AN INVESTIGATION INTO THE NEUROPROTECTIVE PROPERTIES OF MELATONIN

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ABSTRACT

Until the beginning of this decade the neurohormone, melatonin, had been considered as little more than a tranquillising hormone, responsible for regulating certain circadian and circannual rhythms. In the last eight years, a whole new dimension to melatonin's role in biological organisms has emerged. In 1991 it was discovered [1,2] that melatonin exhibited antioxidant properties. Since then, many researchers [3,4] have found melatonin to be a powerful free radical scavenger and antioxidant. In the present study, the ability of melatonin to offer neuroprotection against glutamate, N-methyl-D-aspartate (NMDA), quinolinic acid (QA) and kainic acid (KA) (collectively referred to as the glutamate receptor agonists) was investigated.

It was first shown that stress causes an increase in circulating glucocorticoid concentrations, which resulted in an increase the number of glutamate receptors on synaptic membranes in rat brain homogenate. Melatonin acted to reduce the number of glutamate receptors present on the synaptic membranes, implying that melatonin has neuroprotective properties, as overstimulation of the glutamate receptors leads to excitotoxicity and neurodegeneration.

Further investigations showed that the glutamate receptor agonists induce neurodegeneration in primary neuronal cell cultures. Both co-treatment and post-treatment with melatonin against the glutamate receptor agonists, increased neuronal cell viability in a dose dependent manner. Melatonin also appeared to offer protection against quinolinic acid-induced neurodegeneration following intrahippocampal injections of quinolinic acid.

The mechanism whereby melatonin offered this protection was investigated. The glutamate receptor agonists caused an increase in intracellular calcium concentrations, which is known [5] to be responsible for initiating the excitotoxic response. Melatonin had no effect on regulating intracellular calcium concentrations.

Additional studies indicated that melatonin was effective at scavenging superoxide radicals. Production of superoxide radicals was induced by the glutamate receptor agonists in primary neuronal cultures. Superoxide radicals induce lipid peroxidation, which involves the destruction of lipid membranes by chain reactions. By acting as an antioxidant, melatonin was able to reduce quinolinic acid-induced lipid peroxidation in rat brain homogenate, in a dose dependent manner. Melatonin was also effective at reducing lipid peroxidation induced by the glutamate receptor agonists in primary neuronal cultures.

Melatonin therefore appeared to be offering neuroprotection by removing superoxide radicals and inhibiting lipid peroxidation. It had been reported [6] that melatonin inhibits nitric oxide synthase activity. This enzyme produces the free radical, nitric oxide, and can also produce superoxide radicals. Melatonin was able to reduce nitric oxide synthase activity in a dose dependent manner. This is a novel method of neuroprotection, as melatonin was now acting as an enzyme regulator.

The results obtained demonstrate that melatonin offers neuroprotection against glutamate induced excitotoxicity, by removing free radicals and preventing lipid peroxidation. The neurohormone offers further protection by decreasing the activity of enzymes that aid in the neurotoxic cascade.

Melatonin is the most potent naturally occurring free radical scavenger in the body [3]. During aging, the serum concentrations of melatonin decrease [7]. During the senescence of life, free radical damage to the body is at its highest [8], while at the same time melatonin concentrations are at their lowest. Melatonin therefore shows potential for the treatment of diseases and disorders that exhibit an excitotoxic pathology.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin hormone
AMNH	2-amino-3-[2-(3-hydroxy)-5-methylisoxazol-4-yl]-proprionate
AMOA	2-Amino-3[-(carboxymethoxy)-5-methylisoxolzol-4-yl]-proprionate
AMPA	α-2-amino-3-hydroxy-5-methylisoxazole-4-proprionate
AP5	2-amino-5-phosphonovalerate
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
BHT	Butylated Hydroxytoluene
Bmax	Maximum Number of Binding Sites
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
$CaCl_2$	Calcium Chloride
CCl_4	Carbon Tetrachloride
CNQX	6-cyano-7-nitroquinoxaline
CNS	Central Nervous System
CRF	Corticotropin Releasing Factor
DNA	Deoxyribonucleic Acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
EAA	Excitatory Amino Acid
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin Adenine Dinucleotide
FCS	Foetal Calf Serum
GMP	Guinidine Monophosphate
G-Proteins	Guanine Nucleotide Proteins
4-HDA	4-Hydroxyalkenals
H_2O	Water
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Saline Solution
HEPES	N-[2-hydroxyethyl]piperazine-N'-2-hydroxypropanesulfonic acid
HNO_3	Nitric Acid
HPA	Hypothalamic-Pituitary-Adrenal Axis
KA	Kainic Acid
KCl	Potassium Chloride

Kd	Dissociation Constant
l-BMAA	β-N-methylamino-L-alamine
MDA	Malondialdehyde
MEM	Minimal Essential Media
MK-801	Dibenzocycloheptenеimine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
n	Number of Samples
NADP	Nicotinamide Adenine dinucleotide
$NaHCO_3$	Sodium Hydrogen Carbonate
NBD	Nitro-Blue Diformazan
NBT	Nitro-Blue Tetrazolium
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ns	Not Significant
$O^-_2\cdot$	Superoxide Anion Radical
·OH	Hydroxyl Radical
ONOO·	Peroxynitrite
P	Probability
PCP	Phencylidine
PUFA	Polyunsaturated Fatty Acid
QA	Quinolinic Acid
ROS	Reactive Oxygen Species
s.c.	Sub-cutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
t-ACPD	*trans*-1-aminocyclopentyl-1,3-dicarboylate
TBA	2-Thiobarbituric acid
TCA	Trichloroacetic Acid
Tris	Tris(hydroxymethyl)-aminomethane
USA	United States of America

CHAPTER 1

LITERATURE REVIEW

1.1. MELATONIN

1.1.1. Introduction

Herophilos (325 - 280BC) is credited with discovering the human pineal gland. At the time of discovery he, and other investigators were examining the brain in an attempt to find the soul in the body. For the next 2000 years very little knowledge was accumulated on the pineal gland, although both doctors and philosophers from Europe and Asia considered the pineal to be a prime candidate for the seat of the soul.

By the 19th century, researchers were taking a more scientific and less philosophical approach to their studies on the pineal gland. Investigations in lower vertebrates had revealed that the pineal gland functioned as a photosensory organ. The gradual loss of photosensory apparatus in higher vertebrates was interpreted as phylogenetic regression, and so the pineal glands in higher animals were considered to be little more than the rudimentary remnants of the non-mammalian pineal gland.

It was not until 1930, that Marburg first suggested a hormonal role for the pineal gland. He believed that specific pineal parenchyma cells produced an antigonadotropic hormone that could retard sexual development in infants.

Following the Second World War, there was a great deal of research conducted on pineal anatomy, histology, cytology, biochemistry, physiology and pharmacology. Melatonin, the principal hormone of the pineal gland, was first isolated in 1958 by Lerner *et al* [9]. In the past 50 years, great advances have been made in understanding the role of the pineal gland in human physiology, the role of melatonin in the regulation of circadian and circannual rhythms, and the effect of melatonin on endocrine glands. It

was only in 1991, that Ianas *et al* [1,2] first suggested that melatonin may have antioxidant properties. Since then a great deal of research has been conducted into this aspect of melatonin function.

1.1.2. Structure

Melatonin, 5-methoxy-N-acetyltryptamine (see Figure 1.1) is, evolutionally speaking, a highly conserved molecule that exists in organisms as different as algae and humans [8,10]. Although first isolated in 1958, as far back as 1917, McCord and Allen had reported that pineal extracts contained a substance that lightened the skin of amphibians. The molecule was later termed melatonin, because of its ability to cause aggregation of melanin granules in melanophores.

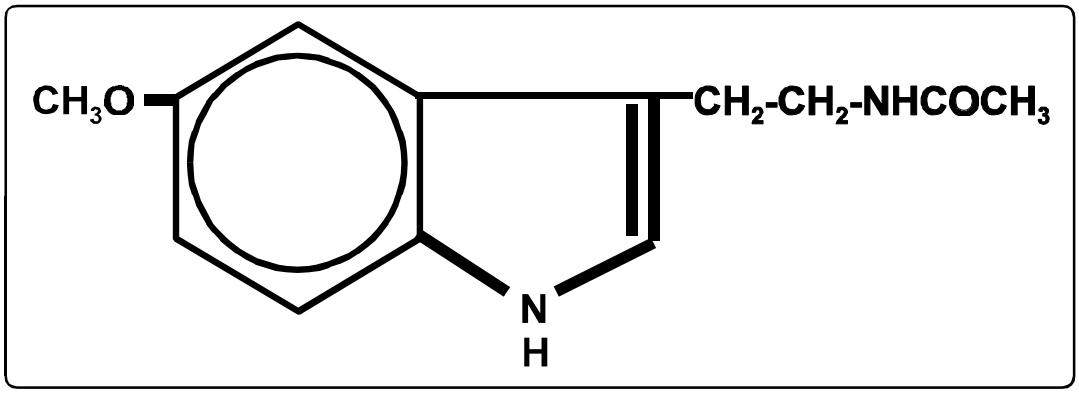


Figure 1.1: Structure of Melatonin

1.1.3. Biosynthesis

Melatonin is the product of tryptophan metabolism by the pineal gland. The production of melatonin is controlled by a visual signal that originates in the retina. During the hours of darkness, the retina initiates the production of melatonin, by passing a signal along unmyelinated fibres [10,11] to the suprachiasmatic nucleus of the hypothalamus [12,13]. The suprachiasmatic nucleus is the body's internal clock which controls circadian rhythmicity of biological events and behaviour [10,14,15]. Light synchronises this clock

to the external environment by daily adjustments in the phase of the circadian oscillation [16].

A pathway then stretches from the suprachiasmatic nucleus, through the lateral hypothalamus to the upper thoracic intermedio-lateral cell column. Impulses are conveyed via the medial forebrain to the spinal cord by preganglionic cell bodies synapsing in the superior cervical ganglion [17]. Finally, post ganglionic fibres approach the pineal together with the pineal blood vessels. When stimulated during darkness, the postganglionic sympathetic fibres release noradrenaline which binds to specific β-adrenergic receptors on the pinealocyte cell membrane [10], so activating melatonin production in the pineal gland.

The biosynthesis of melatonin in the pineal gland follows a two step process. Initially, 5-hydroxytryptophan is transformed to 5-hydroxytryptamine (serotonin) by L-amino acid decarboxylase. Serotonin is allowed to accumulate in the pineal gland, before it is converted to N-acetylserotonin, by N-acetyltransferase [18], during the hours of darkness. This is because N-acetyltransferase is the rate-limiting enzyme in melatonin synthesis [10,19], with its function in the pineal gland being regulated by the release of noradrenaline [20] by the postganglionic synaptic fibres.

The final step in melatonin synthesis, involves the conversion of N-acetylserotonin to N-acetyl-5-methoxytryptamine (melatonin). This conversion is achieved by hydroxyindole-O-methyltransferase using S-adenosylmethionine as a methyl donor [18,20,21] (see Figure 1.2).

Melatonin is not stored in the pineal gland but is rapidly secreted directly into the bloodstream [10]. The normal route of melatonin secretion comprises of the pineal capillaries draining into the surrounding venous sinuses [20]. Melatonin is transported in the blood non-covalently bound to high-capacity, low-affinity binding sites on plasma albumin [22], while in cerebrospinal fluid, melatonin is present in its free form [23]. Due

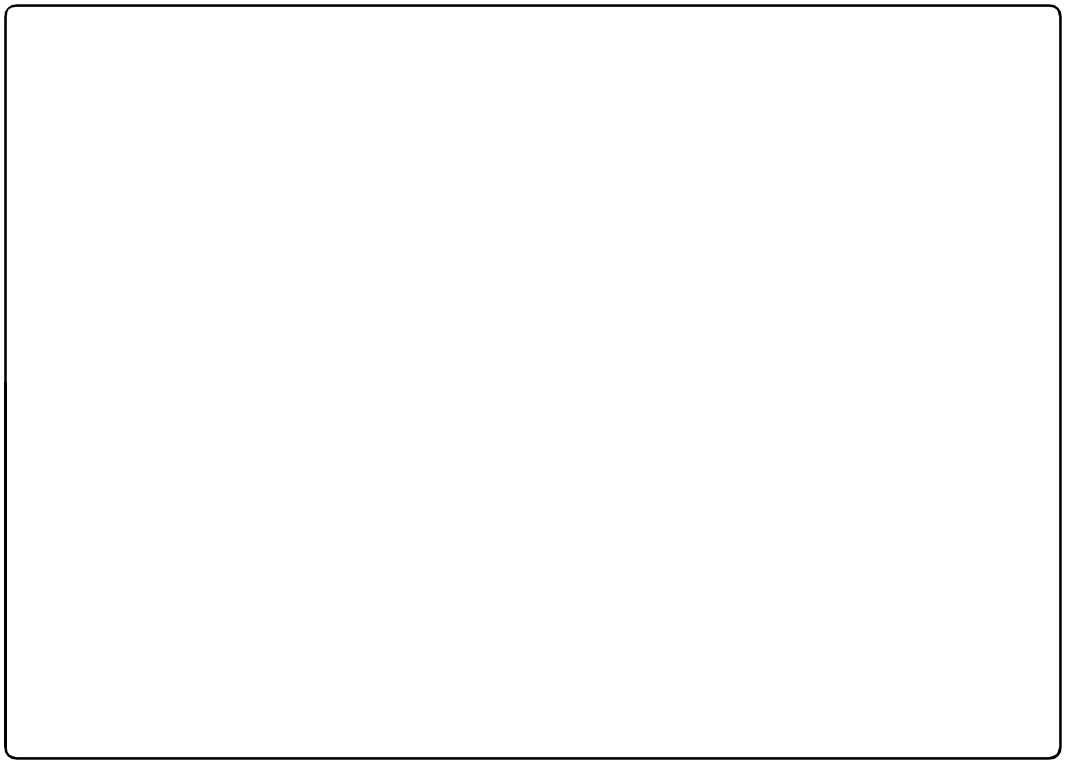


Figure 1.2: Schematic representation of the actions of light and innervation on melatonin synthesis in the pineal gland [20]

to its unique lipophilic and hydrophilic nature [24], melatonin is able to easily diffuse into most tissues and cells in the body.

1.1.4. Metabolism

The half-life of melatonin in rats [25] and sheep [26] is only about 20 minutes. The major site of melatonin metabolism is the liver, where melatonin is converted to 6-hydroxymelatonin by the cytochrome P-450-dependent microsomal mixed-function oxidase enzyme system [27]. Most of the 6-hydroxymelatonin is further conjugated to sulphate, rendering 6-sulphatoxymelatonin as the major urinary metabolite [10,27,28,29], although 6-hydroxymelatonin can also be conjugated to glucuronic acid [24,27].

The metabolic pathway of melatonin in the central nervous system is different from that in the liver [30]. In the brain, indoleamine-2,3-dioxygenase cleaves the pyrrole ring to form N-acetyl-5-methoxykenurenamine which is excreted in the urine [18].

1.1.5. The 24-hour Rhythmicity of Melatonin Production

In all species, the daily period of darkness is associated with a rise in the amount of melatonin produced by the pineal gland [31]. The magnitude of the rise varies among species, but typically night-time values are 2 - 10 times greater than day-time melatonin levels [31]. In humans and the rat [32], melatonin production begins to increase at, or shortly after, the onset of darkness. The production continues to gradually rise, until a peak is reached near the middle of the dark phase. Melatonin levels then begin to drop to reach day-time values at about the time of lights on (see Figure 1.3) [33].

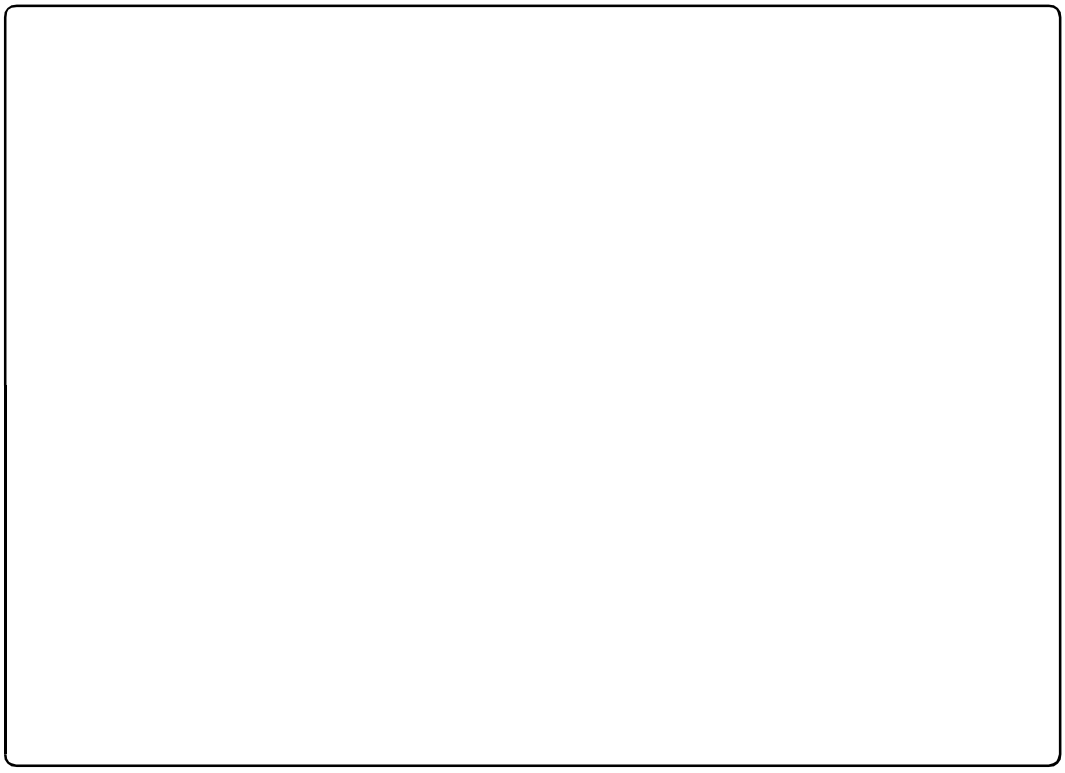


Figure 1.3: Normal Circadian Rhythm of serum melatonin for a male (A) and female (B). Each point represents the mean (±SD) of 10 individuals [20].

1.1.6. Effects of Disturbances to the Diurnal Melatonin Cycle

Because the production of melatonin is regulated by the intensity of the light falling on the retina, the time of the day and even the season of the year [34] affect melatonin

production. Any disturbances that throw the melatonin cycle out of phase with the natural circadian rhythms of the body can harm human well-being [35].

Seasonal Affective Disorder is characterised by periodically recurring depressive episodes [36-39], which normally occur during winter. This disorder is especially prevalent in Scandinavian countries, where it is proposed that a patient's circadian clock does not adjust correctly in accordance with the changes in day length, that occur during the different seasons. Normally the acrophase of the melatonin cycle is either, phase advanced of phase delayed with respect to healthy subjects, in patients with this disorder. This results in a melatonin cycle out of phase with the patient's general environment, and leads to the periods of depression or mania. Treatment involves synchronising the melatonin rhythm with the day / night cycle over a period of 4 - 14 days [40]. This is done by exposing phase advanced patients to 2 500 lux light for 2 hours in the morning, and phase delayed patients to the same amount of light in the evening. Morning appears to be the most beneficial time to give light treatment in most cases, indicating that circadian rhythms are more likely to be delayed in seasonal affective disorder patients when compared to controls [36,40].

Jet-lag results from a similar shift in the melatonin acrophase. Flying over many time zones results in a melatonin circadian rhythm that is out of phase with the general environment [41]. Fortunately most people are able to adjust their circadian rhythms to suit the new environment within a few days [42]. Taking 5 mg of melatonin [43] at the local bed time, for the first two days in the new time zone, can be beneficial for helping to speed up changes in the circadian rhythm. The use of melatonin as a therapeutic agent against jet-lag does not appear to have any undesirable endocrine effects [44], with the only side effect being the promotion of drowsiness [45,46].

It has also been reported [47] that the depressive effects and feelings of disorientation that many night workers experience could be caused by a circadian rhythm that is out of phase with the environment. In this case however, melatonin treatment would not be

therapeutic as the work environment is completely out of phase with the signals from the natural environment that help to regulate the circadian rhythms.

1.1.7. Historic View of the Functions of Melatonin

Up until the discovery of an antioxidant role for melatonin in 1991, researchers had previously thought that melatonin's role was restricted to controlling circadian rhythms, breeding in animals and acting as a tranquillising hormone.

1.1.7.1. Reproduction

The association between the pineal glands and the gonads has been known since the end of the 19th century, when Heubner published a report of a young boy with early onset puberty, who was also suffering from a tumour in the brain tissue around the pineal gland which inhibited melatonin production.

The association between the pineal and sexual development is related to the production of melatonin. Melatonin is able to exert an antigonadal effect [48], and this suggests that secretion is connected with the estrus and menstrual cycles. Melatonin and gonadosteroids appear to function in a feedback loop, as gonadosteroids, estradiols and testosterone given at physiological doses, significantly increase hydroxyindole-O-methyltransferase activity, while castration decreases melatonin production [20].

Melatonin has been shown to regulate the seasonal reproductive cycles of Syrian hamsters by utilising day-length as a primary environmental cue [49]. There are many hypotheses as to how melatonin regulates seasonal breeding, but the internal coincidence hypothesis is the one that most evidence supports [18]. This model proposes that there are two rhythms in the animal body, the melatonin cycle and a rhythm that is sensitive to melatonin. It is only when the melatonin peak coincides with the sensitivity rhythm that gonadal maturation takes place [31] (see Figure 1.4). This

would explain why, for many animals, breeding only occurs at certain times of the year, when the whole breeding population becomes sexually active.

1.1.7.2. Hibernation

Melatonin is also known to play a role in hibernation. When the ground squirrel (*Spermophilus elegans*) is pinealectomised during the summer, hibernation during the subsequent winter is completely abolished [10]. Pinealectomy of other species does not completely abolish hibernation, but does appear to upset the hibernation cycle [10]. Other mechanisms therefore appear to be involved in the control of hibernation.

1.1.7.3. Temperature

All homeothermic animals, including man, exhibit a diurnal rhythm in body temperature [34,42]. The temperature rhythm consistently follows the melatonin rhythm with an inverse amplitude and a delay of about 1.8 hours [50], with melatonin levels starting to increase as the body temperature of man begins to decline during the evening, and as the body temperature starts to rise before dawn, so melatonin levels decrease. Melatonin therefore appears to play a role in circadian thermoregulatory adjustments in body temperature, where it adjusts the set point of body temperature to be consistent with the metabolic rate of the animal. This is supported by work done by Spencer *et al* [51] who observed that pinealectomy drastically reduced the mean core temperature, but did not alter the rhythmicity of the temperature cycle in female rats. This relationship appears to be causal rather than fixed, as some individuals show a temperature rhythm in the absence of a melatonin rhythm [34].

1.1.7.4 Endocrine Effects

It has been reported that the pineal gland restricts and modulates adrenal steroidogenesis under normal and stress conditions [52,53]. Kinson *et al* [54] found, that

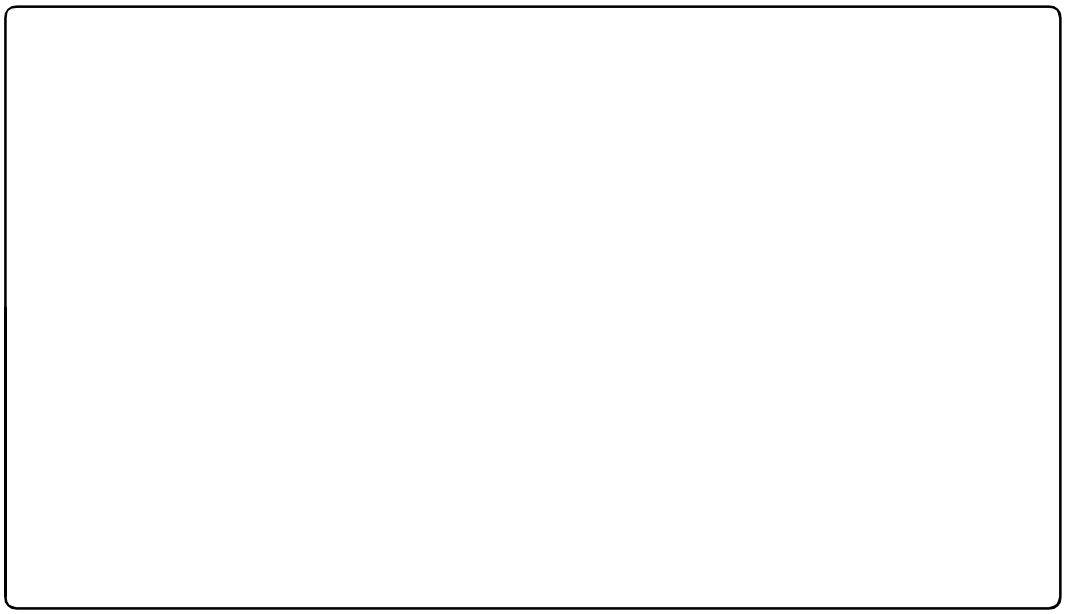


Figure 1.4: Diagrammatic representation of the internal coincidence model [31].

following pinealectomy, the secretion rates of both aldosterone and corticosterone were significantly elevated. The authors therefore concluded that the pineal gland may normally exert an inhibitory effect on the adrenal cortex.

Many researchers [52,55] also proposed that the principal function of the pineal gland was to control, coordinate and regulate the physiological adaption of an organism to stress, where stress was defined as the sum of the biological reactions to any adverse stimulus, whether physical, mental or emotional, that disturbs an organism's homeostasis. Evidence suggests that the pineal gland exerts a general tranquillising effect in stress situations [56]. This is supported by the finding that melatonin exhibits an inhibitory action on the thyroid gland [53]. As the thyroid hormones stimulate general metabolism in most body tissues, an increase in melatonin production would cause a general decrease in body metabolism and make an animal more lethargic.

Therefore, until the beginning of this decade, the pineal gland and melatonin were thought to generally act to synchronise, stabilise and moderate organisms on behalf of several physiological processes [53].

1.2. GLUTAMATE

1.2.1. Role of Glutamate in the Mammalian Organism

L-glutamate is the most common free amino acid in the CNS [57,58], existing in millimolar concentrations in the cytoplasm of most vertebrate neurons [58]. A number of biological functions, including intermediary metabolism of neuronal tissues, detoxification of ammonia in the brain and carbohydrate and nitrogen metabolism [57], involve glutamate. As an amino acid, glutamate, is also an important building block in the synthesis of proteins and peptides.

In 1984, Fonnum [59] showed that glutamate satisfied all the criteria to be called a neurotransmitter. Firstly, glutamate is presynaptically localised in distinct neurons and specifically released by physiological stimuli in concentrations high enough to elicit a postsynaptic response. In addition, intracellular Ca^{2+} is released upon glutamate stimulation and high affinity uptake mechanisms exist to terminate transmitter action rapidly. Finally, cellular and molecular effects of receptor activation are observed, while synaptic transmission can be blocked by glutamate receptor agonists. Glutamate was subsequently classified as one of a number of excitatory amino acids (EAA's). Glutamate and the related EAA's account for most of the excitatory synaptic activity [60,61], and they are released by an estimated 40% of all synapses [59,62]. Of the EAA's, glutamate is the most dominant and it is said to be the predominant fast excitatory neurotransmitter in the central nervous system (CNS) [57].

1.2.2. Structure, Biosynthesis and Metabolism

Glutamate is a non-essential amino acid. In the body there are several precursors for glutamate synthesis as well as many metabolic pools [58]. Glutamate that is used for metabolic processes is derived from glucose via the Krebs Cycle [59,63]. In the neuron the neurotransmitter pool of glutamate is kept separate from metabolic glutamate, and is

localised in the nerve terminals of glutamatergic neurons [57]. Experiments with cortex synaptosomes have shown that 80% of released glutamate is derived from glutamine and less than 20% from glucose [64]. *In vivo* however it may well be that the transmitter pool of glutamate will accept any glutamate available, independent of source, and the synthesis of transmitter itself is not a rate-limiting factor under normal conditions [59].

1.2.3. Glutamatergic Neuronal Transmission

Glutamate is taken up and stored in synaptic vesicles by an adenosine triphosphate (ATP) process [65]. The vesicular glutamate transporter is very specific and does not transport other closely related amino acids, such as aspartate, into the vesicles. When glutamatergic nerve terminals are depolarised, vesicular glutamate is released into the synaptic cleft in a Ca^{2+}-dependent fashion [61,62]. 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 [66,67], but presynaptic terminals also sequester glutamate directly from the synaptic cleft [60]. Within the glial cells, glutamate is converted to glutamine via the enzyme glutamine synthetase [63,68]. Glutamine can then be cycled back to the glutamatergic nerve terminals, where it is converted to glutamate by the mitochondrial enzyme, phosphate-activated glutaminase [63,69] (see Figure 1.5). The rapid removal of glutamate from the synapse is an essential aspect of neurotransmission [70]. Glutamate concentrations are kept extremely low (2 - 3 µM) in the synapse [68,71]. This ensures that postsynaptic neurons are only stimulated following the release of glutamate by the presynaptic neurons.

1.2.4. Glutamate Receptors

Glutamate is termed a “mixed agonist” because it activates several classes of receptors [72]. Each class has a distinct pharmacology and physiology. The receptors are named

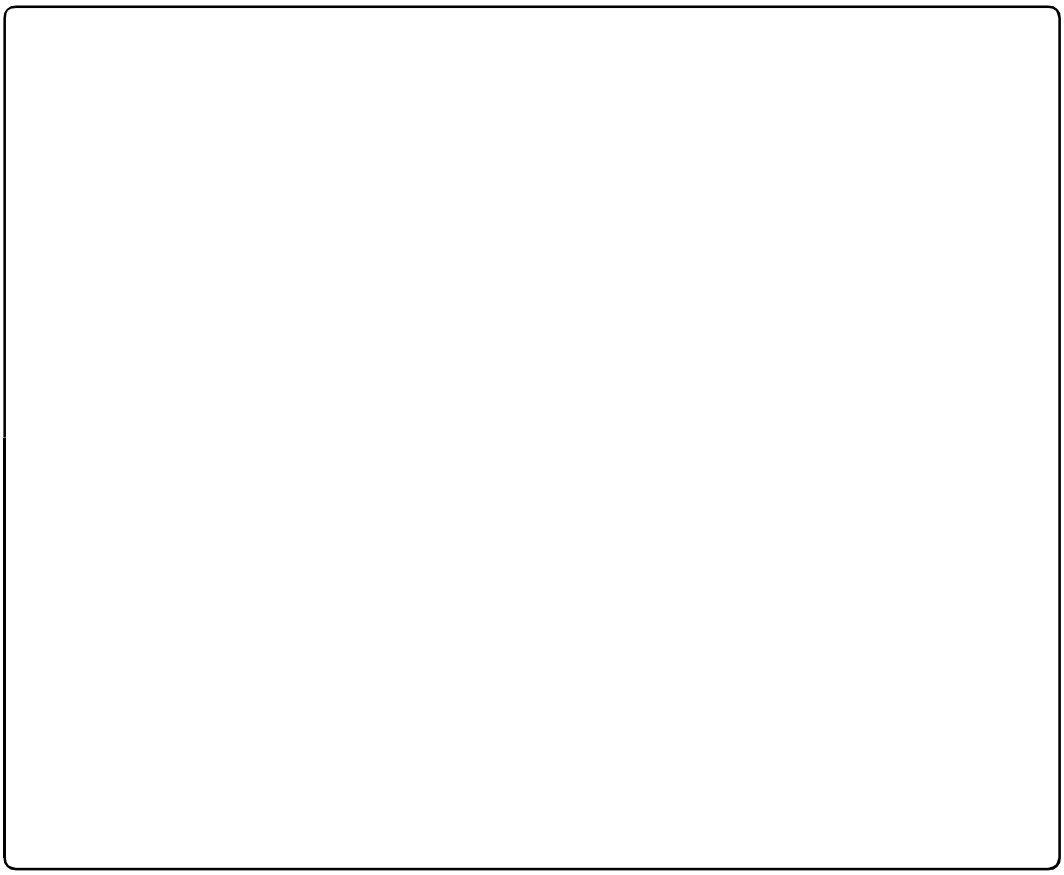


Figure 1.5: Schematic representation of a glutamatergic synapse showing a presynaptic glutamatergic nerve terminal, a postsynaptic neuron, and a glial cell [57].

after the agonist compounds that are specific for eliciting a given pharmacological response. Glutamate receptors are divided into the ionotropic receptors, which are ligand-gated ion channels, and the metabotropic receptors [43], which are linked to G-proteins [62,74,75] and are associated with changes in the cyclic nucleotides or phosphoinositol metabolism [57].

