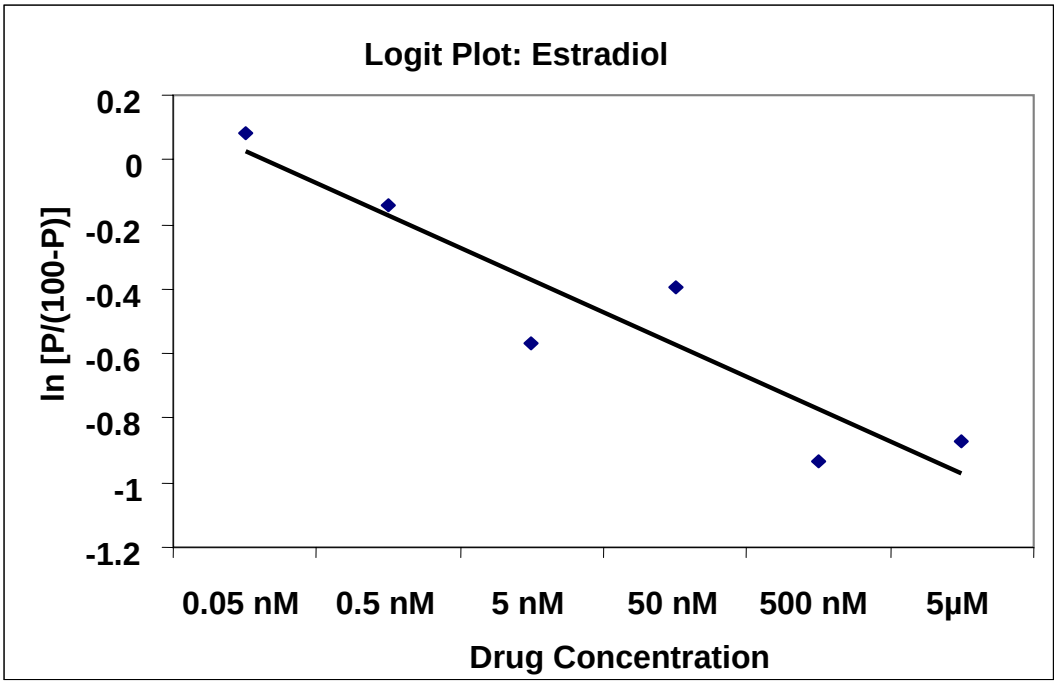


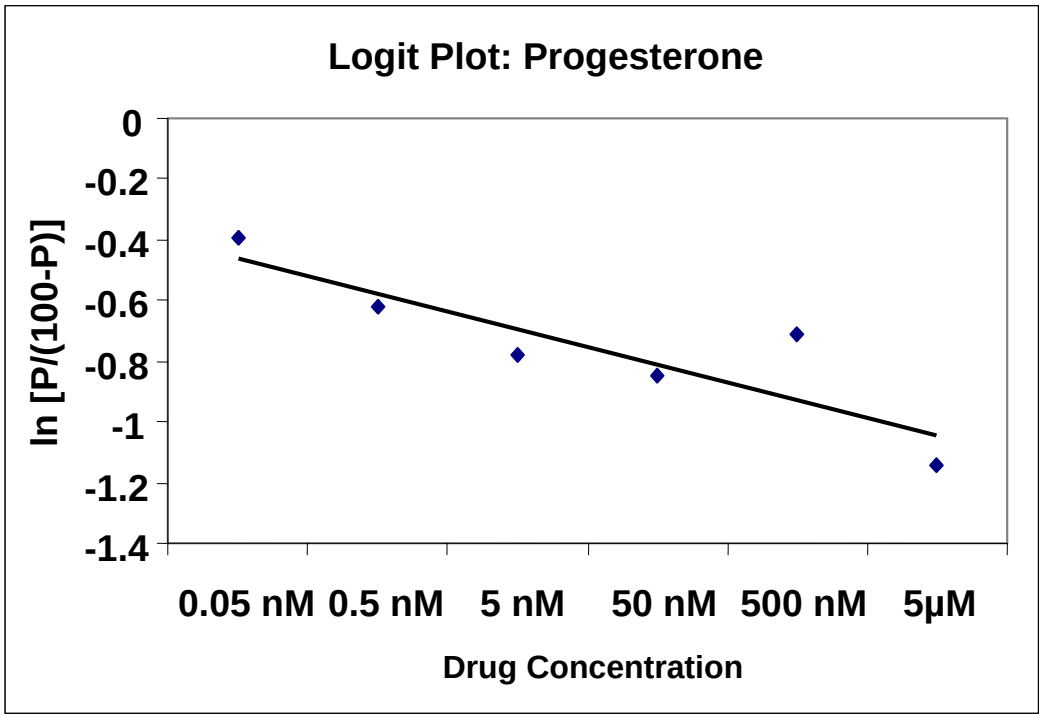
Figure 7.13. Inhibition curves of [H^3]-Glutamate binding in the presence of 17β-estradiol or progesterone or Hcy. The graph shows a greater inhibition of glutamate binding with 17β-estradiol or progesterone as opposed to Hcy as shown by the sharp decrease in percent glutamate binding with both agents.