1.2.4.1. NMDA Receptors

The NMDA receptor has a very complex structure (see Figure 1.6), containing binding sites for both glycine and glutamate [76]. Both the glycine and glutamate sites must be occupied for activation to occur [77,78], and so they are referred to as “co-agonists” of the NMDA receptor [62].

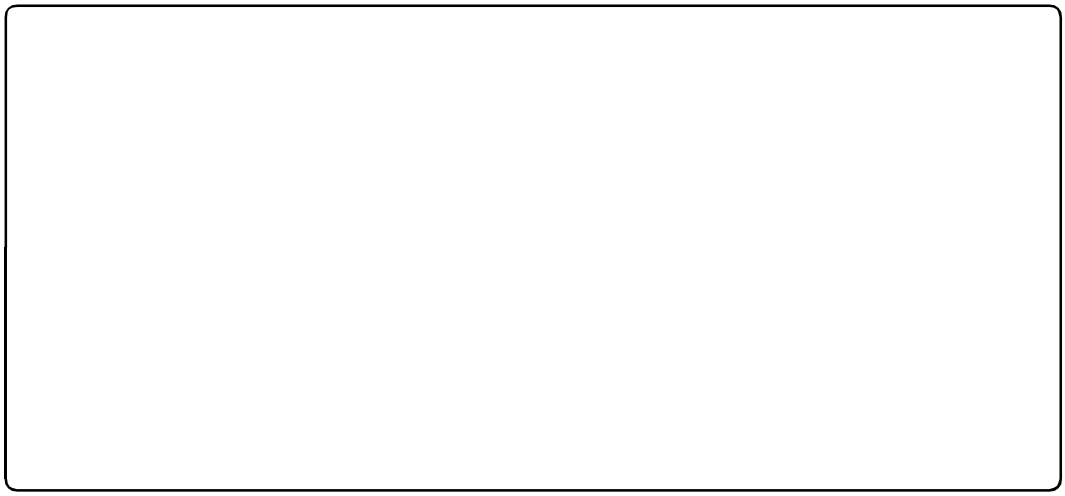


Figure 1.6: Diagrammatic representation of the NMDA receptor [86].

Activation of the NMDA receptor is modulated by polyamines, such as spermine and spermidine [79]. Although the polyamine site need not be occupied for receptor activation, the presence of polyamines increases the ability of glutamate and glycine to open the NMDA-receptor ion channel [80]. One of the most important factors of the NMDA receptor is the voltage-dependent blockade of the receptor ion channel by Mg^{2+} [57,81-84]. At resting potential (about -70mV), normal extracellular concentrations of Mg^{2+} block the NMDA-receptor ion channel and prevent current flow, even when the glutamate and glycine sites are occupied. However, because the Mg^{2+} blockade is voltage-dependent, the degree of Mg^{2+} block is reduced as a neuron becomes depolarised [85] allowing more movement of ions.

The NMDA receptor is ionotropic, permitting an intracellular influx of Ca^{2+}, as well as an exchange of Na^{+} and K^{+} across the cell membrane [58,68,87]. Ca^{2+} permeability of the NMDA receptor is important as the increase in Ca^{2+} concentration activates a number of cellular and second messenger systems, and contributes to the initiation of long-term potentiation [88-90].

Extracellular Zn^{2+} appears to act as an inhibitory modulator of channel function at a site near the “mouth” of the ion channel [58]. A variety of competitive and non-competitive inhibitors exist for the NMDA receptor. Among the more common competitive inhibitors

are 2-amino-5-phosphonovalerate (AP5) which competes directly with glutamate for the binding site [58], while the non-competitive inhibitors dibenzocycloheptenеimine (MK-801), phencyclidine (PCP) and remacemide act within the ion channels to block current flow [57,58]. High concentrations of the NMDA receptors are found in the cerebral cortex and CA1 regions of the hippocampus [82].

1.2.4.2. The AMPA Receptor

The α-2-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA) (see Figure 1.7) receptor is an ionotropic glutamate receptor that appears to be involved in fast excitatory synaptic transmission [91]. This class of receptor was previously called the "quisqualate receptor", but the name has changed following the discovery that quisqualate can activate several receptor classes [86]. The binding of glutamate to the AMPA receptor is associated with influx of Na^{2+} from the extracellular space to the intracellular compartment. K^{+} also passes through the AMPA receptor [58]. Some AMPA [62] and KA [92] receptors have also been reported to be permeable to Ca^{2+}.

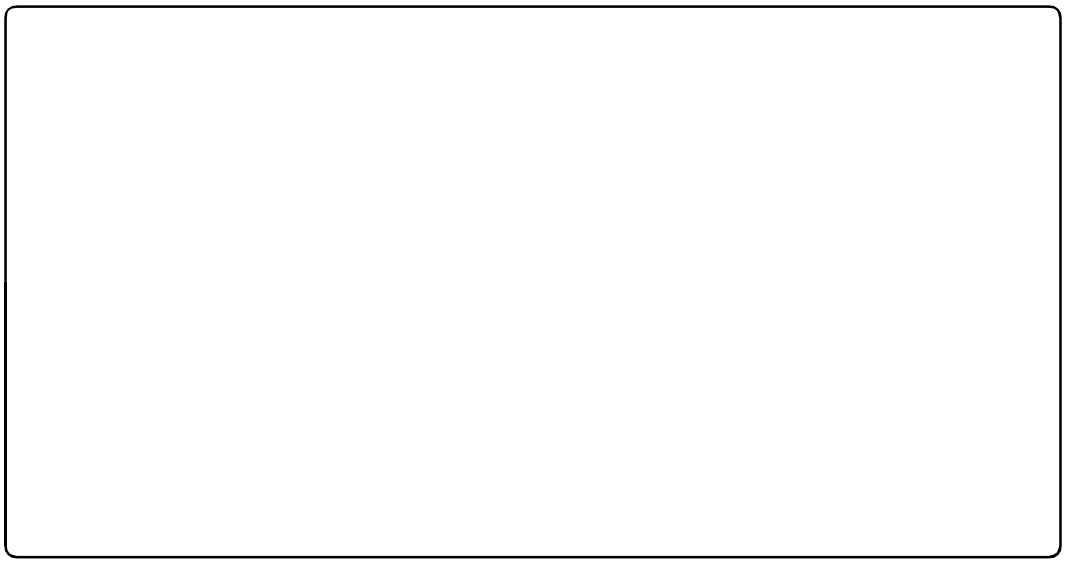


Figure 1.7: Schematic diagram of the AMPA receptor [86].

Several competitive inhibitors compete directly with glutamate for the AMPA binding site. Among these antagonists are 6-cyano-7-nitroquinoxaline (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX). The AMPA receptor is found in relatively high concentrations in the cerebral cortex, molecular layer of the cerebellum, CA1 and CA3 regions of the hippocampus, striatum and the nucleus accumbens [58].

1.2.4.3. The Kainic Acid Receptor

The KA receptor is also ionotropic, and although similar to the AMPA receptor, is differentially sensitive to domoate and KA [93]. As with the AMPA receptor, the KA receptor is predominantly permeable to Na^+ and K^+, but some receptor classes also allow Ca^{2+} to enter the cell [94]. Elucidation of the pharmacology and function of the KA receptor has been hampered by lack of a specific antagonist for this receptor type [57]. 2-Amino-3[-(carboxymethoxy)-5-methylisoxolzol-4-yl]-proprionate (AMOA) and 2-amino-3-[2-(3-hydroxy)-5-methylisoxazol-4-yl]-proprionate (AMNH) are selective inhibitors of the KA receptors [58]. KA receptors are found in their highest concentrations in the CA3 region of the hippocampus [58].

1.2.4.4. Metabotropic Receptors

Metabotropic receptors are not linked to ion channels (see Figure 1.8), but are rather coupled to second messenger systems. Evidence indicates at least five subtypes of metabotropic glutamate receptors, which are coupled to G-proteins [85] and cytoplasmic enzymes. Depending on the receptor subtype and the cell type, metabotropic receptors might mediate [95] inositol phosphate metabolism, release of arachidonic acid, or changes in cyclic adenosine monophosphate levels.

Agonists for metabotropic receptors include *trans*-1-aminocyclopentyl-1,3-dicarboylate (t-ACPD), ibotenic acid, and β-N-methylamino-L-alamine (L-BMAA) [58]. Like KA receptors, the role of metabotropic receptors within the CNS has not been well defined because of a lack of good receptor antagonists [57]. Metabotropic receptors may be

located on glial cells, as well as neurons. The highest concentration of metabotropic receptors is seen in the molecular layer of the cerebellum.

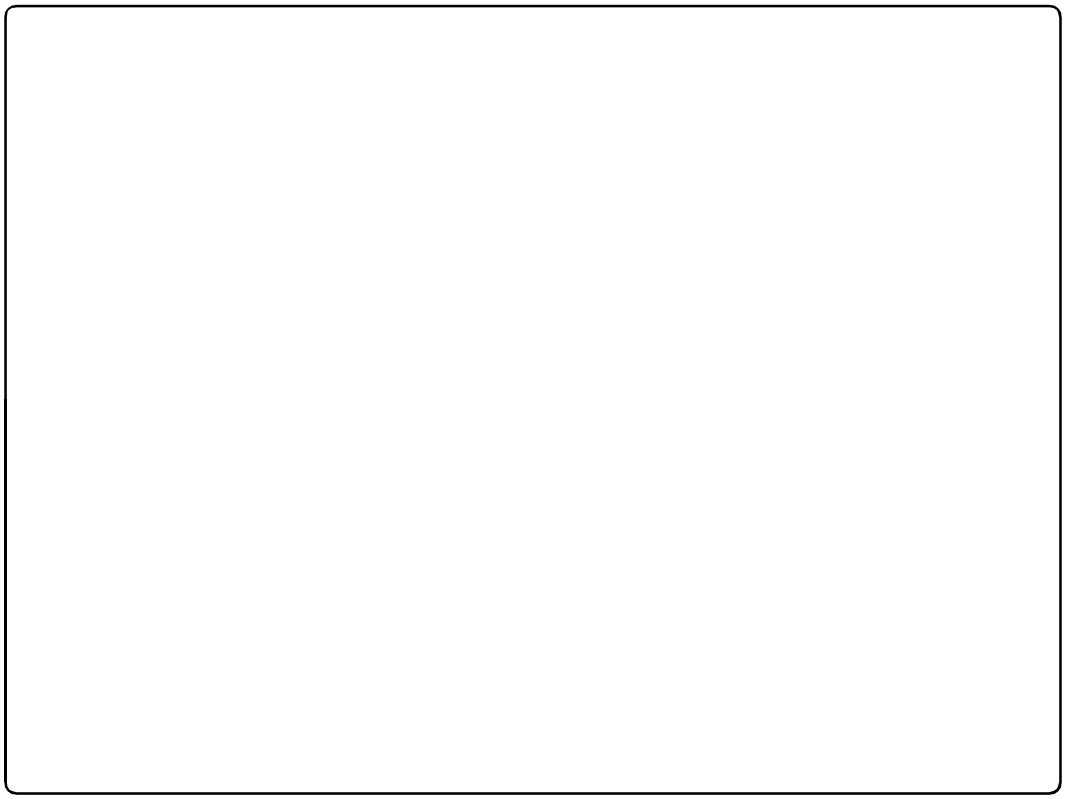


Figure 1.8: Diagrammatic representation of the metabotropic receptor [86].

1.2.5. Glutamate Receptor Agonists

Glutamate is the ubiquitous agonist for all of the glutamate receptors. Among the many glutamate receptor agonists that have been found are NMDA, L-aspartate, polyamines, AMPA, quisqualate, KA, domoate, t-ACPD, t-BMAA and iboterate. For the purposes of this discussion NMDA, QA and KA will be investigated, as these are the agonists that were used throughout the study to promote neurotoxicity in the CNS.

1.2.5.1. N-Methyl-D-Aspartate

NMDA is a synthetically produced amino-acid that selectively binds to NMDA receptors. It is produced by many commercial companies and is neurotoxic if neurons are exposed to it.

1.2.5.2. Quinolinic Acid

QA is a naturally produced metabolite of tryptophan metabolism [96-98]. QA has been shown to be present in normal postmortem human brains at levels similar to those of other species [99], and with concentrations not varying greatly among the different regions of the brain. Heyes and Morrison [97] demonstrated that the brain naturally synthesises QA, and that the rate of QA formation increases in conditions of brain and systemic immune activation. QA concentrations have also been shown to increase during the natural aging process in rats [100].

Foster *et al* [101] showed that there is no active uptake of QA from the extracellular space, nor is there extracellular metabolism of QA. It is therefore unlikely that QA is a neurotransmitter in the classical sense. QA is however a selective agonist for the NMDA population of receptors. Stone and Perkins [102] demonstrated that QA is about one-quarter as active as NMDA, and approximately as active as glutamate and aspartate at stimulating NMDA receptors. It must however be remembered that the latter compounds have a rapid, high-affinity uptake system for their removal from the synapse, while QA does not.

Because QA is not removed from the synaptic cleft, QA will continue stimulating the NMDA receptor. It is thus a very potent neurotoxin. QA is known to induce neuronal lesions after intrastriatatal and intrahippocampal injections in the rat brain [103,104]. These lesions closely resemble those observed in the brains of people with Huntington's disease [105,106].

1.2.5.3. Kainic Acid

Kainic acid activates the KA receptors in the brain and is not naturally produced in the body. It is however produced by a type of kelp (*Digenea simplex*) [93]. KA toxicity first came to the attention of scientists, when they noticed that the inhabitants of Guam and other Pacific Rim Islands, where uncooked seaweed is eaten regularly, suffered from neuropathies and dementias [83].

1.3. EXCITOTOXICITY

1.3.1. Introduction

Apart from glutamate's beneficial function as an excitatory neurotransmitter, glutamate also harbours the potential to be neurotoxic. The concept of glutamate as an excitotoxin developed largely due to the work of Olney and his colleagues [107,108] who, in the 1970's, demonstrated a correlation between the excitatory properties of various glutamate analogues, and their ability to produce neurotoxic damage at high concentrations [93]. Excitotoxicity as defined by Olney [109] refers to the paradoxical property, shown by glutamate and specific EAA analogues, of causing acute neuronal degeneration by excessive stimulation of postsynaptic EAA ionotropic receptors - receptors through which glutamate functions as a transmitter.

Agonists acting at the NMDA and KA receptors are among the most potent agonists able to produce a combination of neuronal excitation and subsequent neurodegeneration. In most cases the initiating factor for neurodegeneration is the accumulation of intracellular Ca^{2+}. Agonists acting at the NMDA receptor induce increases in Ca^{2+} by activating ionic channels through which Na^{+}, K^{+} and Ca^{2+} can enter the cell [110]. KA receptor agonists cause an increase in Ca^{2+} by triggering the release of Ca^{2+} from intracellular stores. It is also possible that under certain pathological conditions such as hypoxia, hypoglycemia, seizure and epilepsy [111], the transport system can be caused to operate in reverse, which results in the release of glutamate into the synapse. High synaptic glutamate concentrations can prove toxic to neurons, as demonstrated by the Ca^{2+} channel blocker nifedipine, preventing damage to cultured neurons by QA and other excitotoxins [112].

1.3.2. Mechanism of Excitotoxicity

Normally the release of glutamate acts as a signal between one neuron and another, triggering the brief opening of receptor-coupled channels, which allows ions to pass

through the cell's protective membrane [8]. High concentrations of glutamate, however, cause these channels to remain open for extended periods of time, permitting an abnormal, prolonged and finally a lethal influx of ions into the neurons.

There are two principle processes in which EAA induced neurotoxicity can lead to cell death [93]. These are distinguished by differences in time course and ionic dependence. The first process, termed the acute process, involves the rapid swelling of neurons caused by excessive depolarisation, ion influx and water entry, while the second, slower or delayed neurotoxic process occurs secondary to Ca^{2+} influx [58].

1.3.2.1. Acute Toxicity

Acute toxicity occurs because of the rapid influx of mainly 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 some circumstances, the neuron may be able to restore osmotic pressure and so survive. This process can be mimicked by depolarising agents like veratridine [113] or by raising extracellular K^+ concentrations. Activation of non-NMDA receptors can cause such neurodegeneration, with both KA and AMPA being examples of potent neurotoxins [114]. The process that takes place may be direct, by over excitation of the neuron leading to prolonged depolarisation 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.

1.3.2.2. Delayed Toxicity

Delayed neurotoxicity involves Ca^{2+} influx mediated by the NMDA receptor. The increase in intracellular Ca^{2+} concentration can set off a cascade (see Figure 1.9) of pathophysiological events that can lead to excitotoxin-mediated neuronal death [5]. In

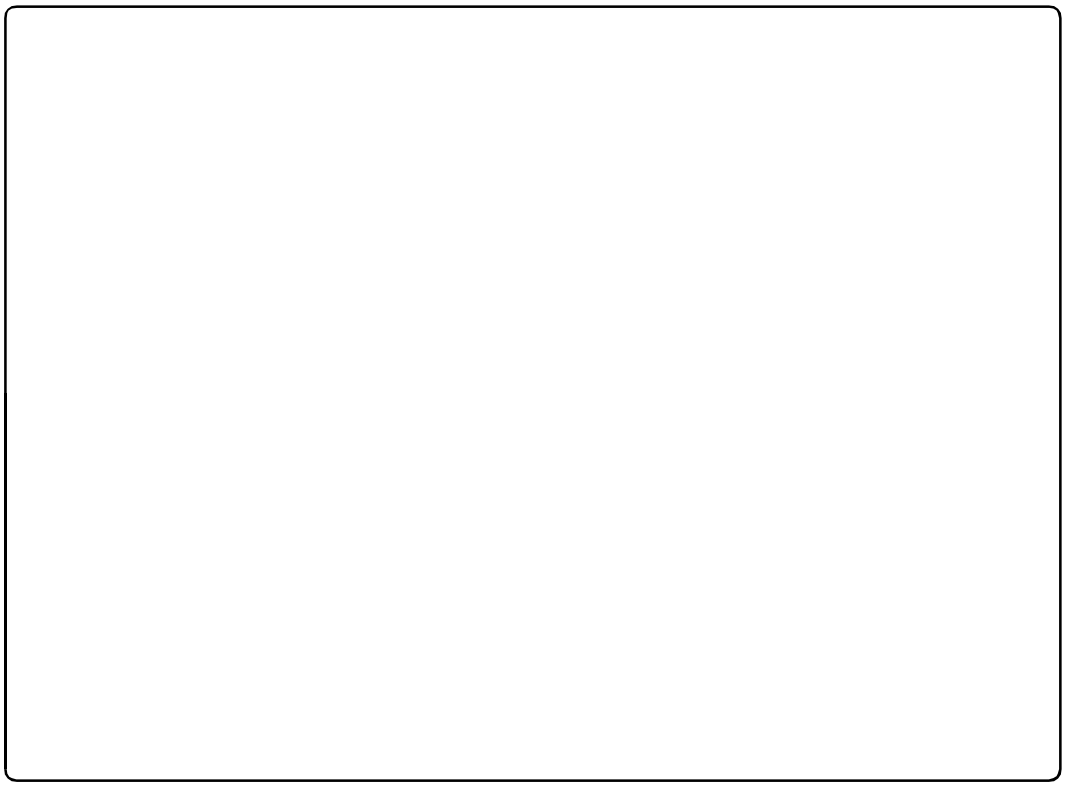


Figure 1.9: Schematic representation of the glutamate-receptor mediated processes that promote oxidative stress [5].

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 [115]. 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 such as calpain-1. These enzymes can degrade major neuronal structural proteins, and induce cytoskeletal breakdown [116]. Calpain also cleaves xanthine dehydrogenase to convert it to xanthine oxidase [93,117]. This enzyme converts hypoxanthine to uric acid while also producing $O^{-}_{2}\cdot$ [5,118].

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

[119]. Among the processes activated are Ca^{2+}-calmodulin-linked protein kinases, which disrupt the cytoskeleton [120], cause mitochondrial dysfunction and activate lipases [121]. Ca^{2+}-calmodulin complexes can also activate nitric oxide synthase (NOS) [121], which produces nitric oxide (NO), a toxin that has been implicated in a number of neurological diseases [122]. Activation of Ca^{2+}-sensitive kinases (proteases) could also result in over-phosphorylation of cytoskeletal proteins such as tau and ubiquitin, hyperphosphorylated forms of which are found in neurofibrillary tangles [123].

Phospholipases [93,124] which are capable of breaking down cell membranes and liberating arachidonic acid may also be activated by Ca^{2+}. This can lead to the breakdown of Ca^{2+} stores within the cell, which can further amplify the excitotoxic response as there will be even higher intracellular Ca^{2+} concentrations. Extracellular arachidonic acid has been shown to reduce glutamate uptake by astrocytes [125] from the synapse [126]. This results in greater glutamate receptor activation, and a further amplification of the neurotoxic process. Endonucleases responsible for breaking down genomic DNA may also be activated [93]. Free radical production has been linked to a loss of cellular Ca^{2+} homeostasis.

Dykens [127] 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 [128,129]. Ca^{2+} is sequestered to the mitochondrial matrix [130-132], driven by the proton electron chemical gradient generated by the electron transport chain [133]. The reduction in the electrochemical gradient [133] caused by the Ca^{2+} influx decreases ATP synthesis [134], 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 [132]. Mitochondrial Ca^{2+} accumulation and the subsequent permeability transition, may therefore be a critical early event specific to the NMDA receptor mediated excitotoxic cascade [135,136].

1.3.3. Free Radicals

1.3.3.1. Structure

All of the processes described in the previous section lead either directly or indirectly to the elevated production of free radicals. The production of oxygen free radicals is a natural consequence of aerobic metabolism, with these molecules being constantly generated in the body by normal metabolic processes [137,138]. By definition, oxyradicals are a molecular species capable of independent existence that contain one or more unpaired electrons [139-142]. Usually, electrons associated with atoms or molecules are paired because this makes atoms relatively stable and unreactive. The loss of an electron leaves a molecule much more reactive than its paired counterpart. If two radicals react, both radicals are eliminated, while if a radical reacts with a non-radical, another free radical must be produced. This characteristic allows free radicals to participate in chain reactions, which may be thousands of events long [143]. Although there are a variety of free radicals produced by molecules, those that are produced from molecular oxygen have received the most investigative interest [140]. The oxygen species that are typically linked to oxidative stress are superoxide anion radical ($O^{-}_{2}\cdot$), the hydroxyl radical (·OH) [144], hydrogen peroxide (H_2O_2) [145], nitric oxide (NO) and peroxynitrite (ONOO·). The collective term for these chemicals is “reactive oxygen species” (ROS), but not all of these species are particularly active in aqueous biological solutions [146].

The mammalian brain is especially vulnerable to oxidative stress [147] as it consumes 20% of total body oxygen, contains large amounts of polyunsaturated lipids, is relatively deficient in protective mechanisms, and readily accumulates iron [146]. Although estimates vary, it is believed that up to 5% of the oxygen [140,148] taken up by an organism may eventually end up as damaging oxygen-based radicals. In the human, this means that there could be the equivalent of 2 kg of $O^{-}_{2}\cdot$ produced each year [140].

1.3.4. Defence Mechanisms against ROS neurotoxicity

The body has many defence mechanisms to deal with oxidative stress [149] (see Table 1.1). These enzyme systems are normally distributed evenly inside cells [143], and under normal circumstances these defence mechanisms can deal with the production of ROS in the neuron. It is only when there is either an increase in oxyradical production, or a decrease in cellular ROS defence systems, that neurotoxicity occurs [139].

Table 1.1: Cellular defence / anti-oxidant mechanisms accessible to neurons to protect against ROS species [146]

Enzymatic	Non-Enzymatic
Cu / Zn - Superoxide Dismutase	Ascorbic Acid (Vitamin C)
Mn - Superoxide Dismutase	α-tocopherol (Vitamin E)
Glutathione Peroxidase	Glutathione
Glutathione-S-Transferase	
Glutathione Reductase	
Catalase	

1.3.5. The Superoxide Radical

The free radical $O^{-}_{2}\cdot$ is generated by multiple enzymatic and non-enzymatic pathways and is often at the start of the oxidative stress cascade. Mitochondria are one of the main producers of other $O^{-}_{2}\cdot$ [143]. During the production of ATP via the electron transport chain [146], the oxygen molecule can be reduced to $O^{-}_{2}\cdot$. The body protects against this by kinetically restricting these reactions. Nevertheless, 1 - 3% of high energy electrons “leak” in close proximity to oxygen resulting in the formation of $O^{-}_{2}\cdot$ [5]. As mentioned earlier, cumulatively this small amount of $O^{-}_{2}\cdot$ leakage leads to the production of up to 2 kg of $O^{-}_{2}\cdot$ during the year.

The $O^{-}_{2}\cdot$ radical can also be generated chemically by auto-oxidative reactions with catecholamines, tetrahydrofolates and reduced flavins. This can lead to a free radical chain reaction as the production of $O^{-}_{2}\cdot$ leads to the autocatilization of more $O^{-}_{2}\cdot$.

It has also been demonstrated that $O^{-}_{2}\cdot$ is produced by the metabolism of arachidonic acid by the cycloxygenases and lipoxygenses [150]. The Ca^{2+}-dependent activation of phospholipase A_2 also yields $O^{-}_{2}\cdot$ through the metabolism or arachidonic acid by the lipoxygenases and cyclooxygenases to form eiconasoids [151].

Most of the $O^{-}_{2}\cdot$ generated in a cell is converted to H_2O_2 by superoxide dismutase [139,142,152]. The H_2O_2 is then metabolised by either catalase, or one of the peroxidases to produce water and oxygen [153].

1.3.6. The Hydroxyl Radical

The ·OH radical is probably the most reactive of the ROS species [8,146] as it will react with almost all molecules in living cells [154]. It is so reactive that no enzyme systems involving it as a substrate exist [155]. The estimated diffusion distance of 0.3 nm, or one-hundredth the diameter of a typical protein [156], means that damage will occur where ·OH is formed. The ·OH radical has been implicated in damage to proteins, carbohydrates, DNA and lipids [140,146,157].

Most of the hydroxyl radicals generated in biological systems are formed when transition metals [158] or biologically active chelators, such as porphyrins and flavins are present simultaneously with an oxidant, such as hydrogen peroxide [8]. The breakdown of hydrogen peroxide is described by the Fenton reaction [5,8] (see Figure 1.10).

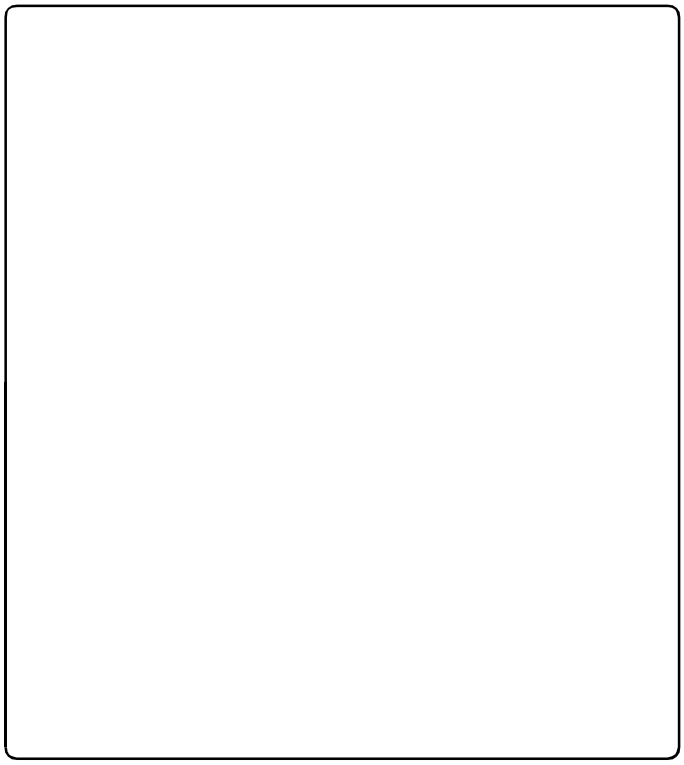


Figure 1.10: The Fenton reaction [8]

1.3.7. NITRIC OXIDE

1.3.7.1. Introduction

Nitric oxide is a ubiquitous and unique biological messenger molecule that mediates all of its biological functions through redox-sensitive interactions. NO's role in the nervous system was first recognised when it was shown that glutamate acting at the NMDA receptor stimulates the formation of NO in cerebral granule cells [85].

1.3.7.2. Physical Properties

At standard temperature and pressure, NO is a gas [159]. However, in most biological systems NO acts as a dissolved non-electrolyte and not as a gas. NO tends to be more soluble in apolar solvents and therefore dissolves selectively in membrane and lipid phases of cells. It also has the ability to diffuse very rapidly from 10 µm (less than a cell size) to several cell diameters [160] in a solution containing proteins and membranes.

Neuroscientists regard NO as a highly reactive molecule owing to its short half life (3 - 6 seconds) in comparison to traditional messenger molecules [161]. As NO is a free radical, it does not remain in it's native form for very long, before it is oxidised, reduced or complexed to biomolecules. It is however relatively less reactive than $O^{-}_{2}\cdot$ or ·OH, and is a relatively stable carrier of the unpaired electron. These properties are exploited by biological systems as a novel class of neurotransmitters.

1.3.7.3. Nitric Oxide Synthase

NO is formed by Nitric oxide synthase [5]. The process involves the conversion of L-arginine to L-citrulline. At least 3 different isoforms of the NOS enzyme have been identified [161]. Neuronal NOS or type I NOS, was first cloned from the rat cerebellum [162]. The other two isoforms of NOS were isolated from macrophages, where NOS is used in the immune defence system, and endothelial cells.

Within the nervous system, NO is responsible for relaxing the pyloric sphincter in the gastrointestinal tract [161], mediating penile erections [163] and vasodilation [164]. NO is also thought to be involved with the regulation of behaviour. Studies with mice that have had the neuronal NOS isoform removed from their genomic make up, exhibit inappropriate sexual behaviour, fight incessantly and do not respond to appropriate submissive posturing by wild-type mice [165]. These behavioural abnormalities may be due to the lack of NOS activity, as there are no detectable differences in brain structure [166], or testosterone levels [165], between wild type and knock out mice.

The catalytically active form of NOS is a dimer, however dimerisation on its own does not lead to electron flow. Neuronal NOS is synthesised as two non-interactive molecules that bind FMN and FAD [167]. These monomers loosely dimerise upon binding tetrahydrobiopterin, haem and L-arginine. Electron transport however, only begins after an increase in intracellular Ca^{2+} has led to the formation of a Ca^{2+}-calmodulin complex. This complex is necessary to bind to the dimer. This causes a conformational change, which results in activation of the enzyme.

One of the most important aspects of NO production is that it has been implicated in neurological damage [167,168]. The glutamate-initiated neurotoxic cascade involves the increased influx of Ca^{2+} into the neurons. This can bind to calmodulin to form the Ca^{2+}-calmodulin complex. As this complex regulates NOS activity, NO production will be initiated [167].

In the absence of the L-arginine substrate the neuronal isoform of NOS is able to produce $O^{-}_{2}\cdot$ and ·OH [169]. This is because the rate of NADPH oxidation for the neuronal isoform is primarily influenced by the Ca^{2+}-calmodulin isoform, and not by L-arginine [161]. Therefore under conditions of reduced L-arginine concentrations, substantial amounts of $O^{-}_{2}\cdot$ can be produced by NOS.

NO is not a very reactive molecule in its native form, but NO can interact with $O^{-}_{2}\cdot$ to form the highly reactive peroxynitrite free radical [5,142,170]. Peroxynitrite is generally considered to be a more toxic species than either NO or $O^{-}_{2}\cdot$ alone [171,172]. The catalytic process triggered by peroxynitrite include initiation of lipid peroxidation, inhibition of mitochondrial respiration, inhibition of membrane pumps, depletion of glutathione, and damage to DNA [173]. Gilad *et al* [173] have demonstrated that melatonin is a scavenger of peroxynitrite. Melatonin was able to inhibit the development of DNA single strand breakages and reduced suppression of mitochondrial respiration in response to peroxynitrite.

1.3.8. Lipid Peroxidation

Most of our current information concerning biological membranes comes from the fluid mosaic model, proposed by Singer and Nicholson in 1972 [174]. They proposed that a membrane consists of a lipid bi-layer, interspersed with proteins and carbohydrates. The membrane is a dynamic system, and the composition depends on where in the cell it is located. Membranes function to compartmentalise the cell, as well as providing a surface from which ATP can be produced, receptors can bind, the transport of

substances can be controlled. The structure and integrity of membranes is therefore essential for the successful functioning of the cell.

Lipid peroxidation is a process whereby membranes are destroyed by oxidative attack from ROS. Of all the major classes of biomolecules attacked by ROS, membrane lipids are probably the most susceptible to oxidative attack, because of the high levels of poly-unsaturated fatty acids (PUFA) present in these macromolecules. Lipid peroxidation is also extremely damaging because self-perpetuating chain reactions are caused by ROS attack. Table 1.2 illustrates the three features which characterise the lipid peroxidation process. During the initiation step (reaction 1) PUFA's are attacked by ROS (In·), resulting in the removal of a hydrogen atom from the PUFA (RH) and the formation of a lipid-derived radical (R·). The propagation step (reaction 2) normally begins with the rapid addition of molecular oxygen to R·, to form the lipid peroxyl radical (ROO·). This radical can attack other lipids, to generate a further lipid radical and a lipid hydroperoxide (ROOH). A self perpetuating autocatalytic reaction then follows with reactions 2 and 3 undergoing a number of cycles [176]. The final step is a termination step, which results when two ROO· radicals react together to form a non-radical product [177]. The removal of ROS by various antioxidant systems is therefore essential to limit lipid peroxidation occurring in cells.

Table 1.2: Chain sequence for free radical auto-oxidation [178]

Initiation	In· + RH ---> InH· + R·	Reaction 1
Reaction	R· + O_2 ---> ROO·	Reaction 2
	ROO· + RH ---> R· + ROOH	Reaction 3
Termination	2 ROO· ---> Non-radical products	Reaction 4

1.4. MELATONIN AS A FREE RADICAL SCAVENGER

1.4.1. Introduction

in 1991, Ianas *et al* [1,2] gave a completely new dimension to melatonin research, when the authors claimed that melatonin had both antioxidant and pro-oxidant actions. In this first experiment the group used luminol and H_2O_2 to generate free radicals and used chemiluminescence to measure free radical production. Using this system the authors found that melatonin was able to quench free radical generation at concentrations above 0.25 mM, while below this concentration melatonin was pro-oxidative.

In 1993 these experiments were repeated by Tan *et al* [3]. This time ·OH was generated by the exposure of H_2O_2 to ultra violet light, and a spin trap employing 5,5-dimethylpyrroline-N-oxide was used to determine ·OH concentrations. The scavenging properties of melatonin were compared to glutathione and mannitol, both well known ·OH scavengers. Under these experimental conditions, melatonin was found to be a far more efficient ·OH radical scavenger, than either glutathione or mannitol. Melatonin was also found to lack pro-oxidant activity.

Various studies soon followed with Poeggeler *et al* [4] showing that melatonin could remove ·OH from a reaction mixture consisting of $FeSO_4$ and H_2O_2, while Pieri *et al* [179] found that melatonin was more effective at scavenging ·OH than either trolox (water soluble vitamin E), ascorbic acid or glutathione. It has been found that melatonin scavenges the trichloromethylperoxyl radical [180], the *tert*-butoxyl and cumyloxyl radicals [181], and the peroxynitrite radical [173].

Melatonin scavenges ·OH by contributing an electron, thereby rendering the radical non-reactive, but becoming itself a radical, the indolyl cation radical (see Figure 1.11). This product is not very reactive and is therefore non-toxic to the cell [182]. It is believed [7] that the indolyl cation radical then scavenges the O^{-}_{2}·, thereby becoming N'-acetyl-N^2-5-methoxykynuramine which is excreted through the urinary system.

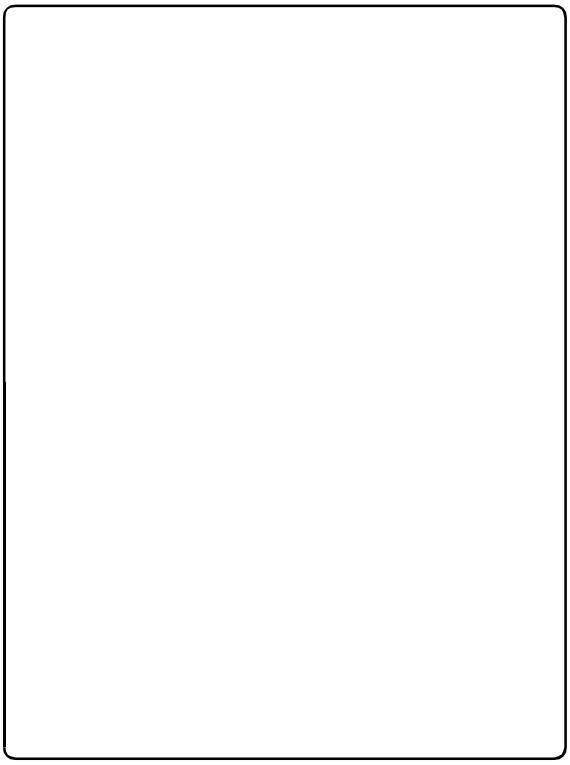


Figure 1.11: The presumed mechanism whereby melatonin reduces the formation of the hydroxyl radical [7].

In contrast to other electron donors such as ascorbic acid, melatonin does not transfer hydrogen to other molecules, nor does it partially reduce oxygen to $O^{-}_{2}\cdot$ or ·OH. Additionally, melatonin does not excite oxygen to generate singlet oxygen. This is due to the great stability of the melatonin molecule, caused by the methyl group which shields the oxygen in the 5 position, while the acetyl group in the side chain enhances the ·OH scavenging capacity of the molecule [3]. This stability allows melatonin to emit and absorb photons, quench singlet oxygen and neutralise other excited molecules [8].

1.4.2. Effect of melatonin on DNA

Earlier Tan *et al* [183] had shown that livers of rats treated with the carcinogen safrole, which damages nuclear DNA by the production of ROS, did not have the same quantity of DNA adduct formation when co-treated with melatonin. It was also found that animals

treated with safrole at night, when physiological levels of melatonin are elevated, had less DNA damage than rats given safrole during the day, when melatonin levels are reduced. This led the authors to conclude that melatonin, even at physiological doses, was able to provide significant antioxidant protection. Ikeda *et al* [184] later demonstrated, through morphological studies, that nuclear degeneration and DNA fragmentation are associated with glutamate toxicity in rat cortical neurons.

As glutamate is known to cause neurodegeneration though the production of ROS, melatonin could offer a neuroprotective role against glutamate induced DNA damage.

1.4.3. Effect of melatonin on lipid peroxidation

Melatonin is the most potent and effective endogenous ·OH radical scavenger yet discovered. This is due to melatonin's lipophilic nature which allows melatonin to scavenge free radicals inside membrane lipid bi-layers [185]. The indolamine can also penetrate most cell compartments and provide onsite protection to other macromolecules [8].

Many studies have been carried out that have demonstrated melatonin to have an antioxidative action within a lipid-rich medium. Melchiorri *et al* [186] demonstrated that melatonin was able to protect against the paraquat-induced increase in the production of malondialdehyde (MDA) and 4-hydroalkenals (4-HDA). These are degraded lipid products from cell membranes, and are taken to be reliable indicators of lipid peroxidation [140]. The same group [187] further showed that paraquat's 24 hour LD_{50} was increased from 79 mg / kg in control rats to 251 mg / kg in rats, co-treated with melatonin.

Sewerynek *et al* [188] used rat brain homogenate to investigate melatonin protection against lipid peroxidation. This team found that melatonin was able to protect in a dose dependent manner against the production of MDA and 4-HDA, induced by H_2O_2.

Likewise, melatonin was able to protect in a dose dependent manner against carbon tetrachloride (CCl_4)-induced lipid peroxidation [189] in hepatic homogenate. Under a number of different experimental protocols [190,191], melatonin has been found to protect against KA neurotoxicity. Other studies have also found melatonin to protect against lipid peroxidation induced by cyanide [192], cysteine [193] and glutamate [194].

1.4.4. Effect of Melatonin on Enzymes

Melatonin has been shown to affect a number of enzymes related to the antioxidative defence system. This action is completely different to the antioxidant nature of melatonin neuroprotection, as in this case melatonin actually affects the activity of enzymes.

1.4.4.1. Glutathione Peroxidase

In 1995, Barlow-Walden *et al* [195] found that exogenously administered melatonin stimulated the activity of this enzyme in the brain. Glutathione peroxidase is involved in the metabolism of H_2O_2 and is therefore an essential antioxidant enzyme. The action of melatonin may have been via nuclear melatonin receptors [196] showing that melatonin even acts at the nuclear level.

1.4.4.2. Glutathione Reductase

Melatonin can also stimulate glutathione production by stimulating hepatic and cerebral glucose-6-phosphate dehydrogenase. This causes an increase in NADPH levels, which promotes the production of glutathione by glutathione reductase [197]. Glutathione is a powerful antioxidant and a cofactor for glutathione peroxidase.

1.4.4.3. Nitric Oxide Synthetase

Benítez-King *et al* [198] found that melatonin binds with a high affinity to calmodulin. This suggests that the hormone is able to modulate cell activity by intracellularly binding to calmodulin at physiological concentrations.

Pozo *et al* [6] demonstrated that physiological concentrations of melatonin were able to inhibite NOS activity. The inhibition was dose dependent and occurred because of melatonin binding to calmodulin. This prevented calmodulin binding to the NOS peptide, thus inhibiting the initiation of NOS activity. These results suggested a new mechanism of action for melatonin, where melatonin is able to stop enzymatic activity by inhibiting receptor substrate interactions.

1.4.5. Melatonin, Free Radicals and Aging

The free radical theory of aging [199] was first introduced in 1956 by Harman. He explained that the generation of free radicals and their associated reactions was the common cause of aging and death in all animals. This theory is supported by work done with animals subjected to a calory restricted diet [200-201]. These diets slow the aging process because there is reduced production of ROS [137] in these animals. It therefore seems certain that the production of free radicals is one of the causative processes in natural aging.

Organisms have a number of antioxidative defence mechanisms which can be use to neutralise the effect of ROS. Under ideal circumstances, ROS production is counterbalanced by the defence system so that cellular damage is kept to a minimum. However damage accumulates with age, as some damage is unavoidable. Also, the rate at which macromolecules within cells accumulate ROS, appears to increase in progressively older animals [7]. In a review of age-related alterations in antioxidant

defence by Matsuo [202] it was found that there was no great deterioration in the antioxidative system of older animals. Matsuo therefore theorised that there may be some, as yet, undiscovered component of the system, that does in fact deteriorate rapidly.

Melatonin concentrations decrease with age. The exogenous administration of melatonin substantially extends the life span of experimental animals [203]. The decline in melatonin production could thus result in the increase in aging experienced later in life.

1.5. GLUCOCORTICOIDS AND THE NEURON

1.5.1. Introduction

When organisms are subjected to circumstances that threaten, or are perceived to threaten their existence, they initiate a stress response. The secretion of glucocorticoids by the adrenal cortex is central to this response [204-206]. In order for the steroid hormones to be produced, an endocrine cascade first has to be initiated.

The cascade begins with the perception of stress in the brain [207]. Within seconds the hypothalamus secretes corticotropin releasing factor (CRF) along with a number of other substances that augment the action of this factor. These collectively stimulate the pituitary to release adrenocorticotropic hormone (ACTH) within 15 seconds [208]. This in turn stimulates the adrenal secretion of glucocorticoids within minutes.

The glucocorticoids divert energy to muscles, promote the breakdown of stored energy [209], increase blood pressure and suppress anabolic processes, such as growth, reproduction, and the immune and inflammatory responses [204]. When exposure to glucocorticoids is prolonged, steroid diabetes, hypertension, impotency and immunosuppression can occur [208,210]. Another undesirable consequence of glucocorticoid overexposure is neuronal damage [210,211].

CNS damage appears to be most pronounced in the hippocampus [212] where neuronal cell loss occurs. This is because the glucocorticoids contribute to neuronal degradation by exacerbating the effects of the EAA's [213,214]. Moghaddan *et al* [215] demonstrated that glucocorticoids cause glutamate accumulation in the hippocampus and, in part, in the prefrontal cortex. The same author, in another paper [216], demonstrated that exposure to physical or pharmacological stress increases the release of glutamate in the hippocampus and prefrontal cortex. These results together, demonstrate that glucocorticoids cause the increased release of glutamate into the

hippocampus. This increases extracellular glutamate concentrations, which can cause overstimulation of glutamate receptors in the neurons.

Neurons consume energy at a high rate and are especially vulnerable to depletion of energy supplies. Neurons have a limited ability to store glycogen, and are therefore heavily dependent on blood glucose as an energy source. Glucocorticoids also inhibit glucose uptake [217,218], so hampering ATP production. Neurons are consequently less able to remove intracellular Ca^{2+}, and so many neurotoxic processes are initiated [219].

1.5.2. Corticosterone and Melatonin

Chronic stress increases the adrenal secretion of corticosteroids, which inhibits the activity of monoamine oxidases to metabolise serotonin. Consequently, more serotonin is present to be converted to melatonin [20]. This leads to an increase in the production of melatonin [220] during times of stress [221]. Conversely pinealectomy enhances corticosterone production in female rats [222]. Long term stress leads to the sustained elevation of glucocorticoids, which down-regulates the control mechanisms of the Hypothalamic-Pituitary-Adrenal (HPA) axis [223], resulting in an inability to terminate the adrenal responses to stress. Chronic melatonin treatment has been shown [220] to affect glucocorticoid receptors in the brain and pituitary. Melatonin decreased the affinity of the receptors for their natural ligand, but this effect was only observed in the presence of corticosterone. Melatonin could thus protect neurons, by decreasing their receptor affinity for their natural ligand during times of enhanced corticosterone concentrations. Melatonin may also prevent hippocampal receptor loss that usually occurs following long term exposure to elevated circulating levels of corticosterone.

1.6. NEUROLOGICAL DISORDERS

1.6.1. Aging

From the day an animal is born it begins to age. Aging is therefore a natural phenomenon, and although the process cannot be stopped there are many physiological factors that can either advance or slow the aging process.

There is much evidence to demonstrate that corticosterone secretion accentuates the aging process. Sapolsky *et al* [212] showed animals that received 3 months of chronic corticosterone treatment, showed many characteristics of aged rats. Adrenalectomy at mid-age has been shown to prevent loss of neurons in the hippocampus, while prolonged exposure to elevated glutamate titres exacerbates the loss [212,224].

There are two ideas as to how aging and stress are related [225]. 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. For instance, aged rats compared to young controls exhibit less efficient HPA axis activity in response to stress [226,227], and are they are unable to terminate the secretion of glucocorticoids at the end of stress [228]. The second idea is that stress can accelerate the aging process by impairing hippocampal functions like learning and memory [229].

The increases in corticosterone concentration need not be large, with Stein-Behrens *et al* [230] demonstrating that even slightly elevated corticosterone levels (typical of those in aged rats) causes greater glutamate accumulation in the hippocampus. Lowy *et al* [205] showed that stress significantly increased extracellular glutamate levels in sham operated rats, while there was 70% less extracellular glutamate in adrenalectomised rats that were exposed to the same stress. A 70% increase in extracellular glutamate is not as great as the glutamate increase that occurs following ischemia. A single acute stress

is therefore not going to produce morphological changes. Watanabe *et al* [231] showed that 3 weeks of stress were needed to cause noticeable damage, demonstrating that there is however a slow accumulation of damage.

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's produced are rapidly removed from the cell by the antioxidant defence 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 [137]. These damages are repaired by enzymes that excise the lesion, however there is a steady increase in the number of lesions as the animal ages. Viani *et al* [232] 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. Other ·OH mediated oxidative damage also accumulates exponentially with age [8].

The reason why the aged brain is more susceptible to ROS damage, is that many of the neuroprotective pathways are no longer as effective as they are in younger animals. Melatonin production is also affected with age [233]. In humans, the baby pineal produces very little melatonin for the first few months, but soon a rhythm is established with maximum melatonin production at night. The amplitude of the melatonin peak is at its greatest in the years before puberty. After adulthood is reached, the peak melatonin level reached at night slowly starts to decrease, so that by old age there is very little variation between night and day time melatonin levels (see Figure 1.12). The basal day-time melatonin levels are not affected by age, but rather there is a decrease in the nocturnal peak, so reducing the amount of melatonin produced in a 24 hour period.

The age related decrease in the ability of the pineal gland to produce melatonin is probably the result of two factors. Firstly, the β-adrenergic receptors on the

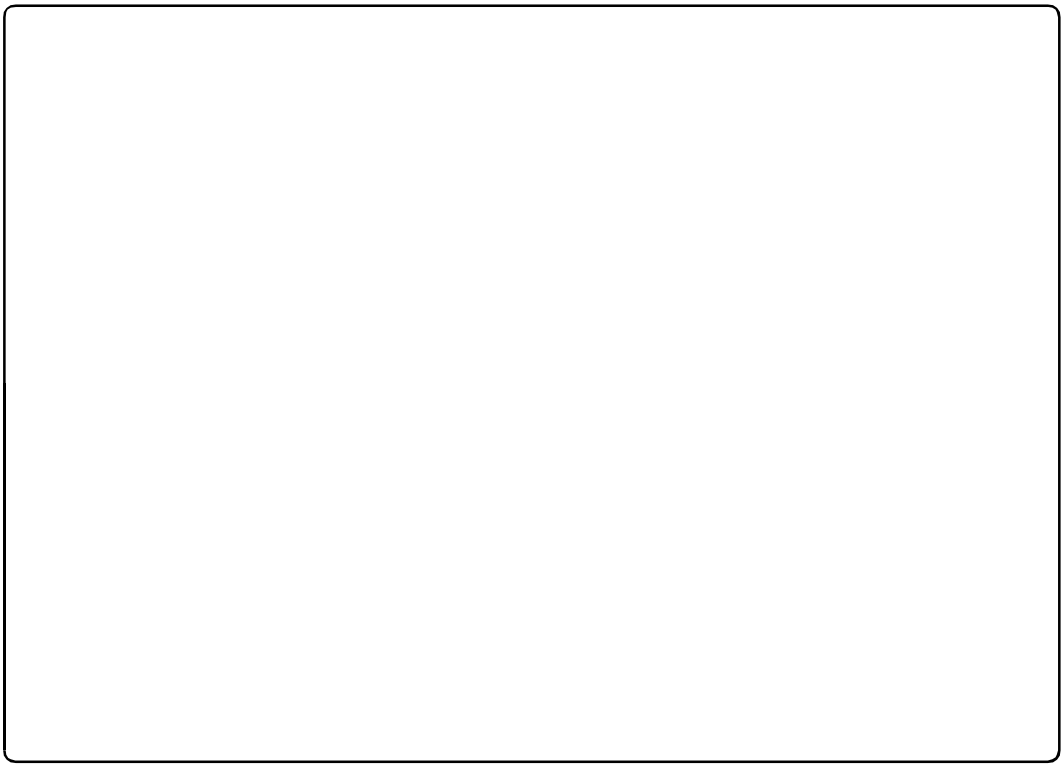


Figure 1.12: Reduction of maximal night time melatonin levels in humans during aging [7].

pinealocyte membranes, which are responsible for mediating the night time increase in melatonin production, are reduced in number in advanced age [10], resulting in a weaker signal being sent to the pinealocyte to produce melatonin. The second reason, is that the neuronal message to the pineal gland in old animals may be weakened by the gradual deterioration in the cells of the hypothalamic suprachiasmatic nuclei, due to the action of EAA's on the neurons at this level [7].

The ability of the pineal gland to produce and release melatonin also depends on the individual. The amplitude of the night time peak in melatonin production appears to be genetically controlled [7]. Those individuals with an inherently low circadian production of melatonin could be more vulnerable to premature aging and other age related diseases [234], than individuals that produce more melatonin.

1.6.2. Parkinson's Disease

Parkinson's disease is a chronic, progressive disorder of late life, which is characterised by rigidity, unintentional tremor and bradykinesia [62]. In Parkinson's disease there is selective degradation of neuromelanin-containing neurons, especially the nigral dopaminergic neurons [235]. Glutamate appears to play a pivotal role in the pathophysiology of Parkinson's disease, as abnormal patterns of glutamatergic neurotransmission are important symptoms of Parkinson's disease [62].

The metabolism of dopamine by monoamine oxidase can yield 6-hydroxydopamine, which is known to yield ROS [236]. Although proof that ROS stress actively causes the loss of monoaminergic neurons in Parkinson's disease is lacking, there is considerable evidence in animals and humans to support the concept [139]. Post mortem studies have revealed that the substantia nigra of patients with Parkinson's disease shows signs of oxidative stress [237], as well as reduced glutathione peroxidase activity and glutathione concentrations [238].

Total iron is increased and ferritin is reduced in the substantia nigra pars compacta in patients with Parkinson's disease. This combination suggests that the transition metal is in a low molecular weight form, capable of catalysing non-enzymatic oxidative reactions, especially the conversion of H_2O_2 to ·OH [239]. Neuromelanin, a product of dopamine oxidation, can serve as a reservoir for iron, promoting the generation of oxyradicals.

The drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is often used to model Parkinson's disease in animals. MPTP [240] destroys dopaminergic neurons by the production of free radicals. ·OH rather than O^{-}_{2}· [241] appears to be the more neurotoxic of the two molecules. Acuña-Castroviejo *et al* [242] demonstrated that melatonin was able to protect against many of the adverse effects of MPTP administration. This suggests that melatonin may have a role to play in the treatment of Parkinson's disease.

1.6.3. Alzheimer's Disease

Alzheimer's disease involves selective and progressive degeneration of lower motor neurons in the spinal cord, and the upper motor neurons in the cerebral cortex. Symptoms normally begin to be apparent in mid life [5]. The disease is characterised by an abundance of senile plaques and neurofibrillary tangles in certain areas of the brain [85]. The evidence implicating EAA's in Alzheimer's disease is circumstantial rather than direct, but non the less supports the concept that excitotoxicity plays a part in the pathogenesis of the disease. EAA metabolism is disturbed in patients with Alzheimer's disease [93], with glutamate concentrations inappropriately high in extracellular compartments [74], suggesting a breakdown in normal uptake mechanisms [115,243]. Although excitotoxicity may not be the initiating event in the disease, it may interact with other factors to fuel continued neurotoxicity and disease progression.

Amyloid β-protein which is derived from the Amyloid Precursor Protein (APP) accumulates in plaques in Alzheimer's disease patients [214,244-246]. Amyloid β-protein has been implicated in neurotoxicity, where it is thought to induce the production of free radicals [213,247,248] and disrupt calcium homeostasis [123]. Mark *et al* [249] showed that amyloid β-peptides induce free radical production, which results in impairment of ion channels and calcium influx [213]. Bioenergetic defects, or amyloid β-protein deposition, could be the final common pathway in neuronal death for this disease. Kirazov *et al* [250] has shown that glutamate induces the secretion of APP from cortical slices in a concentration dependent, but biphasic manner, with the highest release at 50μM L-glutamate and smaller effects at higher glutamate concentrations. The APP molecule could thus be cleaved to produce even more amyloid β-protein.

Melatonin appears as if it could be a neuroprotective agent against Alzheimer's disease. Papolla *et al* [245] reported that melatonin prevents death of neuroblastoma cells exposed to amyloid β-protein and amyloid peptide fragment [251]. It has also been shown that old people and patients with Alzheimer's Disease [34] appear to suffer from a

decrease in the amplitude of the melatonin acrophase [252]. Experiments with rodents have demonstrated that pharmacological treatment with melatonin can increase life span, prevent premature aging and delay the onset of neurological disease [8].

1.6.4. Huntington's Disease

Huntington's disease is an autosomal dominant neurodegenerative disorder that is caused by a genetic defect localised on chromosome 4 [139,253,254] with an onset at about 40 years of age [255].

The disease is characterised by disturbances in movement, psychiatric symptoms and a progressive dementia that leads to severe debilitation and usually death within 15-20 years [139]. The disease also leads to destruction of the brain [256] with the autopsy brain weight of Huntington's disease patients normally about 20% less than average brain weight [254].

The excitotoxic model has been proposed as a possible cause of Huntington's disease. Although there are other factors playing a part in the neurodegeneration that takes place in this disease, it appears that excitotoxic injury plays an important part in a more complicated array of neuropathalogical events, but none the less an important function in the disease process [139]. This is evident by the work of Beal *et al* [105] who, showed that the focal injection of QA in the rat striatum very closely reproduces human Huntington's Disease post-mortem findings, with a relative increase in somatistatin and neuropeptide Y content [93].

1.6.5. Cerebral Ischemia

Many different situations can give rise to ischemia, cardiac arrest, thrombotic stroke and asphyxia. Although all these conditions involve very different pathophysiologies, they are all thought to contribute to the neural degradation that occurs following cerebral ischemia [93].

The ischemic brain damage that occurs can take on several distinct morphological patterns, depending on the particular conditions of the ischemia. Cerebral infarction, which is typically associated with prolonged, focal brain ischemia, is characterised by irreversible damage to neurons, glia, and other supporting cell types in the brain. In contrast, transient global ischemia, such as that accompanying cardiac arrest and resuscitation, principally injures specific populations of neurons known to be highly vulnerable to ischemia [257]. The pyramidal cells in the CA1 zone of the hippocampus, small and medium sized neurons in the striatum, non-cortical neurons in layers 3, 5 and 6, and the cerebellar Punkinje cells are among those neurons most susceptible to ischemic injury [204].

Within three minutes of disruption to cerebral circulation, the extracellular K^+ concentration around neurons rises from ±3 mM to as high as 80 mM [258]. At the same time, astrocytes and neurons can become swollen. The swelling is reversible if the period of ischemia is brief. The reversibility depends on the CNS region and the age of the animal. If the period of ischemia lasts longer than three minutes, necrosis of the neurons may occur. However, neurons that are restored to normal function after a period of ischemia, also succumb to delayed neuronal degradation. This only occurs a few hours [93] to several days [259] after the insult.

Following transient global ischemia, glucocorticoid levels rise. Therefore what is viewed as “expected” neuronal damage in response to focal or global ischemia, may in fact, represent ischemic damage worsened by the acute hypersecretion of glucocorticoids [204]. Reducing glucocorticoid release following ischemia could therefore offer therapeutic value.

The extracellular glutamate concentration is known to rise greatly during ischemia [75,257,260] or anoxia. This is because ATP levels fall [261], and based on thermodynamic grounds, the glutamate uptake carrier is expected to operate backwards, releasing glutamate into the extracellular space [262,263]. Phillis *et al* [264] found that

while ischemia causes an increase in glutamate release into the extracellular space during ischemia, glutamate levels tend to undergo a further, unexpected rise during the initial 30 - 40 minutes reperfusion. This could have resulted from the formation of toxic free radicals, including ·OH. Blockade of NMDA receptors by MK-801, following fluid percussion injury, attenuated some of the neuropathological events that normally follow this injury [265], demonstrating that glutamate must be involved in neurodegeneration. Following reperfusion there is also a burst of ROS production as xanthine oxidase metabolises hypoxanthine that has accumulated during the ischemia [143].

Melatonin has been found to protect against ischemia induced neurotoxicity. Cho *et al* [266] have demonstrated that melatonin protects CA1 hippocampal neurons when administered during, or at least 2 hours following transient forebrain ischemia in rats. Greater neurodegeneration has also been shown [267] to occur in pinealectomised rats following middle artery occlusion and glutamate receptor mediated, epilepsy-like seizures.

1.7. OBJECTIVES

The objectives of this study were firstly to determine whether melatonin could act as a neuroprotective agent under a number of neuropathological conditions, and secondly to attempt to elucidate the mechanism of neuroprotection, should it be occurring.

The potential neuroprotective properties of melatonin were investigated under the following circumstances:

a. Under conditions of stress, when glucocorticoid concentrations increase,

b. During excitatory amino acid insult, under *in vitro* conditions,

c. And against excitatory amino acid insult, under *in vivo* conditions.

Studies involving the EAA's were limited to four different glutamate receptor agonists. Glutamate was used as it is the "mixed-agonist" that acts on all glutamatergic receptors. NMDA was used as it is the main agonist of the NMDA class of receptor, while QA (which also acts at the NMDA receptor) was chosen because it is an endogenous compound that is extremely neurotoxic, and is thought to play a role in the pathology of Huntington's disease. The KA and AMPA receptors, together with the NMDA receptor make up the three classes of ionic glutamate receptors. The KA and AMPA receptors have similar structures and modes of action [268]. KA was therefore used as the agonist of choice for investigations on these classes of receptors.