(a)



(b)



(c)

Figure 7.14. Logit plots generated from inhibition binding studies. [H^3]-Glutamate binding was decreased in presence of 17β-estradiol or Hcy.

7.2.4. DISCUSSION

The results of the saturation binding studies demonstrate the effect of treatment with Hcy alone or in combination with 17β-estradiol or progesterone of both treatment regimes on the ability of glutamate to bind to the receptor. The K_D and B_{max} values determined (Table 7.1) show that the intrahippocampal injection of Hcy results in a reduction in the number of binding sites. Hcy treatment also changed the nature of those receptors that were present on the neurons, in that these had a higher affinity for glutamate than did the control neurons. This could be explained by the histology results in previous chapter (chapter 4), which demonstrate the neurotoxic effects of Hcy and the destruction of the neuronal cells, thus causing a decrease in the number of membrane bound receptor sites. In addition, it has been shown that Hcy itself acts as an agonist at the glutamate binding site of the NMDA receptor (Lipton *et al.*, 1997) and leads to an imbalance in excitatory: inhibitory neurotransmission in the hippocampus (Bleich & Degner, 2000).

Treatment with either 17β-estradiol or progesterone prior to and after the intrahippocampal injection of Hcy results in up-regulation in the number of binding sites of the receptor in comparison to the control and to treatment with Hcy alone. These receptors however show similar affinity for glutamate as do receptors from neurons in the control group. These results demonstrate that 17β-estradiol and progesterone protect hippocampal neurons against Hcy-induced degeneration.

It has been previously reported that 17β-estradiol increases the number of agonist binding sites (Weiland, 1992). Progesterone blocks the glutamate in NMDA neuron (Gentile & McIntosh, 1993; Smith, 1994), thus limiting secondary neuronal death that

occurs due to excessive release of glutamate. However, the displacement binding studies show that both hormones are as effective as Hcy at reducing the amount of glutamate bound. The K_i values obtained for 17β-estradiol and progesterone are not significantly different for Hcy.

In addition to the results of the histological studies, both 17β-estradiol and progesterone protect hippocampal neurons against Hcy-induced damage. This is a direct result of Hcy action on the NMDA receptor. Therefore the protection of either hormone against Hcy-induced damage requires an interaction with the NMDA receptor, thus reducing the amount of Hcy binding to this receptor. Moreover, the results from the displacement studies support the hypothesis that 17β-estradiol and progesterone bind to the receptor with an equal affinity to Hcy, thus competing with Hcy for the receptor and protecting against neuronal damage.

The results imply that 17β-estradiol and progesterone may be able to protect neurons from increase risk of glutamate receptor overstiumlation, by up-regulating the number of glutamate binding sites.

7.2.5. CONCLUSION

The results of the experiments presented in this chapter show that 17β-estradiol and progesterone in both treatment regimes offer protection against Hcy-induced neurotoxicity via a NMDA receptor-dependent mechanism. The histological analysis of the hippocampus of the animals treated with Hcy shows that there is a swelling of neurons in the CA1 region and degeneration of neurons in the CA3 region. However, neurons from both treatment regimes of either 17β-estradiol or progesterone do not

appear to have been affected by Hcy. These results show that both hormones offer neuroprotection against intrahippocampal injections of Hcy. Unfortunately, these studies do not explain the method by which these hormones offer protection, nor the level of protection offered. This would be the subject of another study.

The binding studies attempt to quantify any neurodegeneration that may be induced by Hcy. The reduction in receptor numbers induced by Hcy could be a result of a loss of neurons. Results show that there is a significant increase in the B_{max} values for animals which received either 17β-estradiol or progesterone prior to and following the Hcy injection. The increase in B_{max} values were however similar to those in the control group. However, several authors report that Hcy-induced excitotoxicity is evoked by glutamate and is mediated by NMDA receptors, whose activation leads to massive accumulation of extracellular Ca^{2+} in the cells (Kruman *et al.*, 2000; Lipton *et al.*, 1997).