It was hoped that following this investigation, the potential neuroprotective properties of melatonin would be better understood, and that knowledge gained could ultimately be used by future researchers in the treatment of a number of neurodegenerative diseases.

CHAPTER 2

EFFECT OF CORTICOSTERONE AND MELATONIN ON GLUTAMATE RECEPTORS

2.1. INTRODUCTION

Exposure to acute or chronic stress may be associated with neurological and physiological disorders [269,270]. These could result from the neurotoxic effects of stress on hippocampal neurons. Chronic exposure to stress has been shown to cause hippocampal neuronal loss [271-273], as well as atrophy of dendrites in hippocampal CA3 neurons [231].

Among the many neurochemical changes that occur as a result of stress, the secretion of adrenal steroids [274], and especially corticosterone in rats [275], is thought to play an important role in stress induced neurotoxicity. Glucocorticoids cause glutamate accumulation in the synaptic cleft of the CNS [215], which causes continuous stimulation of the postsynaptic glutamate receptors. This allows excessive movements of Ca^{2+}, Na^{+} and K^{+} into the neurons, which can lead to neuronal death.

In this experiment the effect of melatonin on glutamate receptors was investigated, to determine whether melatonin could give any protection against corticosterone-induced neurotoxicity.

2.2. EFFECT OF CHRONIC CORTICOSTERONE TREATMENT AND MELATONIN ON ^{3}H-GLUTAMATE RECEPTOR BINDING

2.2.1. Introduction

Corticosterone is the main adrenal steroid secreted during stress in rats. Corticosterone increases glutamate release [216], with the result that higher than normal concentrations of the EAA's exist in the synaptic cleft [276]. This can cause overstimulation of the receptor on the post-synaptic neuron, leading to excessive Ca^{2+} influx and neurodegeneration. High circulating levels of corticosterone therefore have the potential to cause severe damage to neurons.

Corticosterone does not cause neurodegeneration directly, but it is known to affect glutamate re-uptake and increase glutamate receptor numbers on neurons [22]. Increasing receptor numbers has the potential to upset the homeostasis of a neuron. This is because an increase in receptor number, results in a greater number of potential binding sites for synaptic glutamate to bind. With more receptors stimulated, there is a greater inflow of ions into the neuron, and the ionic potential is greatly affected. The neuron would have to work harder to extrude the ions that flowed into the neuron. As mentioned earlier, corticosterone is known to increase synaptic glutamate concentrations, and so an increase in receptor numbers would be doubly problematic to the neuron. Firstly, there would be more glutamate to bind to receptors, so a greater stimulus would be received by the postsynaptic neuron. Secondly, there would be more receptors available for glutamate to bind to. As each receptor can allow ions to pass into the cell, more receptors could result in more ions passing into the neuron. This could cause Ca^{2+} levels to rise markedly, which would initiate a number of neurotoxic processes.

Chronic corticosterone treatment should mimic the effects of stress on rat neurons, and so the following experiment was carried out to determine the effect of corticosterone and melatonin treatment on synaptic glutamate receptors.

2.2.2. Materials and Methods

2.2.2.1. Chemicals and Reagents

Corticosterone, melatonin and bovine serum albumin (fraction V powder) (BSA) were purchased from the Sigma Chemical Company (USA). L-[G-^{3}H]-glutamate (1 mCi / ml) was purchased from Amersham International plc (England). Emulsifier Scintillator Plus™ scintillation cocktail and scintillation vials were obtained from the Packard Company (USA). Ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)-aminomethane (Tris) and Triton X-100 were obtained from Unilab (South Africa). All other chemicals were of the highest quality available and were purchased from commercial distributors.

2.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Institute for Medial Research (Johannesburg, South Africa) were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The animal-house temperature was maintained at a constant 20^{o}C to 24^{o}C, while an extractor fan ensured the constant removal of stale air. The rats were housed four per cage with food and water provided *ad libitum*.

2.2.2.3. Corticosterone and Melatonin Treatment Schedule

Rats were divided into 4 groups and treated as shown in Table 2.1. Corticosterone was administered to the animals for 7 days at 12 pm.

Table 2.1: Administration of melatonin and corticosterone to rats.

Group	Melatonin	Corticosterone
Control	0	0
Melatonin	1 mg / kg	0
Corticosterone	0	5 mg / kg
Corticosterone and Melatonin	1 mg / kg	5 mg / kg

The melatonin and corticosterone were suspended in sweet oil and administered separately, so that each animal effectively received two subcutaneous injections each day. Control animals received an equivalent volume of vehicle in each case.

2.2.2.4. Brain Removal

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

2.2.2.5. Preparation of Synaptic Membranes

Each brain was homogenised in 40 volumes of 5mM Tris acetate buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 50 000 x g at 4^{o}C for 20 minutes. The pellets were resuspended and washed a further 3 times with 40 volumes of 50 mM Tris acetate buffer (pH 7.4). The pellets were then resuspended in 20 ml of 0.32 M sucrose and rapidly frozen in liquid nitrogen and stored at -70^{o}C until use.

2.2.2.6. Protein Assay

Protein concentrations were determined using a modified method of Lowry *et al* [277].

The absorbance values obtained were converted to mg / ml from a standard curve (Appendix 1) generated from BSA.

2.2.2.7. Glutamate Binding Assay Procedure

Binding studies were performed using a method similar to that described by Yoneda *et al* [80]. 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^{o}C for 20 minutes. The pellets were resuspended and treated with 0.08% Triton X-100 [278] for 10 minutes. This was followed by centrifugation at 50 000 x g at 4^{o}C for 20 minutes. The pellets were resuspended in 50mM Tris acetate buffer. Protein concentration was determined as in 2.2.2.6. The samples were diluted to a final working protein concentration of 1mg / ml using 50 mM Tris-acetate buffer (pH 7.2). Incubation mixtures consisted of 250 µl membrane preparation, 170 µl of 50 mM Tris-acetate buffer (pH 7.4), 50 µl glutamate ranging from 0 - 400 nM final concentration and 30 µl of ^{3}H-glutamate to give a final concentration of 10 nM. The tubes were incubated for 40 minutes at 24^{o}C and the reaction was terminated by the addition of 4 ml of ice cold Tris-acetate buffer (pH 7.2). This was followed immediately by rapid filtration through Whatman GF/C filters in a 12 place Millipore sampling manifold under negative pressure. The filters were washed a further 3 times with 4 ml of ice cold Tris-acetate buffer, before being dried by negative pressure and placed in scintillation vials containing 3 ml of scintillation cocktail. After 24 hours, the radioactivity in the vials was measured using a Beckman (Model LS 2800) Liquid Scintillation Counter. All results were expressed as fmol ^{3}H-glutamate bound per milligram protein.

2.2.2.8. 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 $P<0.05$.

2.2.3. Results

The specific binding expressed as fmoles ^{3}H-glutamate bound / mg protein, was calculated. Saturation curves and Scatchard plots [279] were derived from the data for each of the 4 groups. The Kd and Bmax values for the plots (Figures 2.1 - 2.8) are shown in Table 2.2.

Table 2.2: Kd and Bmax values derived from the data. (n=4)
([a] P<0.05 vs Control; [b] P<0.001 vs Control)

Test	Bmax (pmoles / mg protein)	Kd (nM)
Control	161 ±2.37	327
Melatonin	153 ±2.83[a]	323
Corticosterone	235 ±3.02[b]	462
Corticosterone and Melatonin	216 ±1.43[b]	455

Corticosterone caused an up-regulation in the number of binding sites when compared to control animals, with Bmax values more than doubled. Melatonin reduced the Bmax values for both control and corticosterone treated animals.

Although melatonin did not appear to be able to bring receptor numbers down to the levels of control animals, it did cause a significant reduction in the number of receptors in those animals treated with corticosterone.

Chronic administration of corticosterone also caused an increase in the dissociation constant for glutamate receptors. Co-treatment with melatonin did not cause a significant change in the dissociation constant, for either control or corticosterone treated animals.

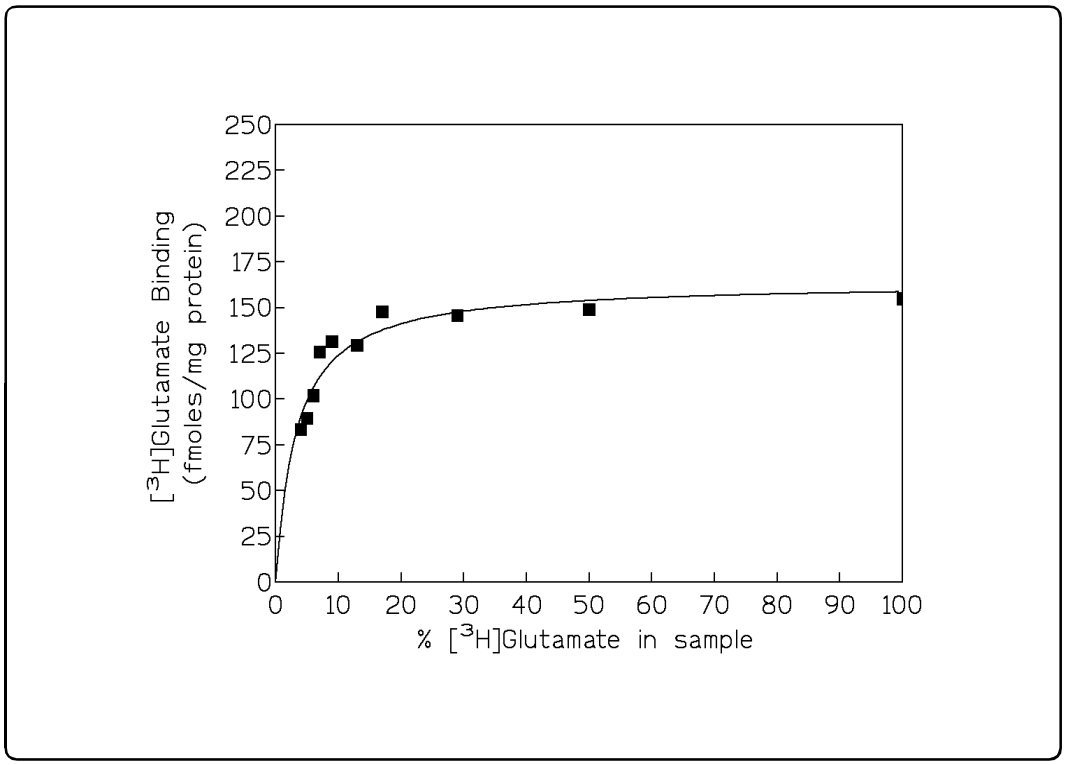


Figure 2.1: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from control rats. (n=4)

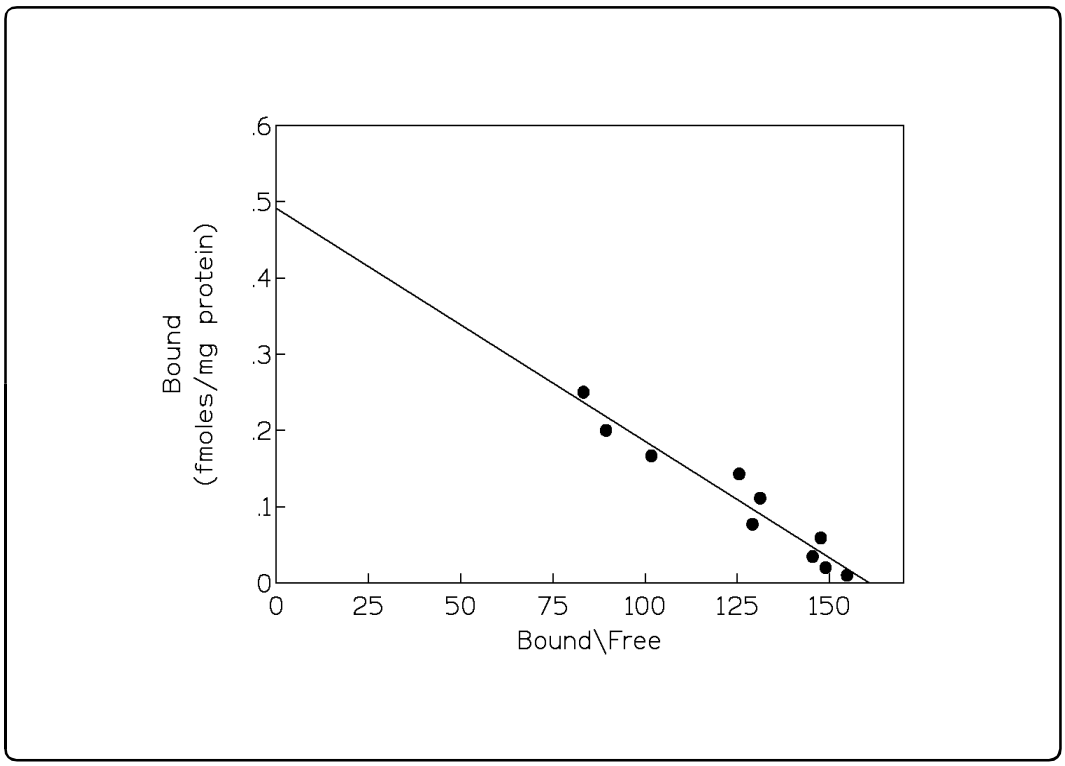


Figure 2.2: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from control rats.

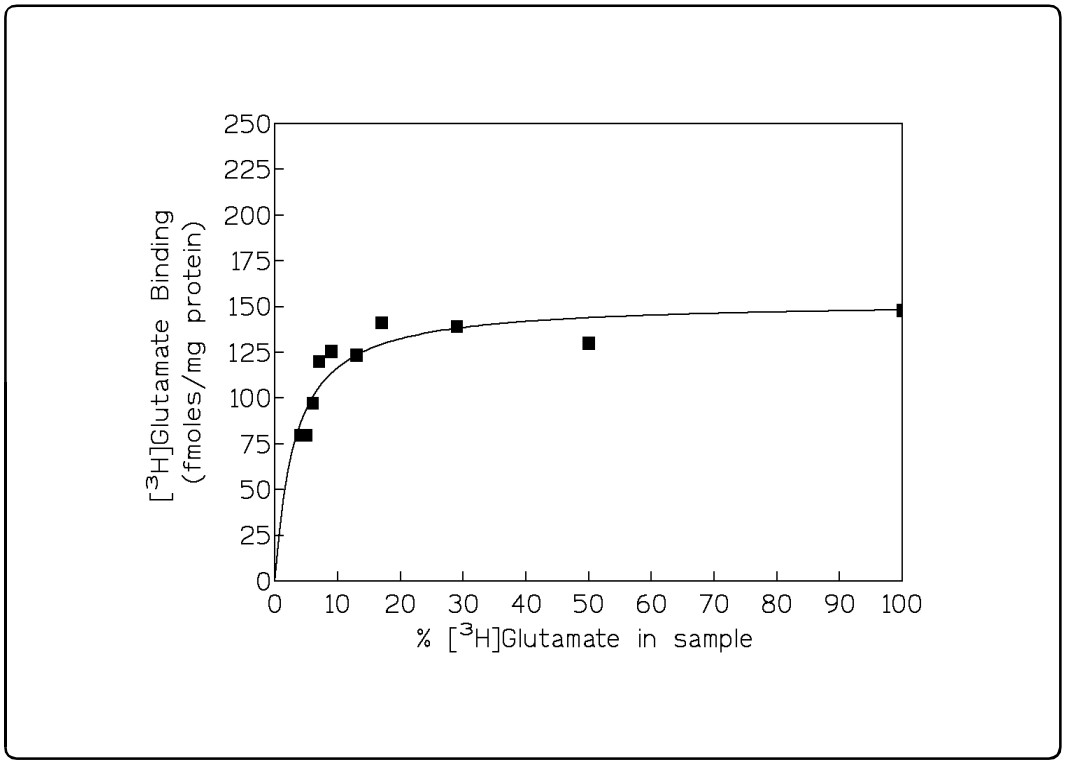


Figure 2.3: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from melatonin treated rats. (n=4)

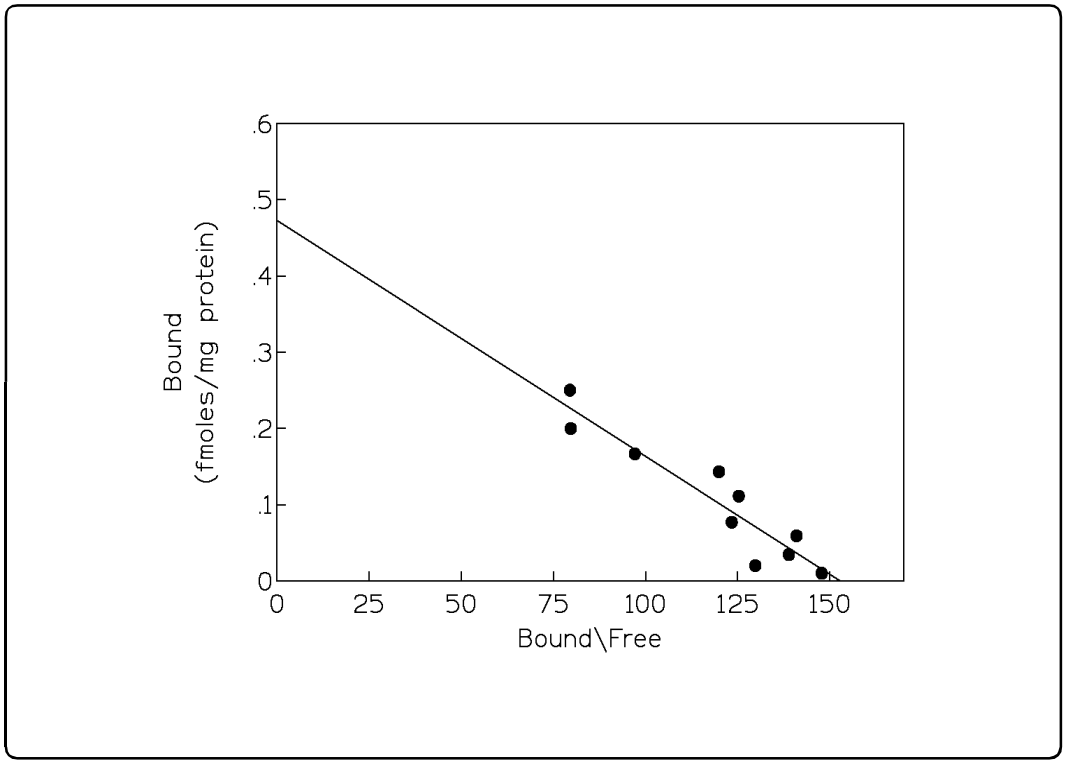


Figure 2.4: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from melatonin treated rats.

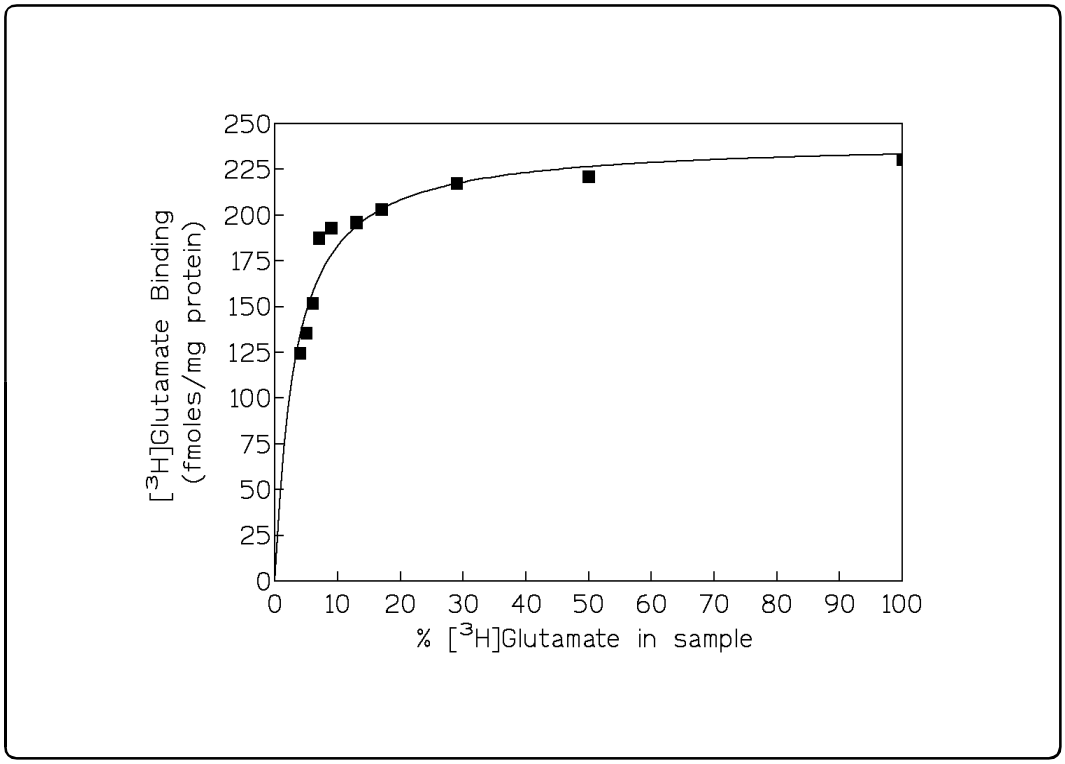


Figure 2.5: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from corticosterone treated rats. (n=4)

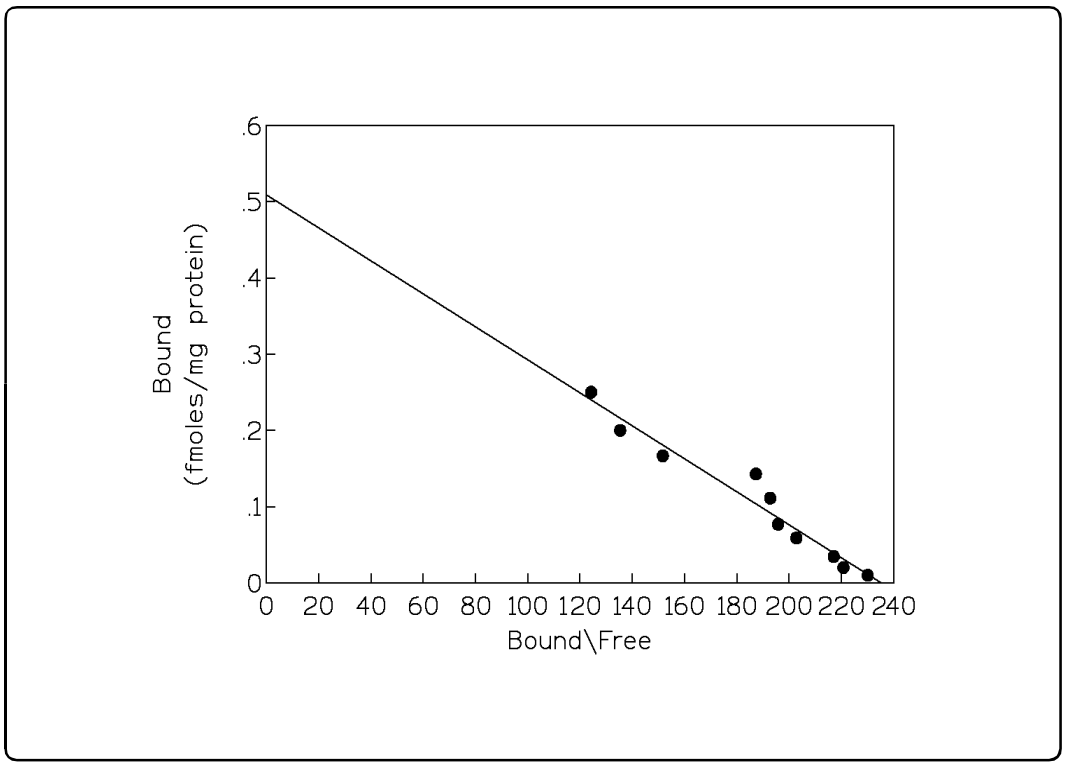


Figure 2.6: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from corticosterone treated rats.

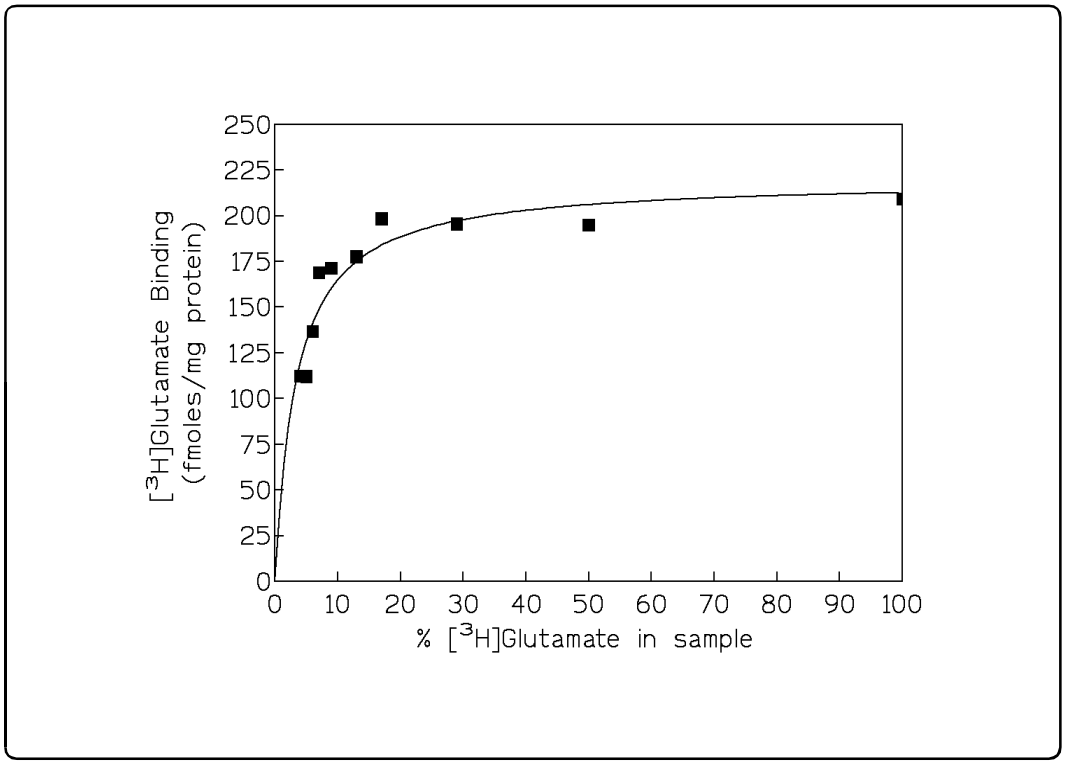


Figure 2.7: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from corticosterone and melatonin co-treated rats. (n=4)

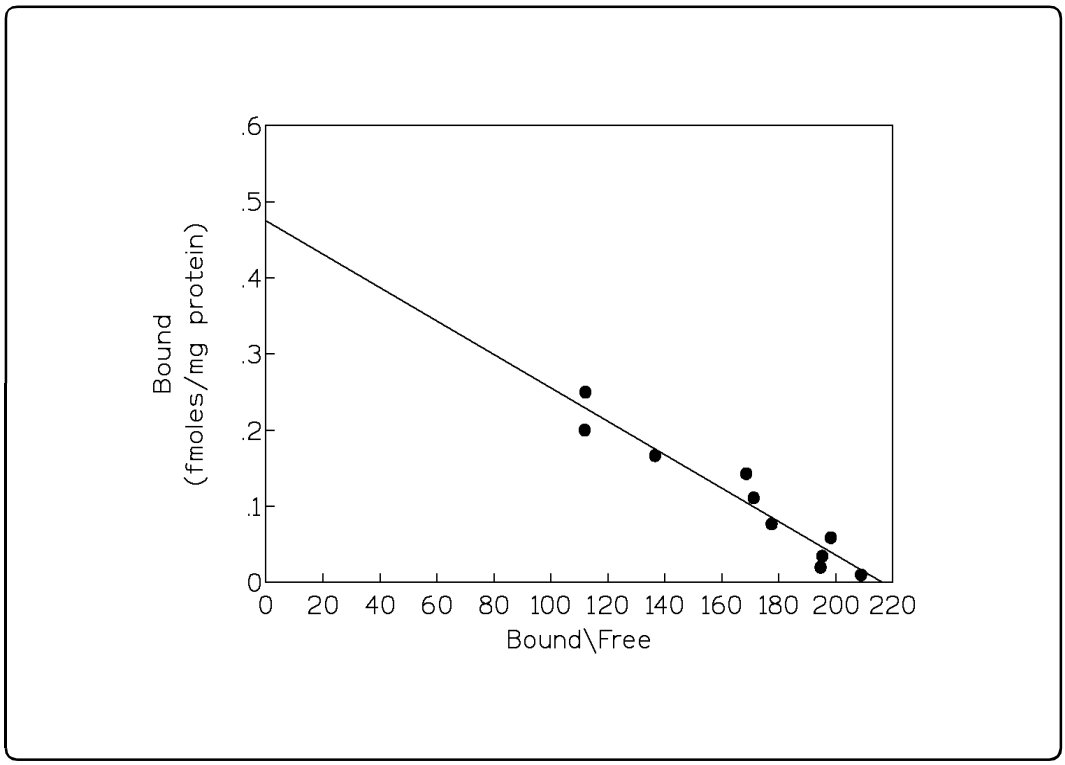


Figure 2.8: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from corticosterone and melatonin co-treated rats.

2.2.4. Discussion

These results demonstrate that melatonin is able to reduce the number of glutamate receptors (Bmax). This appeared to be a general occurrence, as melatonin had the same effect on both control and corticosterone treated animals. The effect of melatonin also appeared to be concerned exclusively with the reduction in receptor numbers, as there was no effect on binding affinity (Kd).

Chronic treatment of rats with corticosterone caused a marked increase in receptor numbers while reducing the affinity of the receptor for its substrate. Co-treatment with melatonin however, appeared to reduce receptor numbers, without affecting the binding affinity of the receptors for glutamate.

Corticosterone appears to cause an upregulation in the number of glutamate receptors on neurons. These receptors do not however show the same affinity for glutamate as do receptors from neurons not treated with corticosterone. Some of the negative effects of greater glutamate receptor numbers on the neurons may therefore be negated by the decrease in affinity of the receptor for its substrate.

Melatonin may be doubly effective at reducing the effects of increased glutamate receptor numbers. First melatonin reduces the number of glutamate receptors on the neurons, and secondly it does not change the affinity of the glutamate receptors for their agonist. Therefore, while reducing receptor numbers, melatonin does not enhance affinity for glutamate by the receptors.

The results imply that melatonin may be able to protect neurons from the increased risk of glutamate receptor overstimulation, by inhibiting the increase in glutamate receptor numbers brought about by chronic corticosterone treatment.

2.3. THE MEASUREMENT OF GLUTAMATE BINDING TO RAT CEREBRAL MEMBRANES FOLLOWING EXPOSURE TO PHYSICAL STRESS

2.3.1. Introduction

The previous experiment had measured the effect of chronic treatment of rats with exogenous sources of melatonin and corticosterone. The following experiment was conducted to investigate whether application of physical stresses to animals, in place of exogenous corticosterone treatment, would give similar results. Pharmacological doses of melatonin were used.

2.3.2. Materials and Methods

2.3.2.1 Chemicals and Reagents

All reagents used were as per experiment 2.2.2.1.

2.3.2.2. Experimental Procedure

Animals were divided into 3 groups of 5 animals each. The animals in the control group did not receive any stress. In addition they were not injected with sweet oil vehicle, as it was thought that the daily handling of the animals may induce a physical stress. The remaining two groups of animals were stressed as shown in Table 2.3. Of the two stressed groups, one group received a dose of 1 mg / kg melatonin immediately prior to the onset of the stress, while the other group received a similar volume of sweet oil vehicle. All stresses were started at 11 am. The temperature in the cold room was 4^{o}C. The loud music consisted of heavy metal music, with lots of high pitched sounds, played at a high volume from speakers directly above the cages. Physical distress was

observed in the animals while the music was being played. Different stresses were used so animals would not become conditioned to any one stress.

Table 2.3: Stresses applied to rats each day

Day	Stress	Duration
1	Cold Room	2 hours
2	Cold Room	2 hours
3	Loud Music	2 hours
4	20^{o}C Swim Test	Till Exhaustion
5	Cold Room + Loud Music	2 hours
6	Loud Music	2 hours

On day 7, the animals were killed and their brains rapidly removed as described in 2.2.2.4. Synaptic membranes were prepared and glutamate binding studies were carried out as described in 2.2.2.5 and 2.2.2.7 respectively.

2.3.2.3. Statistical Analysis

Results were analysed as explained in section 2.2.2.8.

2.3.3. Results

The specific binding expressed as fmoles ^{3}H-glutamate bound / mg protein was calculated. Saturation curves and Scatchard plots were derived for each of the groups. The equilibrium dissociation constant (Kd) and maximum number of binding sites (Bmax) values for the plots (Figures 2.9 - 2.14) are shown in Table 2.4.

Table 2.4: Bmax and Kd values derived from the data. (n=4)
([a] P<0.001 vs Control; [b] P<0.01 vs Control)

Group	Bmax (pmoles / mg protein)	Kd (nM)
Control	121 ±3.64	219
Stress - No Melatonin	171 ±3.85[a]	233
Stress and Melatonin	142 ±4.5[b]	190

The stress caused a significant increase (P<0.01) in the number of glutamate receptors (Bmax) on the synaptic membranes. Animals treated with the same stress, but injected with melatonin, had a lesser increase in the number of glutamate receptors.

The effect of physical stress on the affinity of the glutamate receptors (Kd) for their ligand varied for those treated with melatonin and those that were not treated. Those animals that did not receive melatonin had a lower affinity for the ligand than control animals, while animals that received melatonin, had a higher affinity.

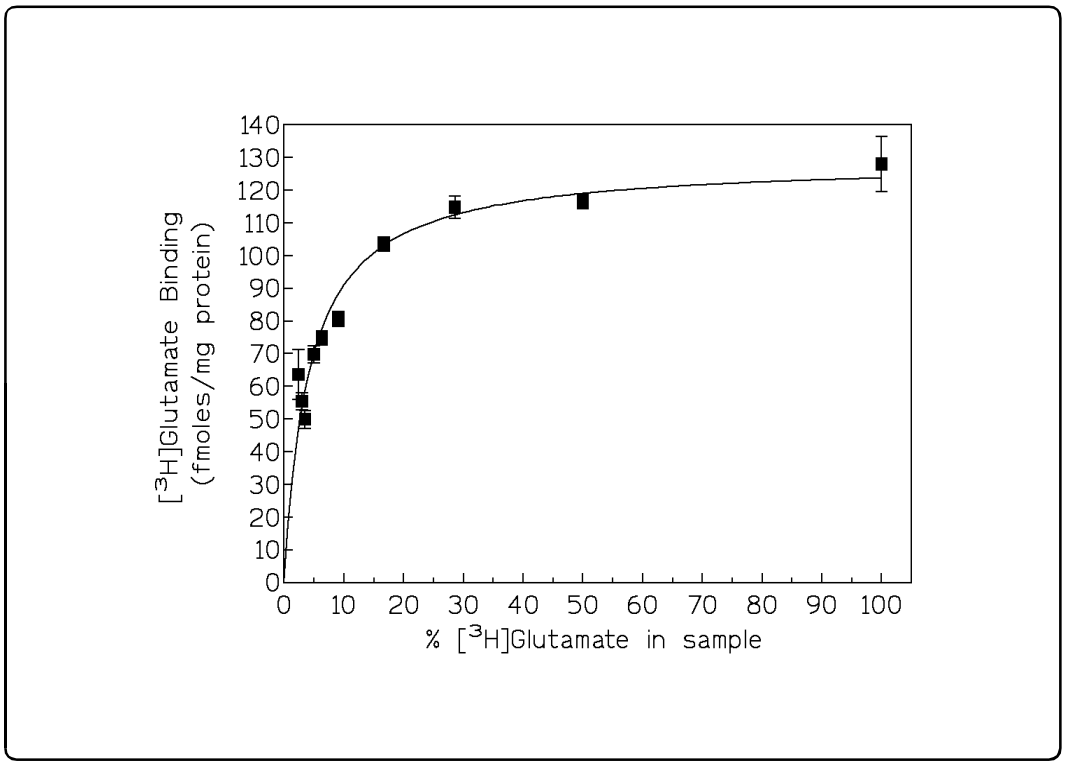


Figure 2.9: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from control, unstressed rats. (n=4)

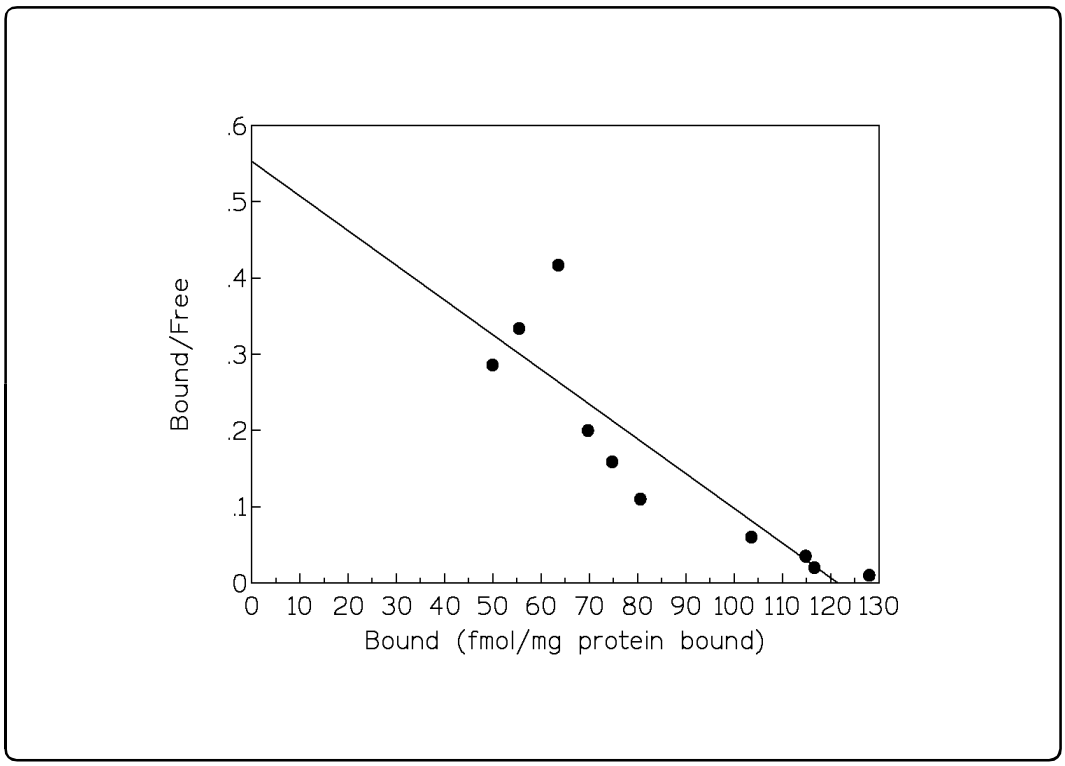


Figure 2.10: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from control, unstressed rats.

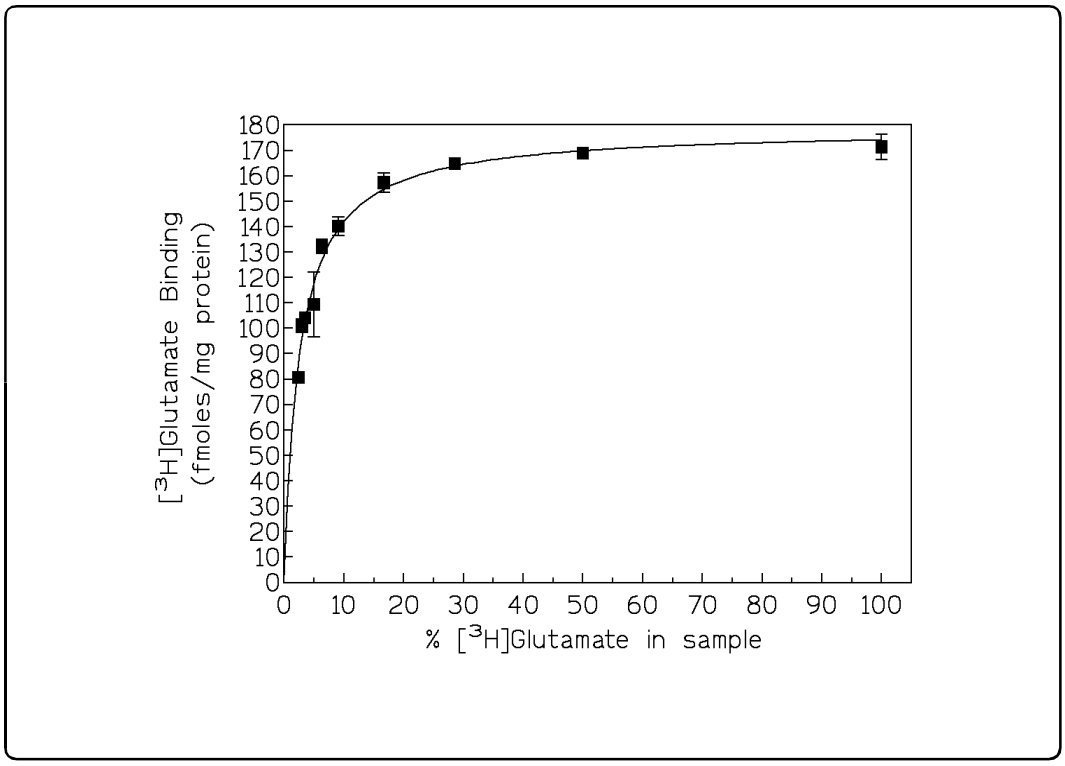


Figure 2.11: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from stressed, untreated with melatonin rats. (n=4)

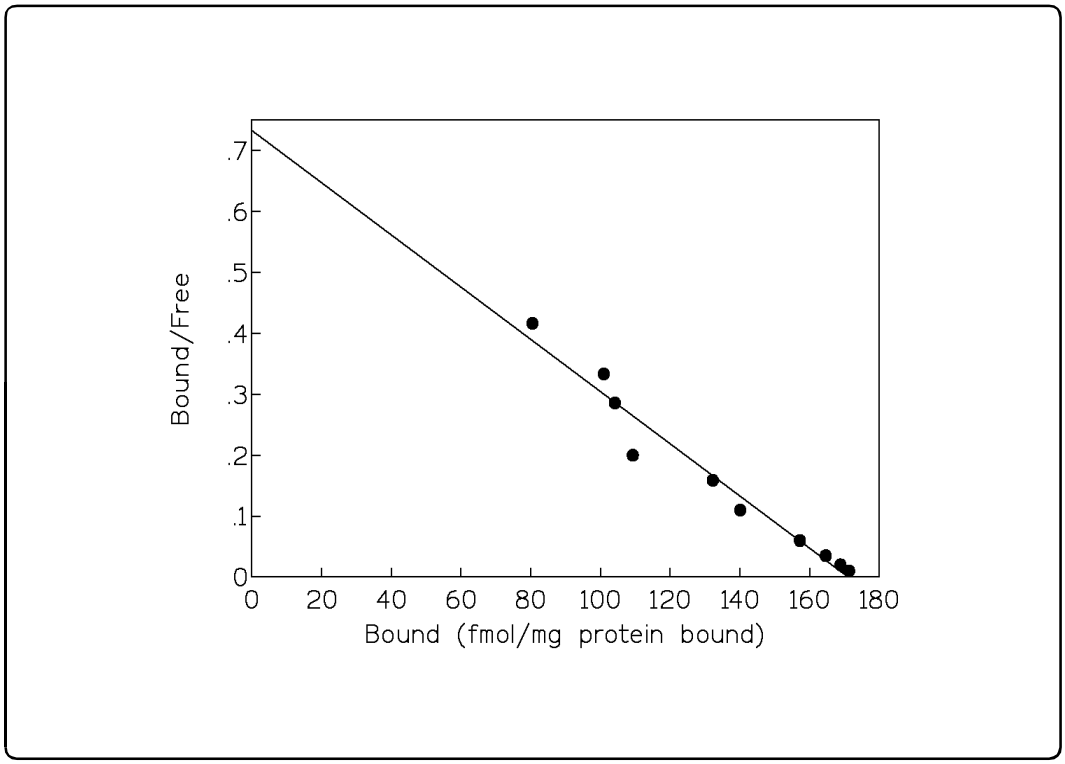


Figure 2.12: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from stressed, untreated with melatonin rats.

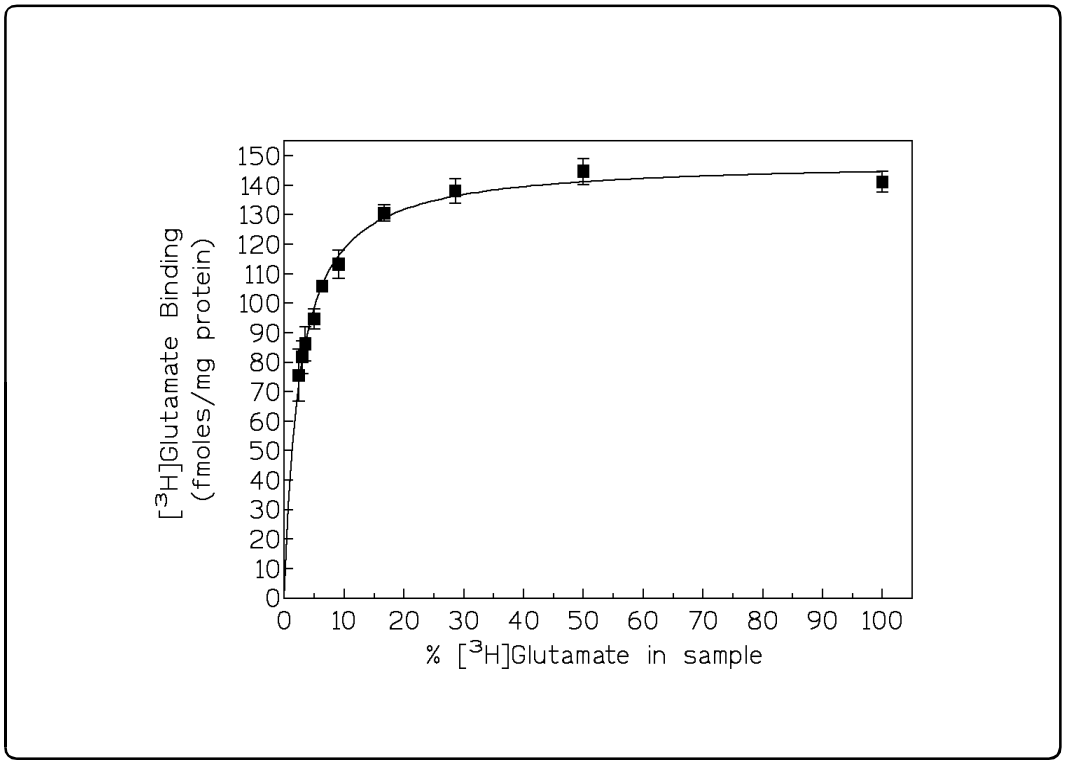


Figure 2.13: Saturation Curve of ^{3}H-Glutamate binding to synaptic membranes from stressed, and melatonin co-treated rats. (n=4)

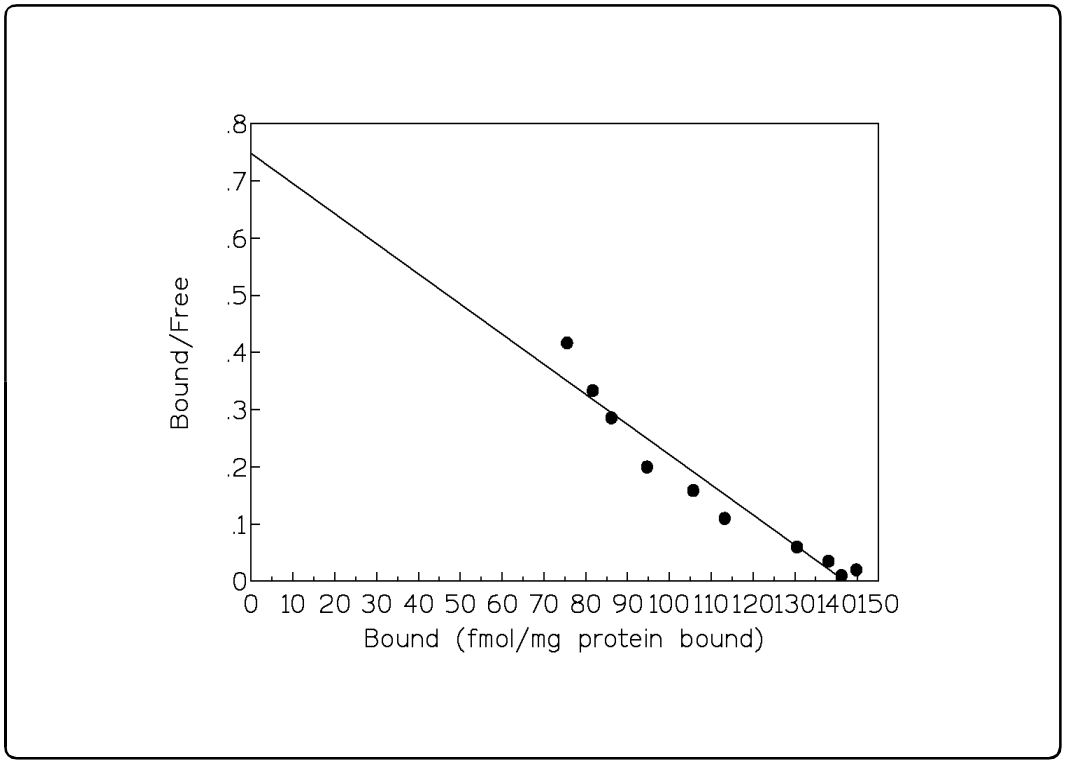


Figure 2.14: Scatchard Plot of ^{3}H-Glutamate binding to synaptic membranes from stressed, and melatonin co-treated rats.

2.3.4. Discussion

The results demonstrate that physical stress was able to cause much the same effects as the injection of corticosterone to the animal. This is shown by the fact that both techniques caused an increase in the number of glutamate receptors. As in the corticosterone treated animals, melatonin was able to lower the number of receptors in stressed animals, but not to control levels.

One difference between the animals injected with corticosterone, and those that were treated with the physical stresses, was that the glutamate receptors from the animals that had received melatonin in the stressed experiment, had a greater affinity for their agonist than either of the groups of animals that did not receive melatonin.

Previously it had been found that melatonin had no effect on glutamate receptor affinity. No explanation for this difference could be found, other than that an *in vivo* system was being used, and the physical stress would have affected more than just the production of corticosterone. One of the other hormones or steroids produced during stress may have interacted with melatonin to cause this effect.

2.4. Conclusions

It has been demonstrated that corticosterone causes an increase in the number of glutamate receptors. This can be brought on by the chronic treatment of animals with corticosterone, or by exposing the animals to physical stresses. The chronic treatment with corticosterone caused a greater increase in receptor numbers than the physical stress. The reason for this is that physical stress may not have raised circulating corticosterone levels to the same highs as the 5 mg / kg injections. The increased number of receptors also led to a decrease in binding affinity of glutamate receptors for their substrate.

Melatonin appears to cause a decrease in the number of receptors. When melatonin treated animals were compared to control, corticosterone treated, and stressed animals, there was a decrease in the Bmax values in all cases. This demonstrates that melatonin must have a general effect of decreasing neuronal glutamate receptor numbers. In the rats treated with corticosterone, melatonin did not affect binding affinity. However in the animals treated with physical stresses, binding affinity was increased, when compared to both control and stressed animals that had not been treated with melatonin. The results from the corticosterone treated animals strengthen the argument that melatonin causes a direct reduction in receptor numbers without affecting the binding affinity of the receptor. The physical stresses that the rats were subjected to in experiment 2.3., would have increased corticosterone levels as well as the concentration of other adrenal steroids. These steroids, together with other hormones that are secreted during stress, may have increased the affinity of the glutamate receptors for their ligands, in melatonin treated, stressed animals.

Corticosterone is a glucocorticoid, and the glucocorticoids like other steroid hormones react with proteins in the cytoplasm of sensitive cells [280] to form a steroid-receptor complex. This complex moves to the nucleus where it stimulates the transcription and translation of specific proteins. It is therefore likely that corticosterone brings about the

increase in the number of glutamate binding sites by inducing an increase in the rate of synthesis of glutamate receptor proteins. How melatonin reduces this process is not known, although it is speculated that melatonin interferes with the transcription and translation of receptor proteins, either by inhibiting the formation of the steroid-receptor complex, or by preventing the steroid-receptor complex from initiating transcription in the nucleus.

An alternative hypothesis is that melatonin may be competing for binding to the glutamate receptors. This would cause a decrease in the amount of ^{3}H-glutamate bound to the synaptic membranes and would indicate that there was a reduction in glutamate receptors. There is however no literature to support this theory that melatonin competitively binds to glutamate receptors.

Glucocorticoids are also known to cause increases in synaptic glutamate, by inhibiting the uptake of the amino acid by glial cells [217]. This leads to higher glutamate levels in the synapse, which can cause overstimulation of the glutamate receptors. Over stimulation of neuronal cells can result in a cascade of neurotoxic events. An increase in glutamate receptor numbers, and an increase in synaptic glutamate concentrations could result in neurons being considerably overstimulated. This could cause changes in neuronal function, or even neuronal death. Glutamate has been implicated in numerous neurodegenerative diseases [75,115,139], and protection against excessive stimulation of the receptors would be vital in protecting neurons.

CHAPTER 3

THE EFFECT OF GLUTAMATE, N-METHYL-D-ASPARTATE QUINOLINIC ACID, AND KAINIC ACID ON NEURONAL CELL VIABILITY

3.1 INTRODUCTION

Excitotoxicity is the term conceived by Olney to denote the group of excitatory amino acids which selectively kill neurons and cell bodies by their depolarising actions [5]. The process is receptor mediated, as it has been demonstrated [281] that EAA antagonists prevent both excitation and toxicity in neurons.

The mechanism by which excessive EAA receptor activation leads to neuronal death involves increased membrane permeability and abnormal Na^+, Cl^- and Ca^{2+} influx into the neuron [282]. The first two ions disrupt the osmotic pressure of the neuron which can lead to lysis of the neuron, while elevated intracellular Ca^{2+} concentrations can lead to the activation of a number of intracellular enzymes and regulator proteins [5,283].

In the brain there are a number of different glutamate receptors that have very distinct pharmacological and physiological properties [72]. EAA receptors have been characterised according to their effect on ionic balance (ionotropic) or secondary messenger systems (metabotropic). The ionotropic receptors [284] are characterised as NMDA, AMPA and KA. Each of these receptors [57] are coupled to ligand-gated ion channels which are permeable to both Na^+ and K^+, and in the case of NMDA receptors, Ca^{2+} as well.

For these experiments it was decided to work with glutamate and the glutamate agonists; KA, NMDA and QA. NMDA is the agonist for the NMDA receptors which are the most populous of the glutamate receptors. KA is the neurotoxic analog of glutamate that

interacts with the KA receptor [285]. When injected into animals, KA typically induces seizures and causes extensive neuronal damage. QA acts at the NMDA receptor [286], but the toxicity that occurs is similar to that observed during KA toxicity. QA is thought to be involved in Huntington's disease, and concentrations of this EAA are known to increase in the brain [100] with age.

In order to test whether melatonin offers any neuroprotection, the neurotoxicity of the EAA's first had to be investigated. This series of experiments was performed to determine the concentrations at which the various agonists prove toxic to neurons, and to determine LD_{50} values for the various agonists.

3.2. EFFECT OF GLUTAMATE ON NEURONAL CELL VIABILITY

3.2.1. Introduction

L-Glutamate is the most abundant free amino acid in the central nervous system [57]. The role of glutamate as a neurotransmitter was only widely accepted in 1974, when Fronnum [59] reviewed the data known at that time and showed that glutamate met all the criteria of a neurotransmitter. In the past decade glutamate has also been shown to be a very potent neurotoxin. The concept of glutamate as an excitotoxin is largely thanks to the work of Olney and colleagues [109], who demonstrated a correlation between the excitatory properties of various glutamate analogues and their ability to produce neurotoxic damage.

In the present study primary neuronal cultures were treated with varying concentrations of glutamate. Trypan blue staining was used to assess the extent of glutamate-induced neuronal cell death. Trypan blue is a dye that is used to measure cell viability. Only dead cells are permeable to the dye, as they have damaged cell membranes. This results in dead cells staining blue, while living cells remain unstained [287].

3.2.2. Materials and Methods

3.2.2.1. Chemicals and Reagents

Glutamate, Eagle's Minimum Essential Medium (MEM), Hanks' Balanced Salts (modified form) (HBSS), Trypan Blue and Cytosine-β-D-arabinofuranoside were purchased from Sigma Chemical Company (USA). Trypsin and trypsin inhibitor were purchased from Boehringer Mannheim (Germany). Foetal Calf Serum (FCS) was obtained from Delta Bioproducts (South Africa). Sodium Benzylpenicillin (Novopen) and Streptomycin Sulphate (Novo-Strep) were supplied by Novo Nordisk (Pty) Ltd (South Africa). Sterile

disposable tissue culture flasks (25 cm^2) were purchased from Corning Costar (USA). All other chemicals were of the highest grade obtainable from commercial sources.

Minimal Essential Media was prepared in Milli-Q water from powder and supplemented with; 10 mM sodium hydrogen carbonate ($NaHCO_3$), 1 mM pyruvate, 20 mM potassium chloride (KCl), Streptomycin (100 mg / L) and Penicillin (100 000 U / L). This solution was later supplemented with foetal calf serum (10% v / v).

Hank's balanced salt solution was prepared from powder in milli-Q water, supplemented with 10 mM $NaHCO_3$ and Streptomycin (100 mg / L) and Penicillin (100 000 U / L).

3.2.2.2. Preparation of Culture Reagents

MEM and HBSS were prepared in batches of multiples of one litre. Both reagents were sterilised by positive filtration (Figure 3.1) through a Millipore filtration unit using the following filters: prefilter, type SM42 "membrane filter" 50K (size 130); a 0.45 µm, 142 mm type NA filter; and a 0.22 µm, 142 mm type GS filter.

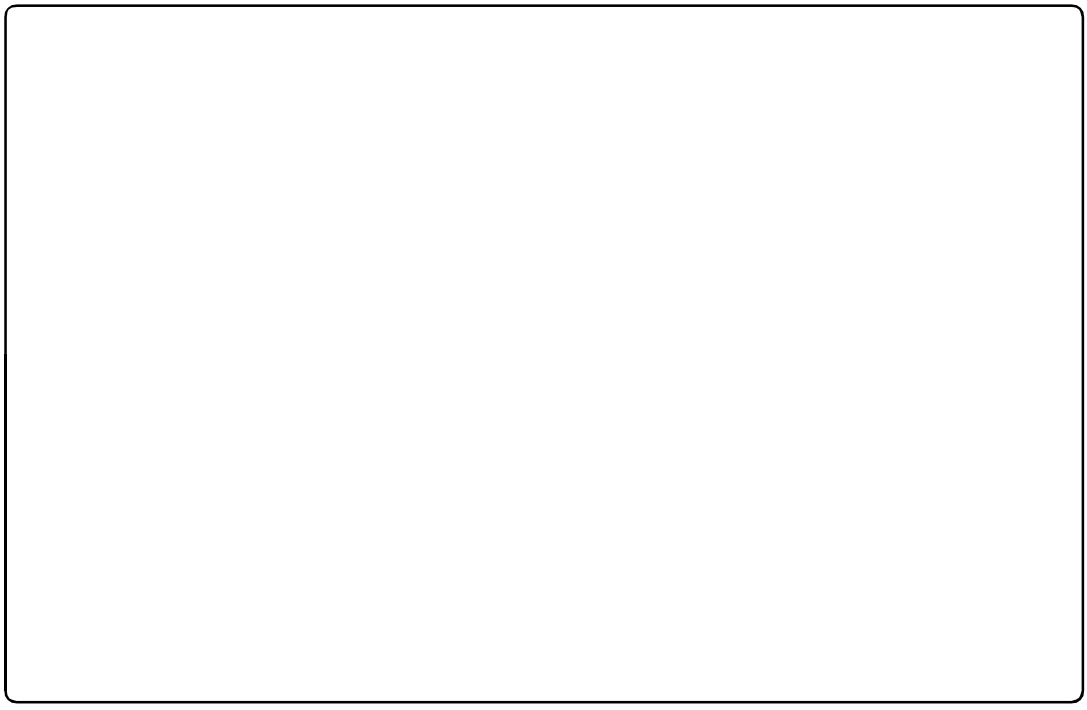


Figure 3.1: Positive pressure filtration system [288]

The reagents were aseptically dispensed into sterile cell culture bottles. Sterilisation of cell culture bottles was carried out by autoclaving for 45 minutes in an Everlight vertical type autoclave. After filtration the reagents were incubated at 37^{o}C for 48 hours to test that no contamination had occurred.

Foetal calf serum was added to the MEM on the third day after filtration through a 0.45 µM Millipore filter using a Swinex-25 holder (Millipore Corporation, USA). The MEM was further incubated at 37^{o}C for 48 hours to check that no contamination had occurred.

3.2.2.3. Establishment of Primary Neuronal Cell Cultures

Primary neuronal cultures were established from the brains of day old rats. The rats were overdosed with ether and then placed in a sterile 250 ml beaker containing cold 0.15 M saline. The heads were removed with scissors and the brains dissected out and transferred to a 50 ml beaker containing cold HBSS. All subsequent steps took place under the laminar flow hood.