Heron *et al*., (2001) showed that 17β-estradiol appears to act directly on NMDA receptors. Weaver *et al*., (1997) has shown that 17β-estradiol inhibits NMDA-induced cell death in glial cells, by reducing Ca^{2+} influx. It has also been shown that 17β-estradiol attenuates neurotoxicity produced by glutamate exposure of primary rat hippocampal cultures (Goodman *et al.*, 1996; Behl, 1995), as measured by cell viability and lysis (Behl, 1995). Furthermore, Weiland *et al.*, (1992) showed that 17β-estradiol may influence learning behaviors and seizure activity by altering the sensitivity of hippocampal neurons to glutamate activation.

Progesterone, on the other hand, blocks the glutamate in the NMDA neurons, which is similar to NMDA antagonists MK-801 (Barth *et al.*, 1990; Gentile *et al.*, 1993; Smith,

1994). Thus progesterone has the potential to limit secondary neuronal death that occurs because of an excessive release of glutamate (Gentile *et al.*, 1993; Smith, 1994). Smith et al., (1994) showed that progesterone may block glutamate via conversion into its metabolite, allopregnanolone, within the brain, which has a weak affinity for $GABA_A$ receptor. Moreover, Misztal et al., (1996) showed that chronic treatment of progesterone provides protection against glutamate-induced excitotoxicity in rat models by acting as a NMDA antagonist.

The results of this study thus show that both treatment regimes of 17β-estradiol or progesterone appear to protect hippocampal neurons from degeneration caused by intrahippocampal injections of Hcy. In conjunction with the results obtained from histological studies in chapter 4, it could be argued that these hormones appear to be effective at protecting neurons from Hcy-induced neuronal damage.

CHAPTER 8

SUMMARY OF RESULTS

8.1. CHAPTER 2

The effects of the pre- and post- treatments of 17β-estradiol and progesterone on the levels of Hcy-induced lipid peroxidation was investigated in the rat brain *in vitro* and *in vivo.* Lipid peroxidation involves the destruction of lipid membranes by a process initiated by ROS. Intrahippocampal injections of Hcy were used to cause neurodegeneration in the *in vivo* study. Both female hormones showed a significant reduction in lipid peroxidation induced by Hcy *in vitro* and *in vivo.*

8.2. CHAPTER 3

The experiment was performed to determine if both regimes of 17β-estradiol or progesterone could act as superoxide radical scavengers, to reduce the superoxide production induced by intrahippocampal injections of Hcy. Hcy is a well known sulphur-containing amino acid which induces generation of $O_2^{\bullet-}$ during auto-oxidation reactions. However, it was theorized that the antioxidant effects of 17β-estradiol and progesterone offered neuroprotection in response to superoxide anions. Under the experimental conditions, both female hormones were able to reduce superoxide anions. Therefore, the extent of protection of both female hormones as antioxidants against oxidative stress in the form of $O_2^{\bullet-}$ was investigated in this chapter.

8.3. CHAPTER 4

Histological techniques were employed in this chapter. Microscopic examination of the hippocampal neurons was performed following intrahippocampal injection of Hcy, and post- and pre-treatment with subcutaneous injection of 17β-estradiol or progesterone. Both regimes of 17β-estradiol or progesterone were shown to protect the neurons in both CA1 and CA3 regions of the hippocampus in Hcy-induced neurodegeneration.

8.4. CHAPTER 5

In Situ Cell Death Detection Kit, POD and light microscopy were used to investigate the ability of Hcy to promote apoptosis in the hippocampus and protection offered by the treatment with 17β-estradiol and progesterone. From the micrographs obtained, both regimes of 17β-estradiol or progesterone were able to protect against the Hcy-induced apoptotic cell death in both CA1 and CA3 regions of the hippocampus. There are no brown stained neurons observed in 17β-estradiol or progesterone treatment which could be observed in the Hcy-treated group.

8.5. CHAPTER 6

These experiments were performed to investigate the effects of Hcy on indirect markers of oxidative stress, SOD and GPx activities, in rat hippocampus *in vivo,* and both regimes of 17β-estradiol or progesterone on the effects of Hcy on SOD and GPx activities. From the results obtained, both regimes of either hormone were able to cause a significant increase in SOD and GPx activities as compared to the Hcy treated group. These results however, correlate with the results obtained in chapter 3, which indicate that these agents are free radical scavengers and antioxidants.

8.6. CHAPTER 7

Radiolabelled glutamate saturation binding studies were performed. The results demonstrate that the intrahippocampal injection of Hcy alone had higher affinity of glutamate to bind to the NMDA receptor as well as causing reduction in the number of binding sites. However, treatment with either 17β-estradiol or progesterone prior to and after the intrahippocampal injection of Hcy resulted in up-regulation in the number of binding sites and showed similar affinity for glutamate receptors from neurons in the control group. These results demonstrate that 17β-estradiol and progesterone protect hippocampal neurons against Hcy-induced degeneration.