The brains were pooled and minced into approximately 1 mm^3 pieces before being transferred to sterile 15 ml tubes containing 5 ml, 0.2% trypsin in HBSS. After 20 - 30 minutes of incubation at 37^{o}C in a shaking water bath, the tissue pieces were collected by centrifugation at 3500 x g for 5 minutes at 4^{o}C, rinsed with fresh HBSS, and incubated for a further 5 minutes at 37^{o}C in 0.1% soybean trypsin inhibitor. After another wash in HBSS, the cells were dissociated by titrating the tissue through the narrow bore of a fire-polished pasteur pipette. Tissue was titrated until most pieces of visible size had been disrupted. Using a sterile pasteur pipette, a suspension of cells was placed onto a haemacytometer and counted. Counts obtained were used to calculate the volume of cell suspension required to seed approximately 1 000 000 cells per 3 ml of MEM containing 10% FCS in a 25 cm^2 culture flasks. Neurons were allowed to attach to the surface of the culture flask for 2½ hours, after which the media was discarded and replaced with 5 ml MEM containing 10% FCS and 10 µM cytosine-β-D-arabinofuranoside.

3.2.2.4. Routine Cell Culture Procedure

All cell culture procedures were conducted on a laminar flow bench which had previously been sterilised by regular swabbing with 70% ethanol and exposure to ultraviolet light. In addition to this, all equipment used under the laminar bench was either purchased sterile, autoclaved or swabbed with 70% ethanol prior to use. When not required for experimental purposes, neuronal cells were incubated at 37^{o}C in 25 cm^{2} flasks containing 5 ml of 10% (v/v) foetal calf serum supplemented media containing 10 µM cytosine-β-D-arabinofuranoside. Media was changed when the nutrients had been exhausted, indicated by a change in the pH of the media.

3.2.2.5. Agonist Exposure

All experiments were conducted in 7 - 10 day old cultures. The MEM was discarded from each flask and the neurons were washed with 4 ml of HBSS. Thereafter the cultures, into which 3ml of fresh MEM containing glutamate had been added, were incubated at 37^{o}C for 20 minutes. Following incubation, the cultures were washed twice with 3 ml HBSS, before 3ml fresh MEM containing 10% FCS and 10 µM cytosine-β-D-arabinofuranoside was added. The neurons were incubated for a further 18 hours at 37^{o}C.

3.2.2.6. Assessment of Neuron Viability

Trypan Blue staining was used to assess neuronal viability. Cultures were incubated in 0.04% trypan blue solution for 5 minutes. Percent viability was assessed by counting the number of dead neurons which stained blue as well as the total number of neurons in five randomly chosen fields per plate using an inverted microscope. Counting was done by an observer unaware of the experimental conditions.

3.2.2.7. Statistical Analysis

Results were analysed as described in section 2.2.2.8.

3.2.3. Results

The effect of glutamate treatment on primary neuronal cell cultures is shown in Figure 3.2. Increasing concentrations of glutamate resulted in a decrease in percent viability. The LD_{50} value was calculated to be 275.5 µM.

3.2.4. Discussion

Glutamate was shown to be a powerful neurotoxin that killed the primary neurons in a dose dependent manner.

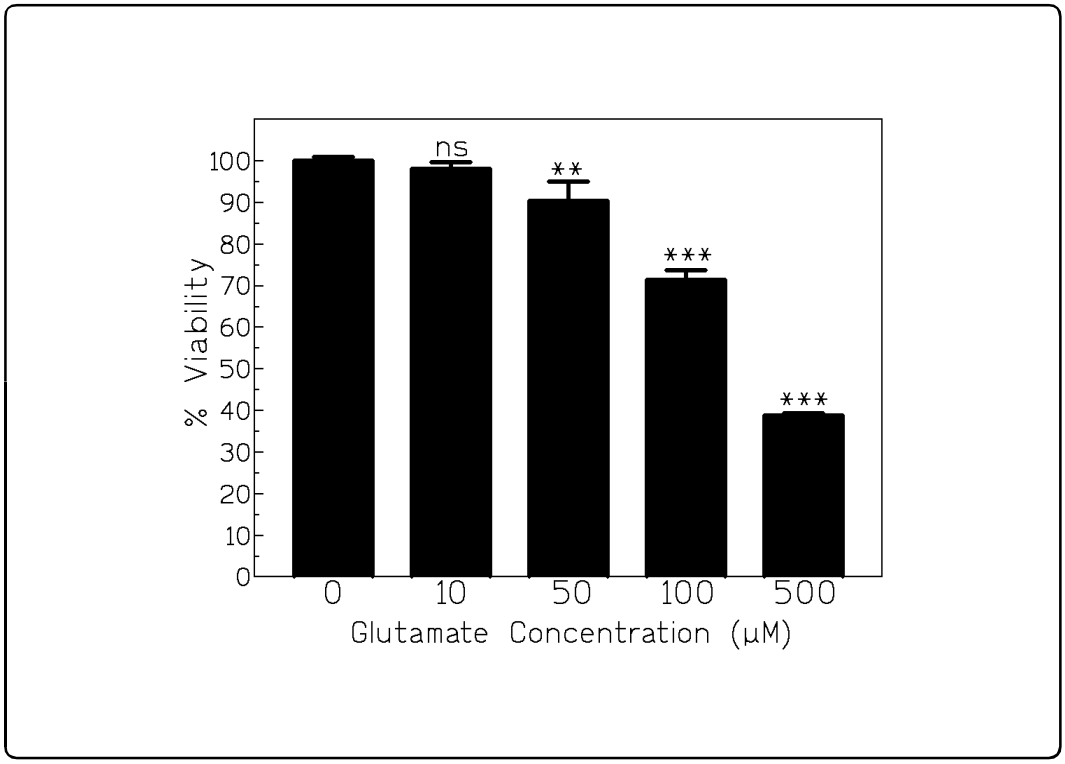


Figure 3.2: The effect of various concentrations of glutamate on cell viability. Values represent the mean ± SEM (n=3-5) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls).

3.3. EFFECT OF N-METHYL-D-ASPARTATE ON NEURONAL CELL VIABILITY

3.3.1. Introduction

NMDA is an agonist of the NMDA class of glutamate receptor. NMDA receptors are the most common of the glutamate receptors and are widely distributed in the brain. Much interest has focussed on the NMDA receptor, because in animal experiments blockade of the receptor by aminophosphonocarboxylates (AP5 and AP7) or by the non-competitive antagonists (MK-801, phencyclidine, ketamine), resulted in the symptoms of many neurological disorders caused by hypoxia, ischemia and traumatic brain injury [289] being dramatically reduced.

In the present study effect of various doses of NMDA on neuronal viability was investigated using trypan blue staining.

3.3.2. Materials and Methods

NMDA purchased from Research Biochemicals International (USA) was used in place of glutamate. Otherwise the method used was exactly the same as that in experiment 3.2.

3.3.3. Results

The effect of NMDA treatment on primary neuronal cell cultures is shown in Figure 3.3. Increasing concentrations of NMDA resulted in a decrease in percentage viability.

3.3.4. Discussion

The neurotoxic properties of NMDA were able to cause a concentration dependent decrease in neuronal cell viability. The LD_{50} value for NMDA could not be calculated, because a 50% decrease in cell viability was never achieved with the concentrations of agonist used.

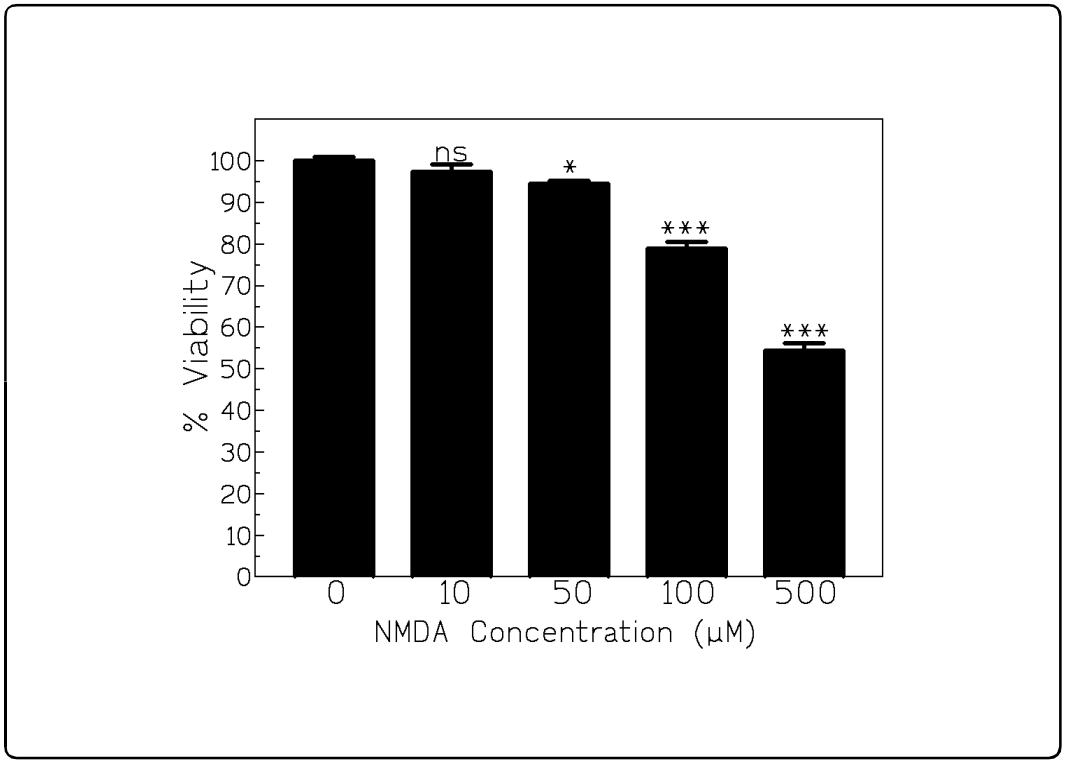


Figure 3.3: The effect of various concentrations of NMDA on cell viability. Values represent the mean ± SEM (n=3-5) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{***}P<0.001$ in comparison to zero controls).

3.4. EFFECT OF QUINOLINIC ACID ON NEURONAL CELL VIABILITY

3.4.1. Introduction

Quinolinic acid is a neurotoxic metabolite of the tryptophan-kynurenine pathway [97,98]. It is usually present in the brain in nanomolar concentrations [110]. Substantial increases occur in a broad spectrum of infections and inflammatory neurological diseases, e.g. ischemia brought on by strokes [110]. Heyes *et al* [97] recently reported that microglia and macrophages may be an important source of this neurotoxin. QA acts at the NMDA receptor [290], but the toxicity that occurs is similar to that produced by KA toxicity. This agent has been shown to induce neuronal lesions after intrastriatal and intrahippocampal injections in the rat brain [103]. These lesions closely resemble those observed in the brains of individuals with Huntington's Disease.

In this study, the effect of increasing doses of QA on primary neuronal cultures was investigated.

3.4.2. Materials and Methods

QA which was purchased from the Sigma Chemical Company (USA) was used in place of glutamate. Otherwise the method was the same as in experiment 3.2.

3.4.3. Results

The effect of QA treatment on primary neuronal cell cultures is shown in Figure 3.4. Increasing concentrations of QA greatly decreased the percent viability of the neuronal cultures. The LD_{50} value was calculated to be 218.4 µM.

3.4.4. Discussion

QA is a very potent neurotoxin. The viability of the neuronal culture was greatly reduced by treatment with increasing doses of quinolinic acid.

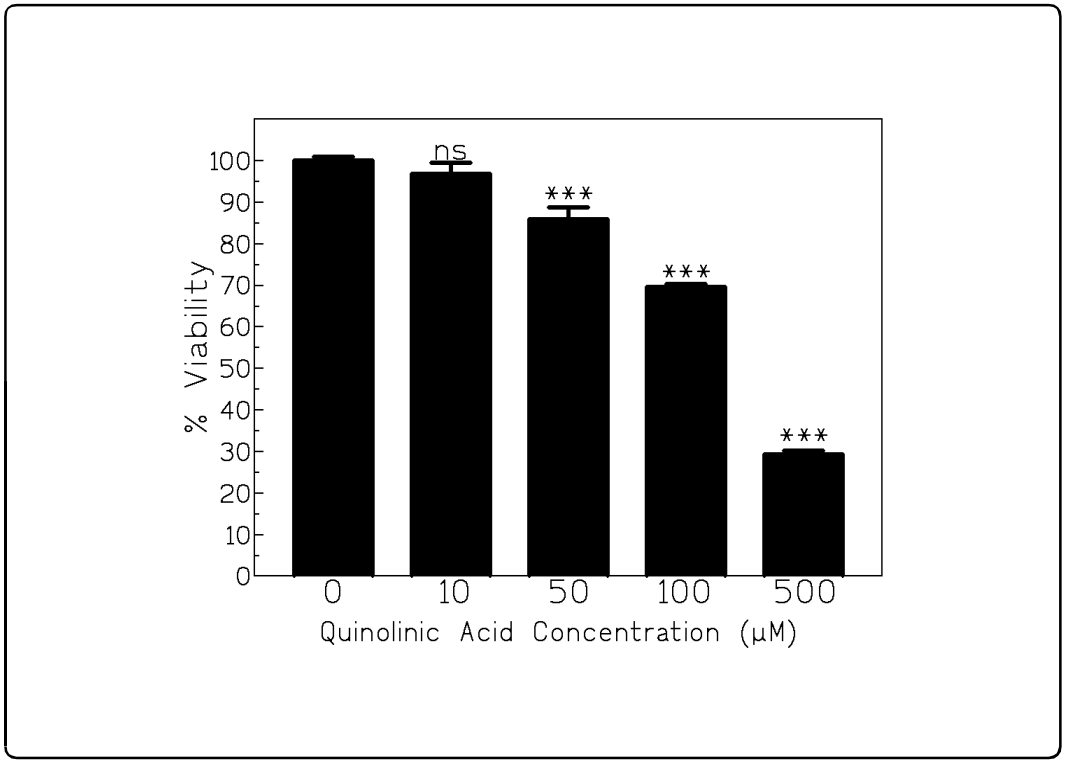


Figure 3.4: The effect of various concentrations of QA on cell viability. Values represent the mean ± SEM (n=3-5) ($^{ns}P>0.05$; $^{***}P<0.001$ in comparison to zero controls).

3.5. EFFECT OF KAINIC ACID ON NEURONAL CELL VIABILITY

3.5.1. Introduction

Kainic acid is an agonist for a subtype of non-NMDA glutamate receptors [291]. The neurotoxic action of KA was discovered by Olney *et al* [292] while they were investigating the neurotoxic effects of glutamate. In addition to inducing lesions directly, KA also triggers epileptiform activity and secondary brain injury that can be reduced by anticonvulsent drugs [293].

In the present study we wished to investigate whether KA too, could prove toxic to primary neuronal cultures.

3.5.2. Materials and Methods

KA which was purchased from Research Biochemicals International (USA) was used in place of glutamate, otherwise the method was as in experiment 3.2.

3.5.3. Results

The effect of various concentrations of KA on primary neuronal cell cultures is shown in Figure 3.5. Increasing concentrations of KA resulted in a decrease in cell viability when compared to control cultures. The LD_{50} value was calculated to be 359.6 µM.

3.5.4. Discussion

The glutamate agonist KA was found to be a very effective neurotoxic agent. Cell viability was decreased as the concentration of KA was increased.

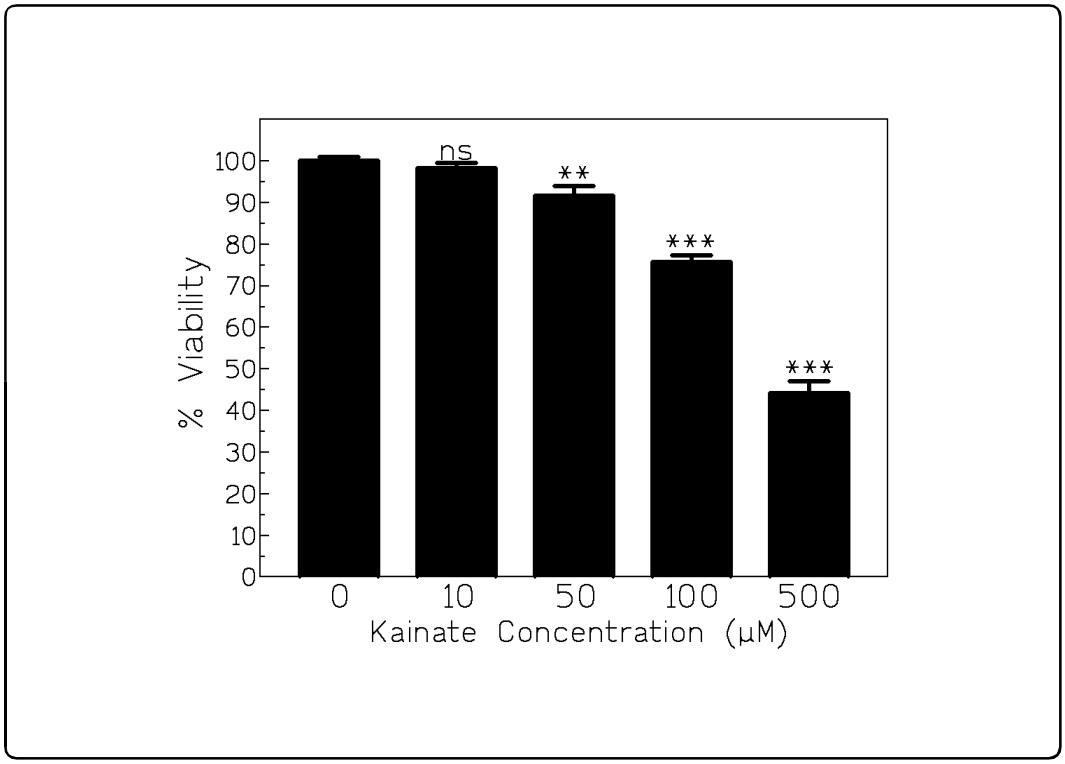


Figure 3.5: The effect of various concentrations of KA on cell viability. Values represent the mean ± SEM (n=3-5) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls).

3.6. Conclusions

The neurotoxic effects of glutamate and the glutamate agonists; NMDA, QA and KA was investigated. The results obtained confirm other reports that glutamate [72,294-297], NMDA [294,295,341,342], QA [289] and KA [295,297] are toxic to neurons when present in high concentrations. In all cases treatment resulted in death to a number of neurons, which was expressed as a decrease in cell viability compared to untreated control neurons.

Of the various agonists tested, QA was found to be the most neurotoxic. This is demonstrated by the fact that only 29.22% of neurons survived in flasks treated with 500 µM of QA. Similar doses of glutamate, KA and NMDA yielded survival rates of 38.72%, 44.18% and 54.31% respectively when compared to control cultures.

The results obtained demonstrate that all of the agonists tested are toxic to neurons. This provides a basis in order to test the hypothesis that melatonin is able to protect against the neurotoxicity induced by the different agonists. The experiments in Chapter 4 are therefore concerned with investigating whether melatonin is able to protect against neurotoxicity caused by the different agonists.

CHAPTER 4

EFFECT OF TREATMENT ON PRIMARY NEURONAL CELL CULTURES WITH MELATONIN AND GLUTAMATE RECEPTOR AGONISTS

4.1. INTRODUCTION

The neurohormone melatonin, 5-methoxy-N-acetyl-tryptamine, is primarily synthesised in the pineal gland and released into the bloodstream in a diurnal rhythm, peaking at night [31]. Melatonin has many important physiological functions, including the regulation of seasonal reproduction and circadian rhythms [24]. Recently, much work has focussed on the antioxidant properties of melatonin [180,234]. Melatonin has been reported to rapidly scavenge hydroxyl- [3] and peroxyl radicals [299], and it has been reported to be a more potent free radical scavenger than either glutathione, mannitol, or vitamin E [299]. Among the properties attributed to melatonin are the suppression of the development of cataracts in newborn rats treated with the glutathione depleting agent, buthionine sulfoximin [300] the protective effect of melatonin against cyanide-induced seizures and lipid peroxidation in mice [192], as well as a decrease in DNA damage in rats treated with the carcinogen safrole [183].

Much of the toxicity associated with glutamate receptor activation is the result of excessive elevation of intracellular neuronal Ca^{2+} levels [58]. There are many possible ways in which Ca^{2+} overload may kill cells, including activation of intracellular proteases and lipases, impaired mitochondrial functions, and the generation of free radicals [301]. Free radicals, and products of free radical reactions, can cause damage to macromolecular targets such as DNA, proteins, and cellular membranes [302,303]. A particularly important consequence of free radical damage in cells, is the peroxidation of polyunsaturated fatty acids, which results in the formation of lipid peroxides and aldehydes [304]. These products can cause extensive damage to membrane structure and integrity, and it is this damage that can result in death to neurons.

Melatonin has unique physiological properties which distinguish it from other antioxidants. In contrast to all other known low molecular weight antioxidants, the melatonin molecule, once oxidised, cannot be reduced or regenerated. This built in safety mechanism protects against auto-oxidative free radical generation and toxic redox cycling [8].

It has been proposed that glutamate neurotoxicity enhances free-radical production, thereby causing neuronal death in a variety of age-related diseases. It was therefore decided to investigate the neuroprotective properties of melatonin against glutamate and the three neurotoxic glutamate agonists; NMDA, QA and KA, that had been found to be neurotoxic in Chapter 3.

4.2. INVESTIGATION OF THE POTENTIAL NEUROTOXIC ACTIONS OF MELATONIN SUPPLEMENTATION

4.2.1. Introduction

Before an investigation could be made into whether melatonin was able to protect against glutamate receptor induced neurotoxicity, it was necessary to investigate the effect of melatonin on primary neuronal cell cultures.

4.2.2. Materials and Methods

4.2.2.1. Chemicals and Reagents

Melatonin was purchased from Sigma Chemical Company (USA) and dissolved in ethanol. The final ethanol concentration in the culture flasks was 1.5%. The same concentrations of ethanol were added to all control incubations.

4.2.2.2. Cell Culture Technique

Cell culture reagents and primary neuronal cells were prepared as described in section 3.2.2.2. and 3.2.2.3.

4.2.2.3. Procedure for Melatonin Treatment

Ten day old cultures were used in this experiment. The MEM was discarded and the cells washed with 4 ml HBSS. Thereafter, 5 ml fresh MEM was placed in the culture flasks. Appropriate aliquots of melatonin stock and 50% ethanol stock solutions were added to the flasks so as to give final melatonin concentrations of 0 - 1000 µM, while keeping ethanol concentrations constant. Final ethanol concentrations did not exceed 1.5%. Cultures were returned to the 37^{o}C incubator for 20 minutes. These were then

washed twice with 3 ml HBSS before the addition of 3 ml of fresh MEM. The neurons were incubated at 37°C for a further 18 hours. Trypan blue staining, as described in section 3.2.2.6. was carried out in order to assess the neuronal viability of the cultures. Each experiment was carried out in duplicate.

4.2.2.4. Statistical Analysis

The results were analysed as described in section 2.2.2.8.

4.2.3. Results

The effect of melatonin treatment on primary neuronal cell cultures is shown in Table 4.1. Increasing concentrations of melatonin had no significant (P=0.5165) effect on neuronal viability.

Table 4.1: The effect of various concentrations of melatonin on Neuronal Cell Viability (n=5)

Melatonin Concentration (μM)	% Viability	SEM
0	99.39	0.60
10	98.75	1.25
150	99.01	0.61
500	99	1.02
1000	100.07	0.52

4.2.4. Discussion

The exposure of primary neuronal cultures to melatonin, at concentrations as high as 1 mM, did not significantly alter the viability of the neuronal cultures. The use of ethanol as a vehicle also did not appear to have any neurotoxic effects.

Since melatonin did not have any effect on the neurons, it was decided to determine whether the hormone could protect neuronal cell cultures from neurotoxicity induced by the glutamate receptor agonists tested in Chapter 3.

4.3. EFFECTS OF MELATONIN CO-TREATMENT WITH GLUTAMATE, N-METHYL-D-ASPARTATE, QUINOLINIC ACID AND KAINIC ACID ON NEURONAL VIABILITY

4.3.1. Introduction

In Chapter 3, it had been demonstrated that glutamate, and the glutamate receptor agonists; NMDA, QA and KA, were very strong neurotoxins. Previous experiments [57,190] had demonstrated that melatonin was able to protect against KA induced neurotoxicity. However Giusti *et al* [295] also found that melatonin was not able to protect against NMDA induced neurotoxicity.

In this experiment, an investigation was carried out to determine whether melatonin is able to protect neurons against the above mentioned neurotoxins. Cultures were co-treated with varying concentrations of melatonin and a fixed concentration of agonist. The percent neuronal viability was assessed by trypan blue staining.

4.3.2. Materials and Methods

4.3.2.1. Chemicals and Reagents

Melatonin was prepared in ethanol as described in section 4.2.2.1. The ethanol concentration was kept the same in all culture flasks. Glutamate, NMDA, QA and KA were dissolved in Milli-Q water. All other reagents and chemicals were prepared as described in 3.2.2.1.

4.3.2.2. Experimental Procedure

Primary neuronal cultures were prepared as described in section 3.2.2.3. Agonists

(420 µM) were applied as described in section 3.2.2.5, except that melatonin (0 - 500 µM) was added to the culture medium at the same time.

The percentage viability of cultures was determined using the trypan blue assay as described in section 3.2.2.6.

Results were analysed as described in 2.2.2.8.

4.3.3. Results

The addition of melatonin to neuronal cultures treated with glutamate receptor agonists caused an increase in the survival rate of neurons with all the agents tested (see Figures 4.1 - 4.4). In the absence of melatonin, viability of neuronal cultures ranged from as low as 48.8% for QA treated cultures, to as high as 67.4% for NMDA treated cultures.

Melatonin was able to protect the neurons in a dose dependent manner. Maximum protection was offered in all cases when 500 µM of melatonin was applied to the culture medium. Melatonin was able to offer significant protection from 100 µM of KA and QA, while concentrations as low as 10 µM offered significant protection in the NMDA treated cultures.

Glutamate treated cultures required 250µM of melatonin before significant protection was achieved.

In no case did the survival rates of the cultures reach 100% after melatonin treatment.

4.3.4. Discussion

The results show direct evidence of a dose-dependent neuroprotective effect of melatonin against the four agonists investigated. Melatonin was able to offer maximum

protection at 500µM concentration. Melatonin may have offered greater protection at a higher dose, however the aim of this experiment was not to determine melatonin dosage regimes, but rather to investigate whether melatonin did indeed increase survival rates of primary neuronal cultures.

The doses of agonist used, led to a significant reduction ($P>0.05$) in viable cells when compared to untreated control cultures. As Giutsi had demonstrated [295], melatonin was indeed able to protect neurons against KA induced neurotoxicity. It was however also found that melatonin afforded protection against glutamate, NMDA, KA and QA-induced neurotoxicity. This was significant in that Giutsi *et al* [295] had not found melatonin to be neuroprotective against NMDA induced neurotoxicity. There were however differences in the techniques used between this experiment and those used by Giutsi *et al*. These differences in technique may have resulted in melatonin proving protective in these results, while it was not protective when Giutsi performed a similar experiment.

It has thus been shown that melatonin is able to increase neuronal viability following neurotoxic insults initiated by glutamate, NMDA, KA and QA.

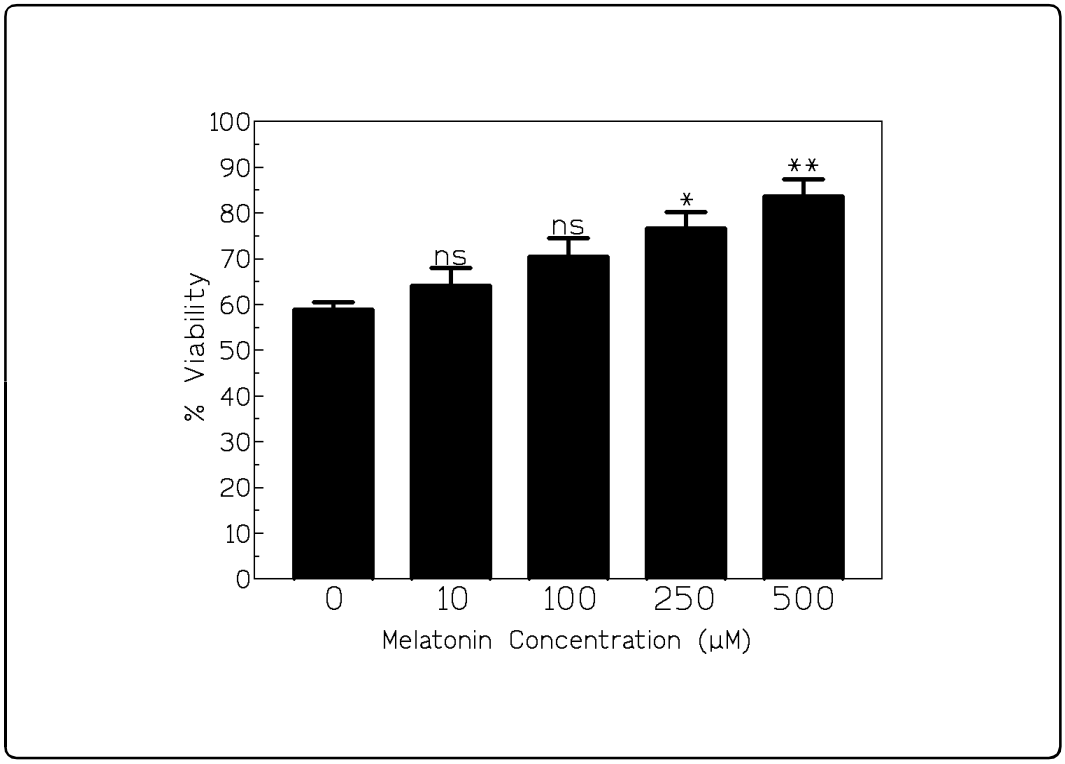


Figure 4.1: Effect of melatonin on neuronal viability when co-treated with 420 µM Glutamate. Values represent the mean ± SEM (n = 6 - 9) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$ in comparison to zero controls)

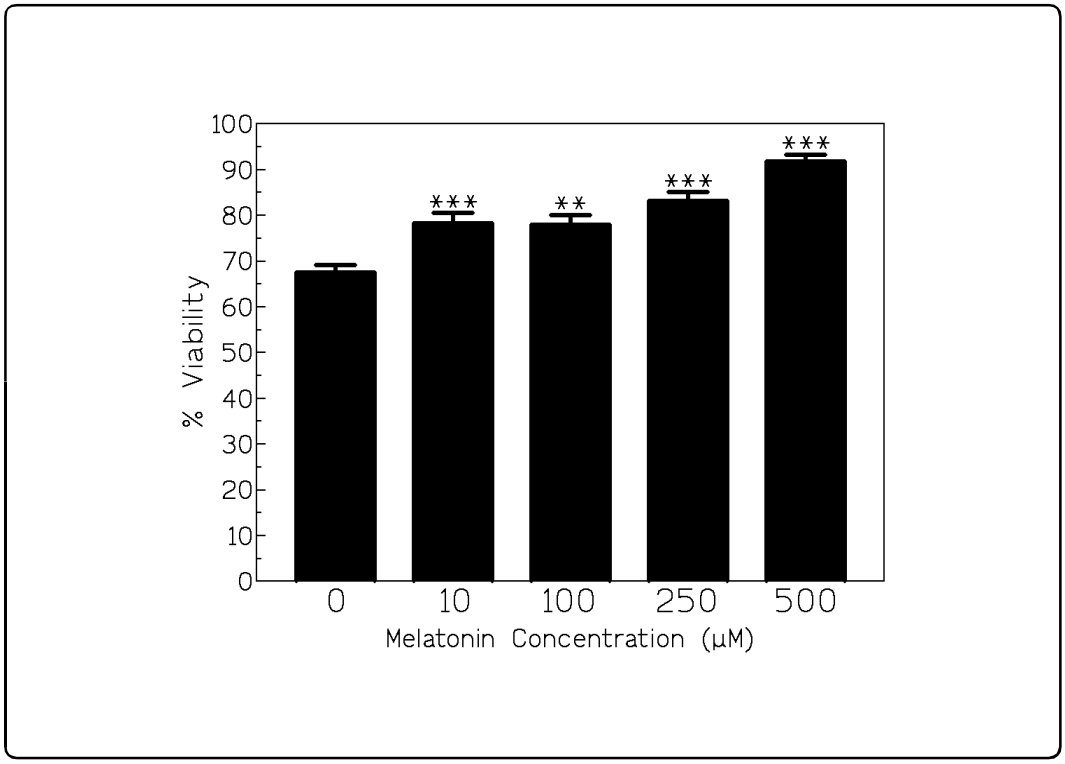


Figure 4.2: Effect of melatonin on neuronal viability when co-treated with 420 µM NMDA. Values represent the mean ± SEM (n = 6) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls)

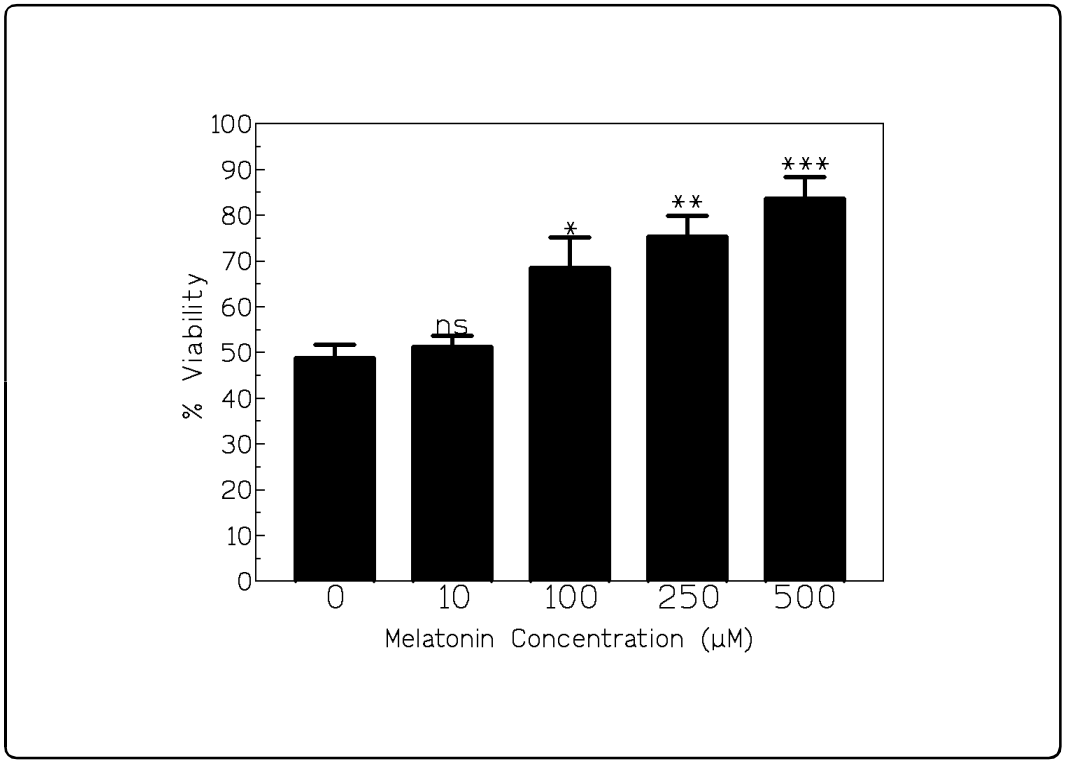


Figure 4.3: Effect of melatonin on neuronal viability when co-treated with 420 µM QA. Values represent the mean ± SEM (n = 6) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls)

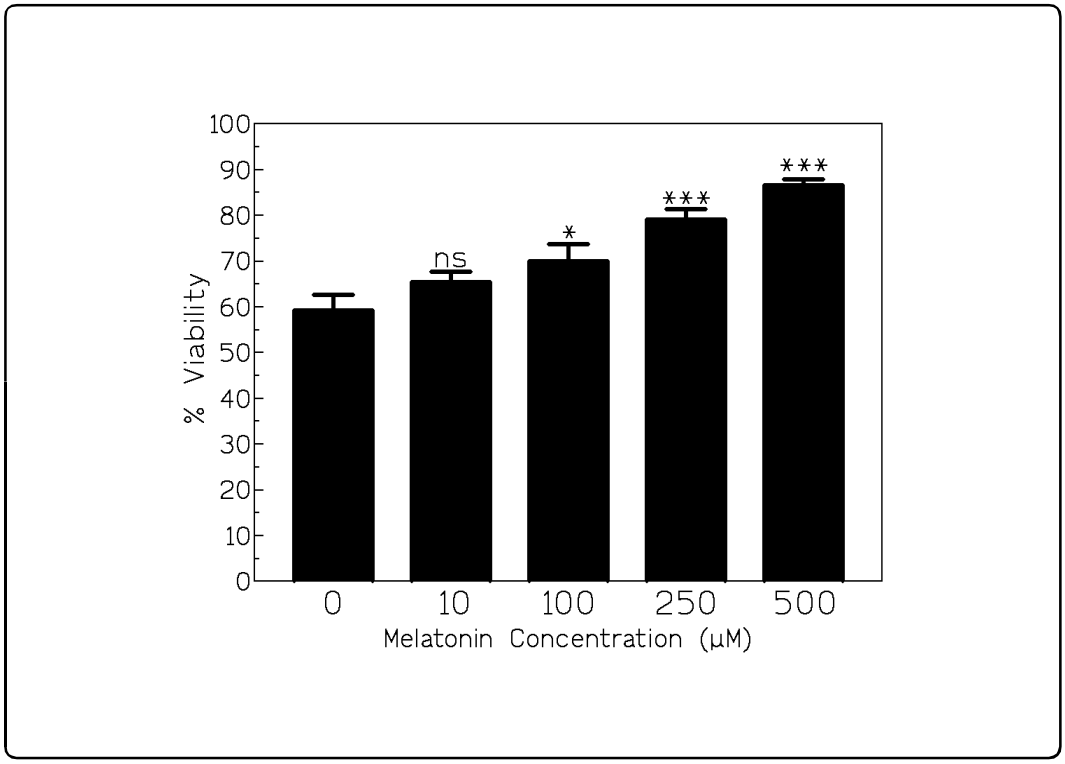


Figure 4.4: Effect of melatonin on neuronal viability when co-treated with 420 µM KA. Values represent the mean ± SEM (n = 7) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{***}P<0.001$ in comparison to zero controls)

4.4. INVESTIGATION TO DETERMINE WHEN TO ADD MELATONIN TO ACHIEVE MAXIMUM NEUROPROTECTION

4.4.1. Introduction

Melatonin has been suggested as a neuroprotective agent against a number of neurological disorders. Among the disorders mentioned are Alzheimer's disease [245,251] and Cerebral Ischemia [266]. Each of these disorders involves different mechanisms that lead to neurotoxicity. It was hoped that by investigating when, in relation to the agonist insult, melatonin was able to offer protection, an idea of the mechanism of the neuroprotective action could be determined.

It had been demonstrated (in section 4.3.) that melatonin was able to protect against neurotoxicity induced by glutamate, NMDA, QA and KA. However it was not known when it was best to administer melatonin to achieve maximum protection.

This experiment was aimed at determining whether melatonin should be administered prior to the onset of excitotoxicity as a preventative measure, or whether co-administration of melatonin during glutamate excitotoxicity offered greater protection. In addition, the effect of melatonin as a means of treating neuronal damage, after glutamate toxicity had occurred, was also investigated.

4.4.2. Materials and Methods

4.4.2.1. Chemical Reagents

Chemicals used were prepared as in 4.3.2.1, except that a lower dose of agonist (100µM) was used, as this was the lowest dose of agonist that gave significant

decreases in cell viability in Chapter 3. A low dose of melatonin (150µM) was also used so that subtle changes in neuronal survival could be better observed.

4.4.2.2. Experimental Procedure

Primary neuronal cultures were prepared as described in section 3.2.2.3. Agonists were applied as described in section 3.2.2.5, except that neurons were either pre-treated, co-treated of post-treated with melatonin (see Table 4.2). Neurons pre-treated with melatonin had sufficient melatonin added to the existing growth media to make up to the correct melatonin concentration 20 minutes before the addition of the media containing the agonist. Cultures co-treated with melatonin during the agonist insult, had melatonin added to the incubation media, together with the agonist. Finally, in cultures post-treated with melatonin, the cultures were treated with agonist as per normal, and melatonin was added to the fresh medium that was put into the flasks following the insult. Neurons were then incubated for 18 hours in the presence of melatonin.

The percentage viability was assayed as described in section 3.2.2.6, and the results were analysed as explained in 2.2.2.8.

Table 4.2: Experimental parameters used to determine when best to administer melatonin to achieve maximum neuroprotection

Time of Treatment	Melatonin (150µM)
Pre-Treatment	Added 20 minutes prior to addition of agonist
Co-Treatment	Added during 20 minute exposure to agonist
Post-Treatment	Added 20 minutes after inducing agonist toxicity (18 hours)

4.4.3. Results

The results (see Figures 4.5 - 4.8) demonstrated that no protection was offered to neurons that were pre-treated 20 minutes prior to the insult. In those flasks where cultures were co-treated with agonist and melatonin, there was significant protection offered, except in the case of the flasks treated with glutamate, where no significant increase in cell viability was recorded in comparison to control cultures. Neurons post-treated for 18 hours with melatonin, following the 20 minute agonist insult, all showed significant increases in cell viability.

4.4.4. Discussion

Melatonin given 20 minutes prior to the agonist insult was not effective at protecting neurons against agonist-induced neurotoxicity. The melatonin was removed from the culture medium before the addition of the agonist, so that only melatonin that had been taken up by the neurons would have remained in the culture. These results suggest that melatonin must act as an antioxidant, as melatonin given before the insult, and production of ROS, is not able to offer protection.

Apart from the neuronal cultures co-treated with glutamate, all the other cultures co-treated or post-treated with melatonin had their viability increased. This would suggest that melatonin is protecting the neurons from the post insult production of ROS.

Co-treatment with glutamate and melatonin caused an increase in cell viability, although it was not statistically significant. Experiment 4.3. had demonstrated that at high melatonin and glutamate concentrations, co-treatment could result in a significant increase in neuronal viability. It was therefore decided that all future experiments would use co-administration of the agonist and melatonin, as the method of preference.

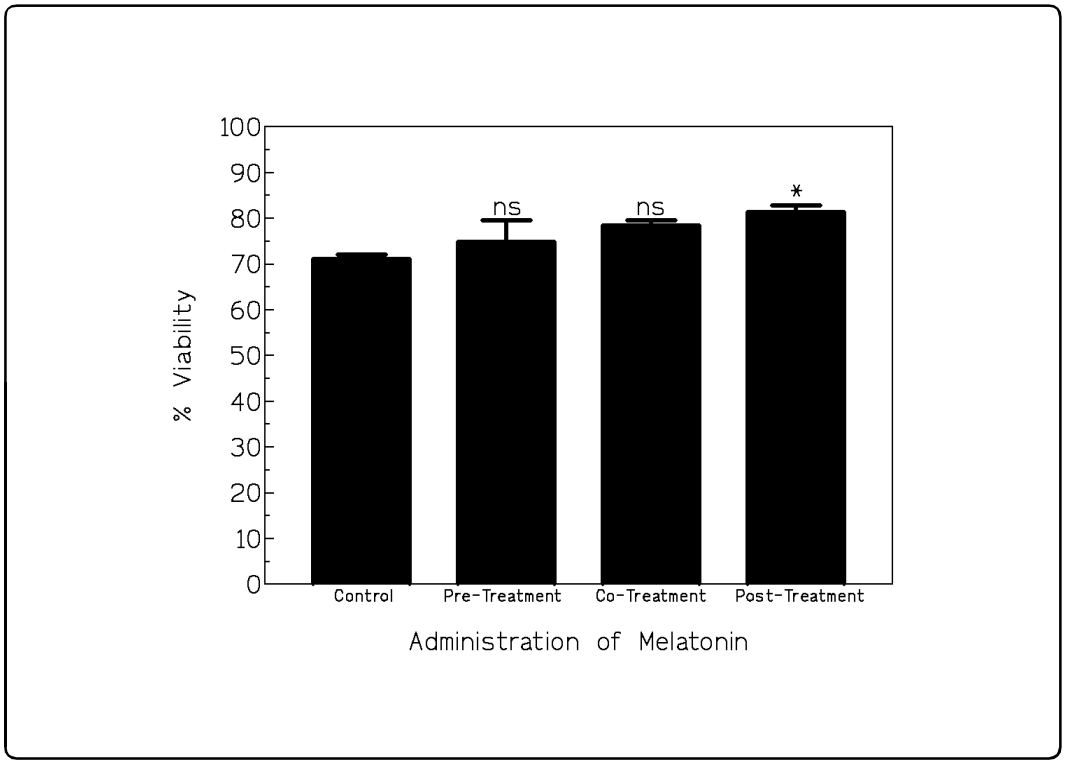


Figure 4.5: Effect of time of melatonin administration on the neuroprotection against glutamate toxicity. Values represent the mean ± SEM (n= 4 - 5) ($^{ns}P>0.05$; $^{*}P<0.05$ in comparison to zero controls)

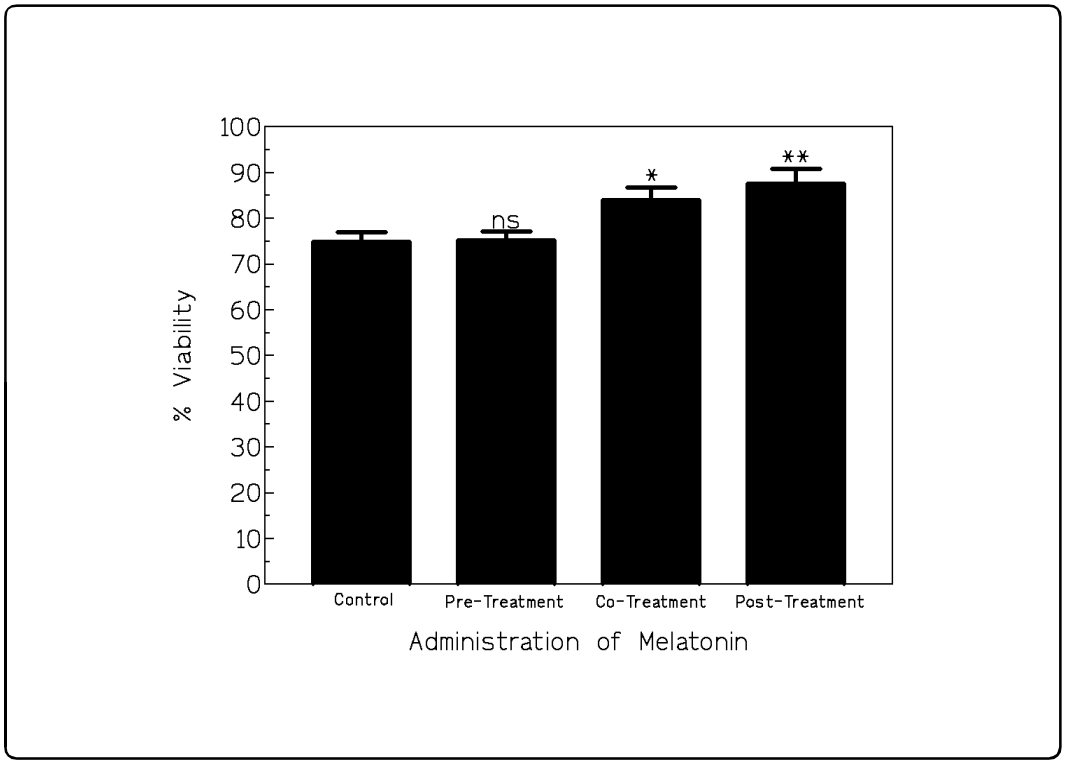


Figure 4.6: Effect of time of melatonin administration on the neuroprotection against NMDA toxicity. Values represent the mean ± SEM (n= 3 - 5) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$ in comparison to zero controls)

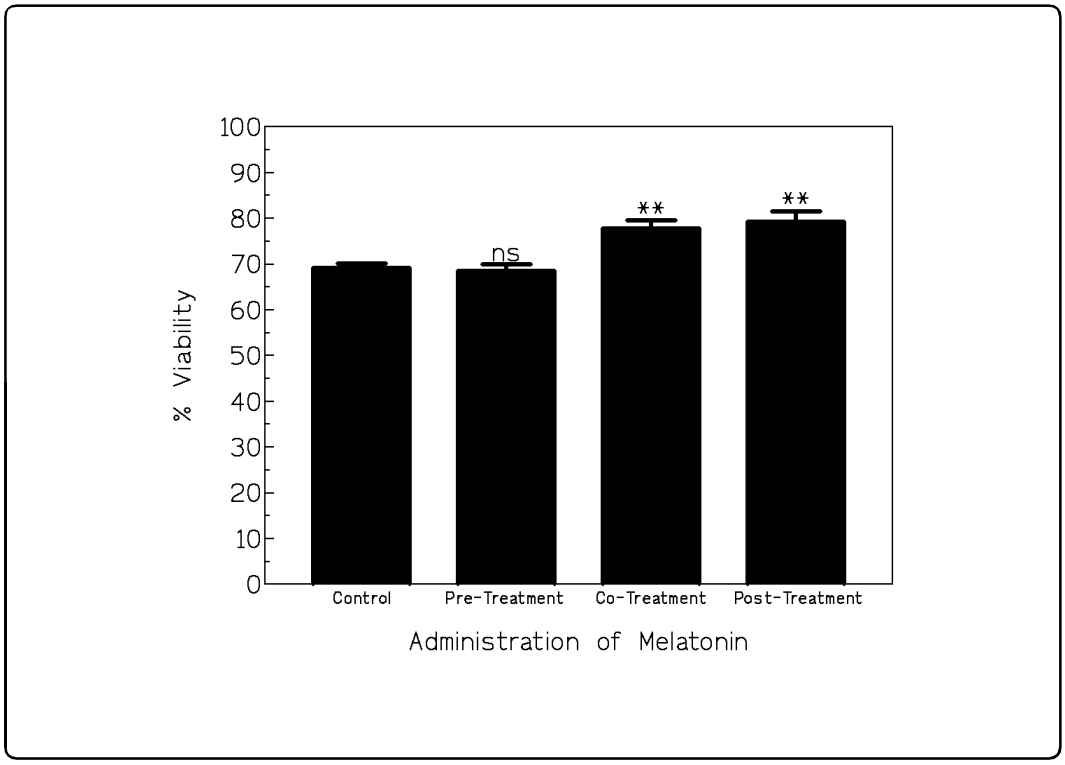


Figure 4.7: Effect of time of melatonin administration on the neuroprotection against QA toxicity. Values represent the mean ± SEM (n = 5) ($^{ns}P>0.05$; $^{**}P<0.01$ in comparison to zero controls)

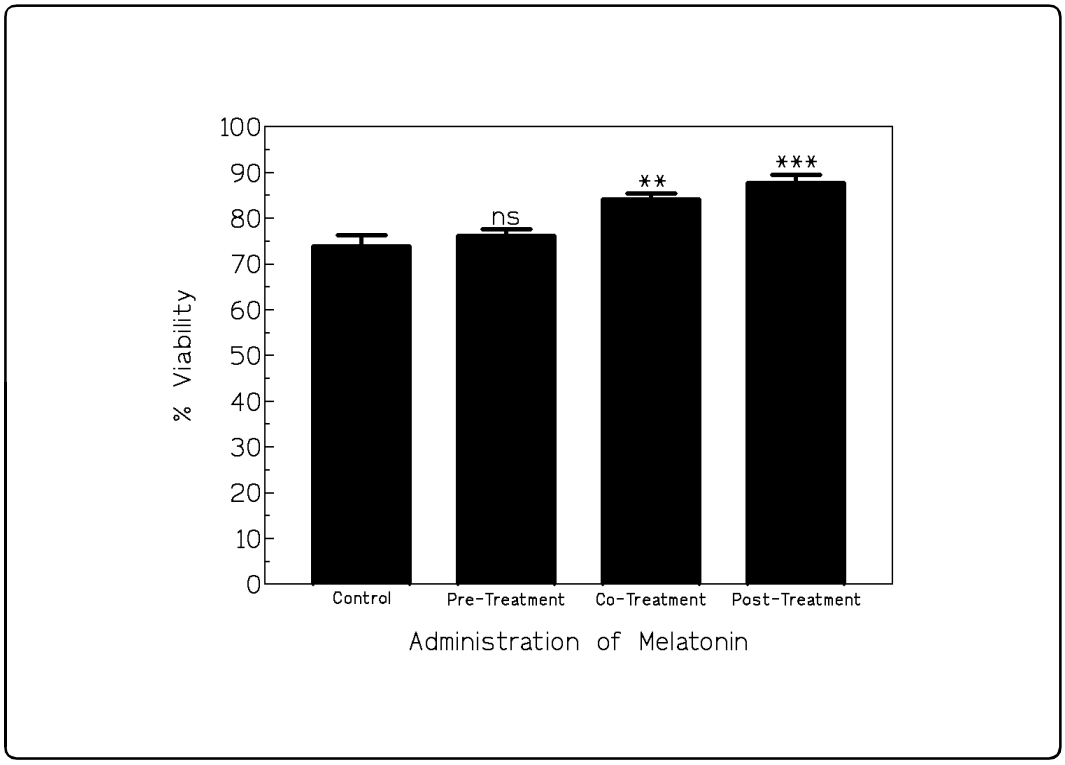


Figure 4.8: Effect of time of melatonin administration on the neuroprotection against KA toxicity. Values represent the mean ± SEM (n = 5) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls)

4.5. MOVEMENT OF ^{3}H-MELATONIN INTO NEURONS

4.5.1. Introduction

The previous experiments demonstrated that melatonin is effective at reducing neuronal cell death induced by the various glutamate agonists. It is not known how melatonin protects the neurons, but it is proposed that melatonin acts as a neuroprotector by removing free radicals within the cell. In order to do this, melatonin would first have to enter the neurons, as neurons cannot naturally produce melatonin.

In this experiment the accumulation of melatonin within neuronal cells was investigated.

4.5.2. Materials and Methods

4.5.2.1. Chemical Reagents

[O-methyl-^{3}H]melatonin (1 mCi / ml) was purchased from Amersham International plc (England). Triton X-100 was purchased from Unilab (South Africa).

Melatonin was prepared in absolute ethanol and diluted with Milli-Q water to obtain the correct stock concentration. Final ethanol concentrations in the flasks did not exceed 1.5%.

^{3}H-melatonin was diluted in MEM, and a working stock was prepared so that it would give a final concentration of 0.1 µCi / ml media, once present in the incubation flask.

4.5.2.2. Preparation of Primary Neuronal Cultures

Primary neuronal cultures were established as explained in section 3.2.2.3.

4.5.2.3. Experimental Procedure

A modified method of Sorg *et al* [125] was used to measure melatonin uptake. Seven to ten day old primary neuronal cell cultures were washed with 4 ml of HBSS, after which 3 ml of MEM was added. To this was added 100 µl of melatonin to give a final concentration of 500 µM, and 100 µl of ^{3}H-melatonin in MEM to give a final concentration of 0.1 µCi / ml. The flasks were then returned to a 37^{o}C incubator.

Samples were removed at 0, 10 , 20 and 30 minutes after the addition of melatonin to the flasks, to measure ^{3}H-melatonin uptake into the cells.

To assay for ^{3}H-melatonin, the MEM containing the radiolabelled melatonin was immediately poured out of the flasks, and the flasks were washed with 4 ml of ice-cold 50 mM Tris-HCl Buffer (pH 7.4) containing 5 mM melatonin. This was poured off and 2 ml of 10 mM NaOH containing 0.1% Triton X-100 was added to the flasks. The flasks were left at room temperature for 10 minutes, before being gently agitated and the contents being poured into new reaction tubes. A 500 µl aliquot of the solution in the reaction tube was added to 3ml of scintillation cocktail. After 24 hours, the radioactivity in the scintillation vials was measured using a Beckman (Model LS 2800) Liquid Scintillation Counter. All radioactivity was expressed as fmol ^{3}H-glutamate / mg protein. An aliquot of the solution left in the reaction tube was used to determine the protein concentration using the modified method of Lowry *et al* as explained in section 2.2.2.6. All results were analysed as described in section 2.2.2.8.

4.5.3. Results

^{3}H-melatonin uptake increased significantly as the incubation time increased (see Figure 4.9). After a 30 minute incubation in the presence of melatonin, there was a 32.1% increase in intracellular melatonin concentration compared to controls (25.7 fmol / mg protein).

4.5.4. Discussion

Melatonin accumulates in the interior of the neuron. Being a lipid soluble molecule [305], melatonin could simply diffuse into the interior structure of the neuron through the cell membrane, or there may be transport proteins to allow melatonin to enter the neurons.

The aim of this experiment was not to determine the mechanism by which melatonin enters the neuron, but rather to determine whether melatonin accumulates within the neuron. The neurons that were used had been in a melatonin free environment for 7 - 10 days, and so there should have been no intracellular melatonin present in the neurons prior to the application of the melatonin in the MEM.

The fact that melatonin can be found intracellularly, shows that melatonin is present, should it be needed to perform a neuroprotectant role.

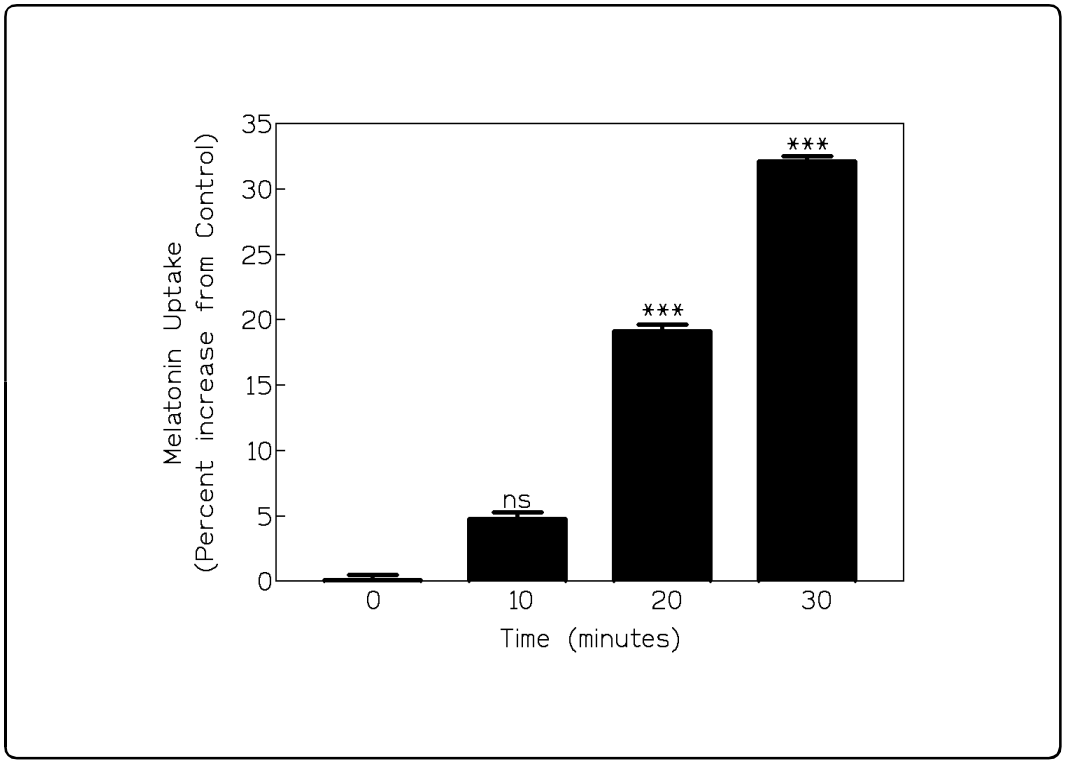


Figure 4.9: ^{3}H-Melatonin uptake over time. Values represent the percent increase (± SEM) compared to zero controls. (n=4) (^{ns} P>0.05; ***P<0.001 in comparison to zero control)

4.6. CONCLUSIONS

Chapter 3 had demonstrated the neurotoxic effects of glutamate, NMDA, QA and KA on primary neurons. In this chapter melatonin was investigated to see if it afforded any neuroprotection against the agonist induced neurotoxicity.

It was first demonstrated that melatonin dissolved in an ethanol vehicle was not neurotoxic to neuronal cultures. No significant changes in neuronal viability occurred even at melatonin concentrations of 1 mM.

When primary neuronal cultures were co-treated with both melatonin (0 - 500 µM) and an agonist (420 µM), the melatonin was able to protect in a dose dependent manner. Melatonin was also found to only be neuroprotective when administered either during or after the agonist insult on the neurons. This would indicate that melatonin is offering protection by inhibiting neurotoxic processes that occur after the agonist insult.

The excitotoxic model of neurotoxicity proposes that when the NMDA or KA receptors are stimulated, Ca^{2+} moves into the neuron. Over stimulation of these receptors would thus lead to excessive Ca^{2+} influx [118]. Ca^{2+} stimulates a number of pathways that can give rise to the production of free radicals. Mitochondria attempt to accumulate the Ca^{2+}, but this affects the membrane potential and decreases the efficiency of the mitochondria. This can increase ROS production.

Melatonin could well be acting, either to inhibit Ca^{2+} influx into the neurons, or as an ROS scavenger. Chapters 6 - 9 will investigate these possible neuroprotective pathways.

CHAPTER 5

EFFECT OF QUINOLINIC ACID AND MELATONIN ON HIPPOCAMPAL NEURONS

5.1. INTRODUCTION

In the previous two chapters it had been demonstrated that glutamate and the glutamate agonists, NMDA, QA, and KA, induce neuronal cell death in primary neuronal cell cultures. It had also been shown that melatonin was able to protect neurons to a certain degree from EAA-induced neurotoxicity. In this experiment, an investigation was made to determine whether melatonin was able to protect neurons in an *in vivo* situation.

Quinolinic acid is an agonist of the neuronal NMDA receptor [102,306], which has been detected in both the human and rat brain [79]. Intrastriatal and intrahippocamal injections of QA into the rat brain have been shown to induce neuronal lessions [103,104]. These lesions closely resemble those observed in the brains of individuals with Huntington's Disease [105,106].

Previous experiments had demonstrated QA to be the most potent of the glutamate agonists tested. At high concentrations melatonin had been shown to partially protect cultured primary neurons. An investigation was thus made to investigate whether melatonin could protect hippocampal neurons against insults induced by QA. The hippocampus was selected as it is very densely populated with glutamate receptors, and is also known to be susceptible to QA attack.

5.2. HISTOLOGICAL INVESTIGATION

5.2.1. Introduction

Histology is derived from the Greek word for web or tissue, and involves the examination of preserved, sectioned and stained tissues. Most of our knowledge of internal tissue structure has come from this branch of science [307].

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

5.2.2. Materials and Methods

5.2.2.1. Chemicals and Reagents

Quinolinic acid and melatonin were purchased from Sigma Chemical Company (USA). 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. Sodium Pentobarbitone was purchased from Merck (Germany) and was made to a working concentration of 60 mg / kg , by diluting in water. All other chemicals were of the highest quality available and were purchased from commercial distributors.

5.2.2.2. Animals

Adult, male Wistar rats were cared for as described in section 2.2.2.2.

5.2.2.3. Surgical Procedures

i. Bilateral Intrahippocampal Injections of Quinolinic Acid

Male rats were anaesthetized with sodium pentobarbital (60 mg / kg i.p.) and placed in a stereotaxic frame. The rat skull was orientated according to the König and Klippel stereotaxic atlas [308]. After a sagittal cut in the skin of the skull, the bregma suture was located and holes were drilled with an electrical trepan drill at the following co-ordinates; 4.4 mm anterior, 2 mm lateral of the sagittal suture. Care was taken not to lesion the meninges. A Hamilton syringe, with a cannula of diameter 0.3 mm, was used to inject 1 µmol of quinolinic acid in 2 µl of PBS, 3 mm ventral of the dura. The injection was administered at a rate of 1 µl per minute and the cannula was left *in sutu* for a further 3 minutes following the drug injection, to allow for passive diffusion away from the canulla tip and to minimise spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals were kept in an incubator (37^{o}C) until they recovered from the anaesthesia.

ii. Sham Lesioned Rats

Rats to be used as controls were subjected to the surgical procedures outlined in section 5.2.2.3.(i). However, stereotaxic injections into the hippocampus were free of QA, and comprised only of PBS.

5.2.2.4. Treatment Regimes

Animals were divided into three groups as shown in Table 5.1.

Sweet Oil was administered sub-cutaneously at the same dosage as that given to the mel (+) rats. QA was dissolved in PBS made up to pH7.4.

Table 5.1: Treatment regime for each group of animals

Group	Received 20 mins prior to operation (s.c.)	Intrahippocampal injection consisted of:	Received daily doses for 5 day after operation
Control	Sweet Oil	PBS	Sweet Oil
Mel (-)	Melatonin in Sweet Oil (10 mg / kg)	1µmol QA in PBS	Melatonin in Sweet Oil (10 mg / kg)
Mel (+)	Sweet Oil	1µmol QA in PBS	Sweet Oil

Animals were treated for 5 days following surgery. Injections of sweet oil or melatonin were administered at the same time (14h00) each day, so as to increase circulating melatonin concentration, when levels should have been physiologically at their lowest.

5.2.2.5. Brain Removal

Rats were sacrificed and their brains were removed as described in section 2.2.2.4.

5.2.2.6. Histological Techniques

i. Fixing the brain

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.

Brains were removed from the skull as in section 2.2.2.5, and 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.

ii. 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 5.2.

Table 5.2: Procedure for embedding brains in paraffin wax

Step	Processing Agent	Time
1	70% Ethanol	1 hour
2	90% Ethanol	1 hour
3	Absolute Ethanol I	1 hour
4	Absolute Ethanol II	1 hour
5	Xylene I	1 hour
6	Xylene II	1 hour
7	Melted Paraffin Wax I (57^{o}C)	1 hour
8	Melted Paraffin Wax II (57^{o}C)	1 hour

iii. Blocking Out

The brain material was fixed into a block so that it could be cut with a microtome. A mould was formed from two L-shaped metal bars. These were coated with ethanol-glycine to prevent the block sticking to the mould. A little molten wax was added to the mould and allowed to harden. 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 whole mould was then immersed in a large dish of cold water. This was to enable the wax to solidify quickly, and prevent the formation of crystals that might have disrupted the tissue. The blocks were left overnight in cold water to ensure that the wax had completely solidified.

iv. Sectioning

The wax block was trimmed with a razor blade so that two of the sides were parallel, while the other two converged slightly (see Figure 5.1). 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.

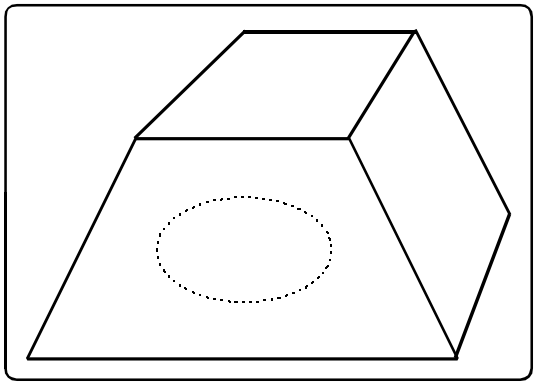


Figure 5.1: Diagram of wax block ready for sectioning with brain embedded in the centre

Sectioning was done using a rotary microtome. The microtome was set to cut sections of 10 µm thickness. As sections were cut they 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^{o}C) using a paint brush.

v. 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.

vi. Staining

The sections were Nissl stained using cresyl violet. This stains Nissl substances intense purple, the nuclei purple, and leaves the background clear [309].

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 5.3.

Table 5.3: Procedure for dewaxing and rehydrating brain sections

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

Sections were stained by placing in a 0.1% cresyl violet solution for 2 hours. 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 5.4.

Table 5.4: Procedure for dehydrating brain sections after staining

Step	Processing Agent	Time
1	Absolute Ethanol I	5 minutes
2	Absolute Ethanol II	5 minutes
3	Xylene I	5 minutes
4	Xylene II	5 minutes

vii. 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.

5.2.2.7 Photo-microscopy

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

5.2.3. Results

Neurons were investigated in an area adjacent to the site of the quinolinic acid injection. Care was taken not to select samples that may have been physically damaged by the cannula. All damage that resulted was therefore of a neurochemical nature and not a result of physical damage caused by the cannula. Neurons in the CA1 and CA3 regions of the hippocampus were examined. Neurons in the control group from both the CA1 region (Figure 5.2) and those in the CA3 region (Figure 5.5) appeared to be undamaged. The neurons in the CA1 region (Figure 5.4) and those in the CA3 region (Figure 5.7) of those animals treated with melatonin, also appeared to be undamaged. Neurons from the QA treated animals showed signs of degeneration by virtue of their roundness and swelling in the CA1 region (Figure 5.3), while the CA3 region (Figure 5.6) showed signs of degeneration.

5.2.4. Discussion

These results demonstrate that melatonin appears to protect against QA-induced neurotoxicity. QA acts at the NMDA receptors, allowing Ca^{2+}, Na^{+} and K^{+} to enter the neurons. The influx of these ions can upset the ionic balance of the neurons, leading to swelling and lysis [5,103]. This phenomenon appears to be occurring in Figure 5.3.

The influx of Ca^{2+} into the cell can also activate many free radical producing pathways, which can prove toxic to the neurons [310]. A combination of changes in osmotic pressure and free radical damage could have resulted in the neurodegeneration observed in Figure 5.6.

Melatonin appeared to protect neurons from the neurotoxic effects of QA as neurons co-treated with melatonin did not appear to swell (Figure 5.4), nor was there the neurodegeneration observed in the Mel (-) cultures in the CA3 region (Figure 5.6) when melatonin was administered (Figure 5.7).

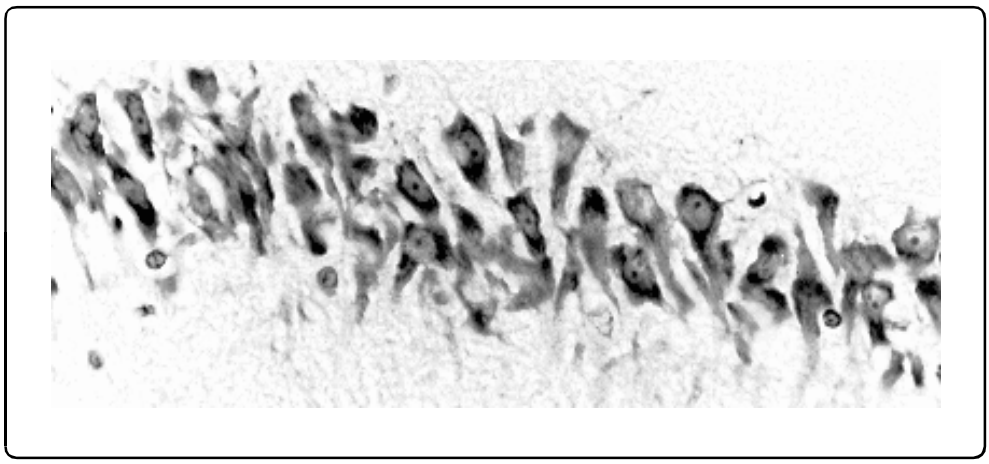


Figure 5.2: Micrograph of cells in the CA1 region of the hippocampus from a control animal. (Bar = 10µm)

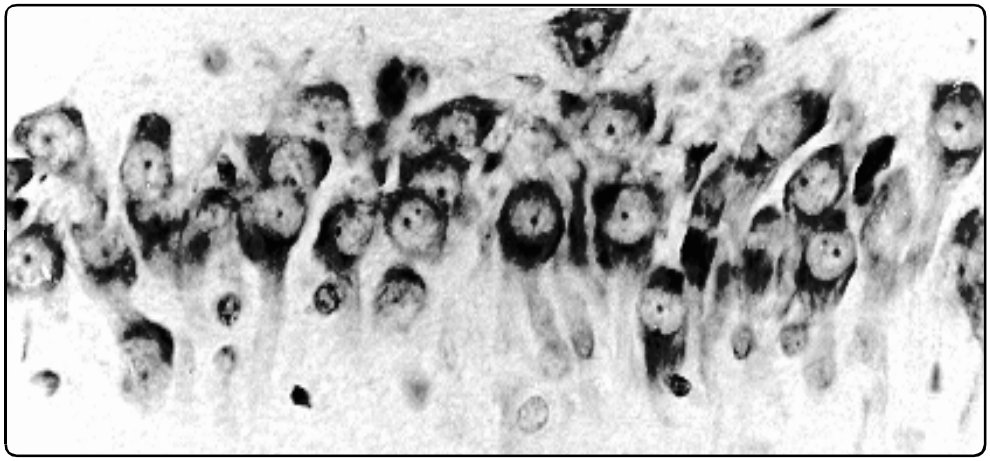


Figure 5.3: Micrograph of cells in the CA1 region of the hippocampus from an animal treated with quinolinic acid only. (Bar = 10µm)

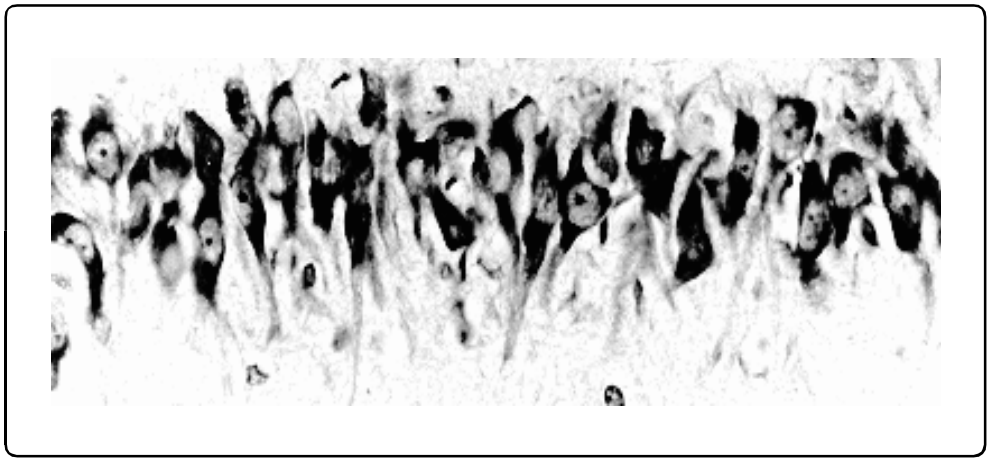


Figure 5.4: Micrograph of cells in the CA1 region of the hippocampus from an animal treated with quinolinic acid and melatonin. (Bar = 10µm)

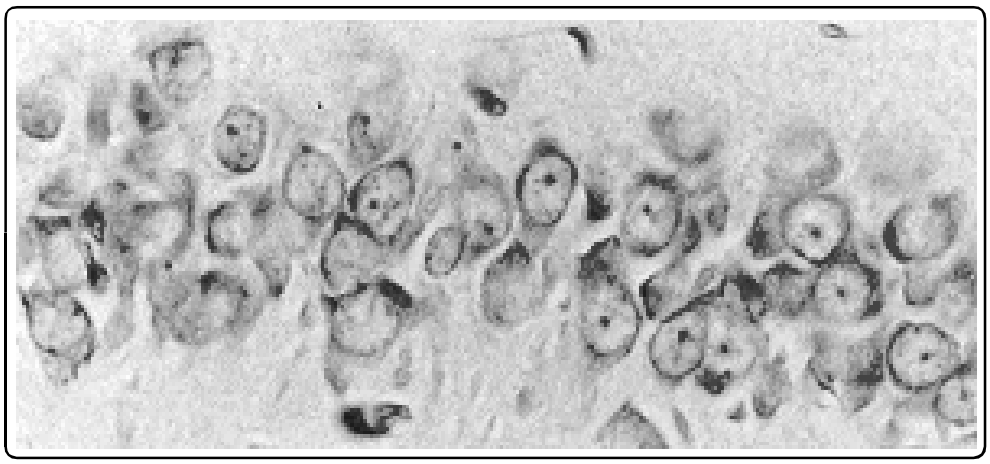


Figure 5.5: Micrograph of cells in the CA3 region of the hippocampus from a control animal (Bar = 10µm)

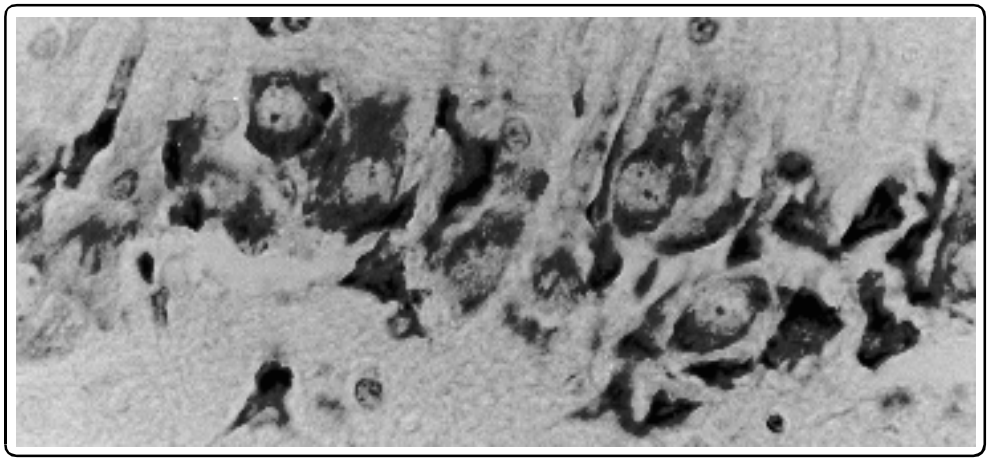


Figure 5.6: Micrograph of cells in the CA3 region of the hippocampus from an animal treated with quinolinic acid only. (Bar = 10µm)

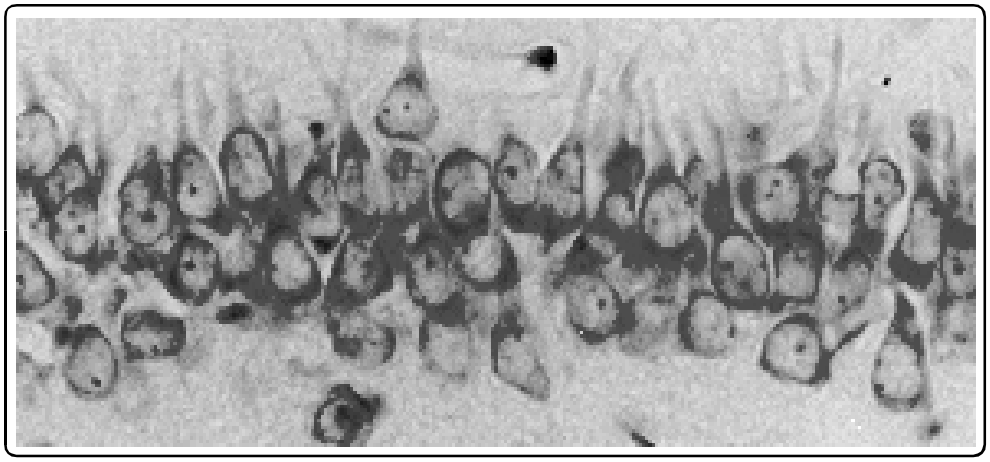


Figure 5.7: Micrograph of cells in the CA3 region of the hippocampus from an animal treated with quinolinic acid and melatonin. (Bar = 10µm)

5.3. EFFECT OF INTRAHIPPOCAMPAL ADMINISTRATION OF QUINOLINIC ACID ON ^{3}H-GLUTAMATE RECEPTOR BINDING

5.3.1. Introduction

In the previous experiment it was determined that melatonin appeared to offer protection against QA induced neurotoxicity in the hippocampus. Neurons of rats treated with melatonin, both before and after the intrahippocampal administration of QA, did not appear to swell in the CA1 region, nor undergo neurodegeneration in the CA3 region. The previous studies were however not able to quantify the protection offered by melatonin.

It was therefore decided to conduct receptor binding assays on hippocampal neurons of animals treated as in experiment 5.2. Glutamate receptors are known [175] to be the most abundant neuroreceptor found in the hippocampus, and it was therefore decided to determine whether there was a reduction in the number of glutamate receptors. If there was a reduction, it would demonstrate that there could possibly be a reduction in the number of viable neurons in the hippocampus. If receptor numbers remained constant, this would demonstrate that the number of viable neurons had remained the same after the QA injection.

5.3.2. Materials and Methods

5.3.2.1 Chemicals and Reagents

All chemicals used in the intrahippocampal injections were as described in section 5.2.2.1., while chemicals used for the glutamate binding studies were the same as those used in section 2.2.2.1.

5.3.2.2. Preparation of Synaptic Membranes

Rats were treated as described in section 5.2.2.4. On the fifth day following the operation, the rats were sacrificed and the brains removed as described in section 2.2.2.4.

The hippocampi were dissected from the brains according to the method of Glowinski and Iversen [311]. Synaptic membranes were prepared from the individual hippocampi using the method described in section 2.2.2.5.

5.3.2.3. Glutamate Binding Assay Procedure

The Glutamate binding assay was performed as described in section 2.2.2.7.

5.3.2.4. Statistical Analysis

The results were analysed as per the method in section 2.2.2.8.

5.3.3. Results

Saturation isotherms were plotted for control (Figure 5.8), Mel (-) (Figure 5.10), and Mel (+) (Figure 5.12) glutamatergic receptors on hippocampal synaptic membranes. The

data was converted to Scatchard plots so as to determine the Bmax for each of the treated groups (see Figures; 5.9, 5.11 and 5.13).

The Bmax values obtained in each case are shown in Table 5.5. QA caused a significant ($P<0.001$) decrease in the number of glutamate binding sites, while those animals which received a combination of QA and melatonin (mel (+)) showed a less dramatic decrease in binding sites compared to control animals.

At the same time, the receptors of animals that received melatonin in conjunction with QA injections had very similar Kd values to those of control animals. The receptors of QA treated animals that did not receive melatonin had a much higher glutamate binding affinity than either control or melatonin treated animals.

Table 5.5: Comparison of Bmax values obtained from glutamate receptor binding studies. (n=3) ([a] $P<0.001$ vs Control; [b] $P<0.01$ vs Control)

Test	Treatment	Bmax (fmol ^{3}H-glutamate /mg protein)	Kd
1	PBS (Control)	97 ± 1.86	116
2	Quinolinic Acid	29 ± 2.63[a]	86
3	Quinolinic Acid and Melatonin	80 ± 2.52[b]	119

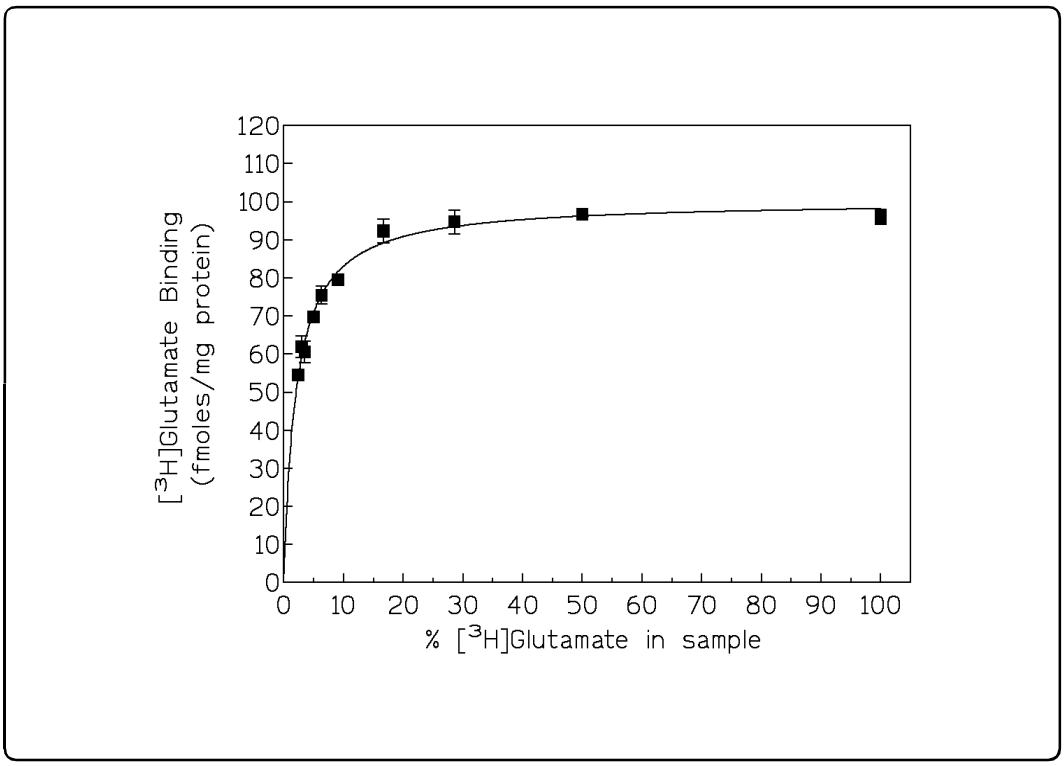


Figure 5.8: Saturation isotherm of ^{3}H-glutamate binding to hippocampal synaptic membranes from control rats. (n=4)

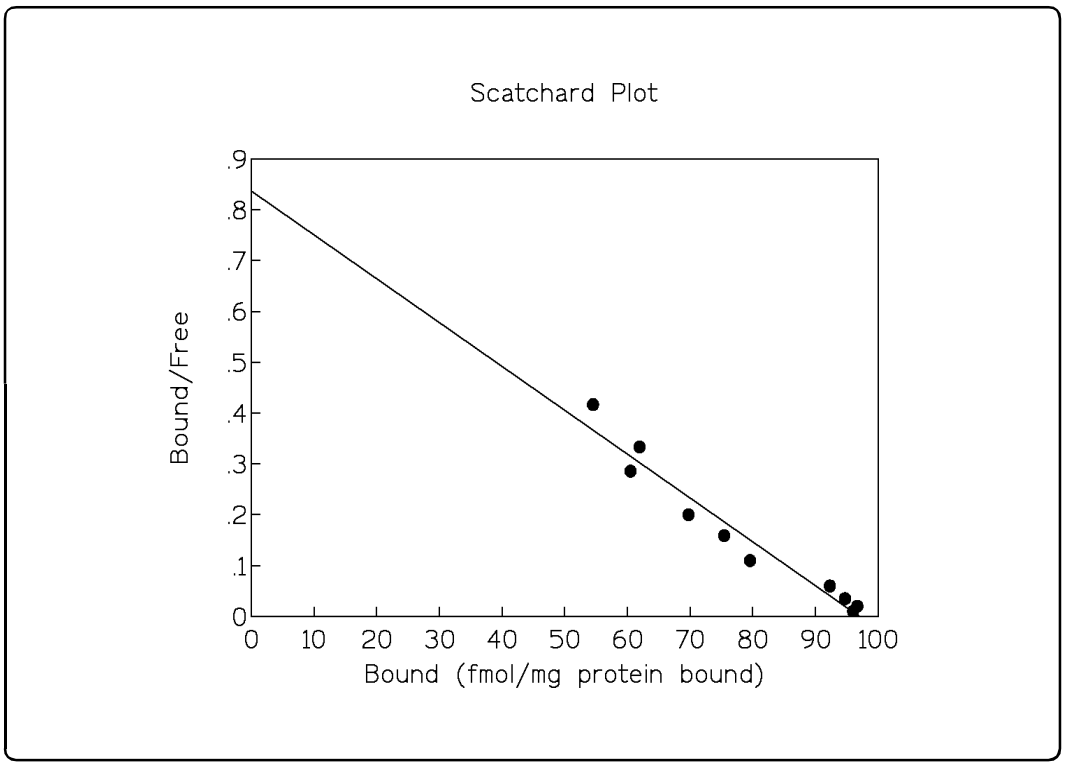


Figure 5.9: Scatchard plot of ^{3}H-glutamate binding to hippocampal synaptic membranes from control rats.

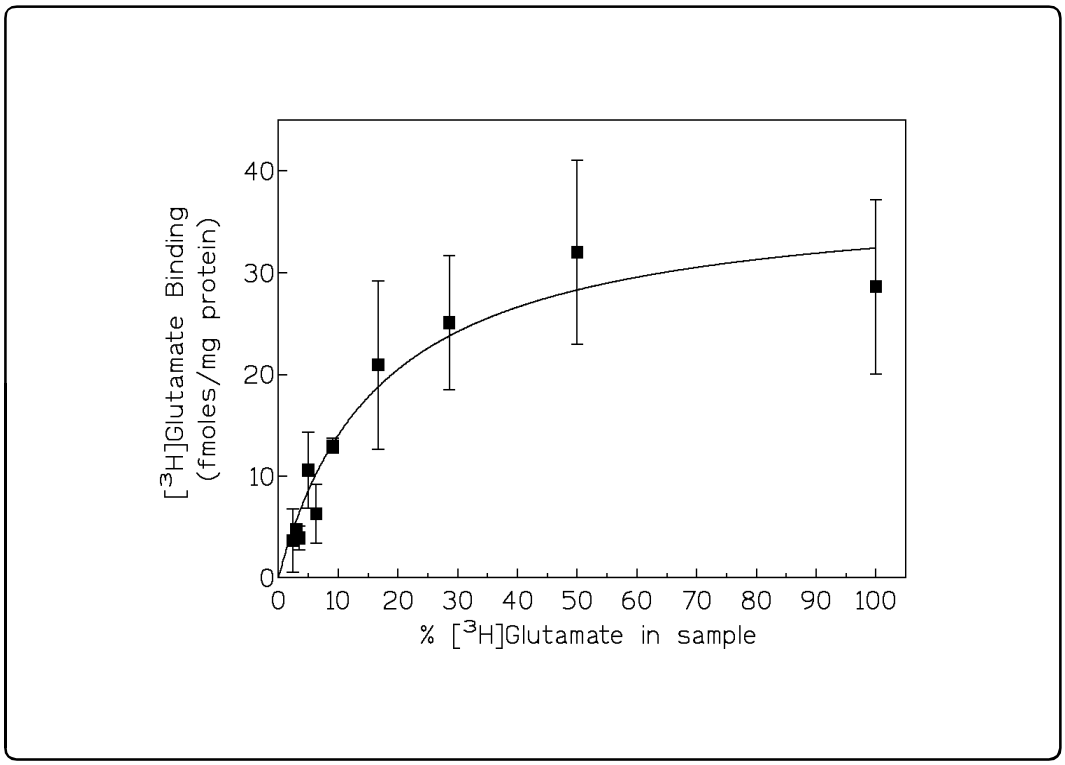


Figure 5.10: Saturation isotherm of ^{3}H-glutamate binding to hippocampal synaptic membranes from quinolinic acid treated rats (mel (-)). (n=4)

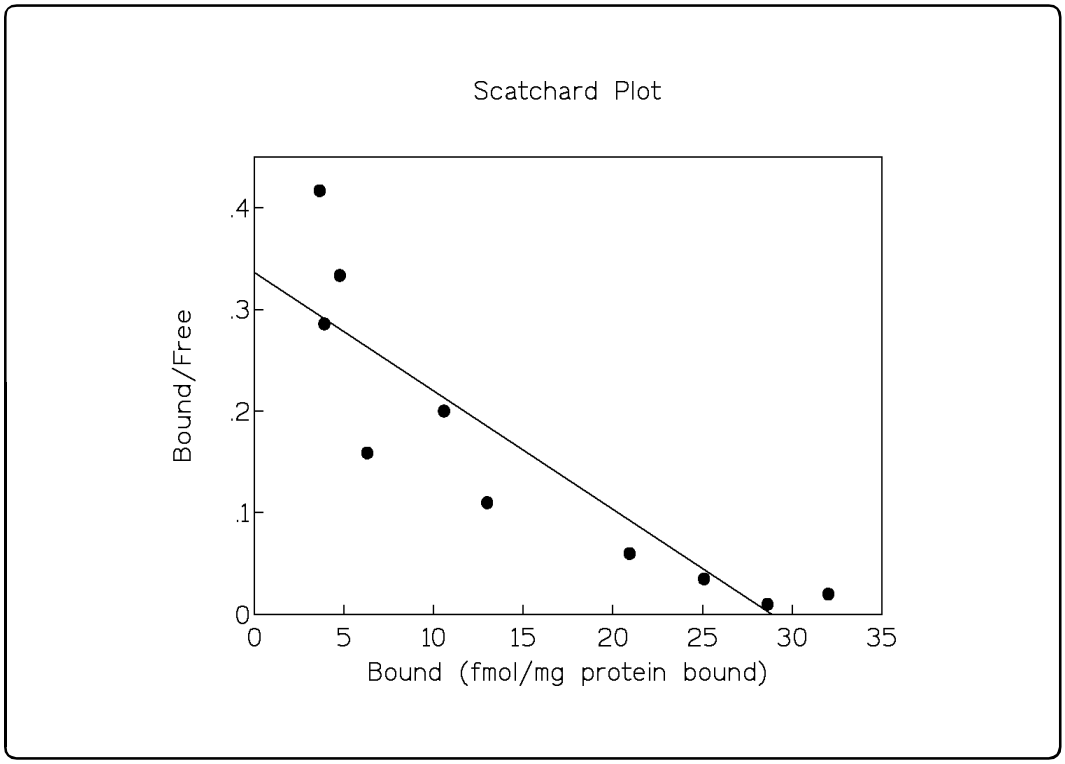


Figure 5.11: Scatchard plot of ^{3}H-glutamate binding to hippocampal synaptic membranes from quinolinic acid treated rats (mel (-)).

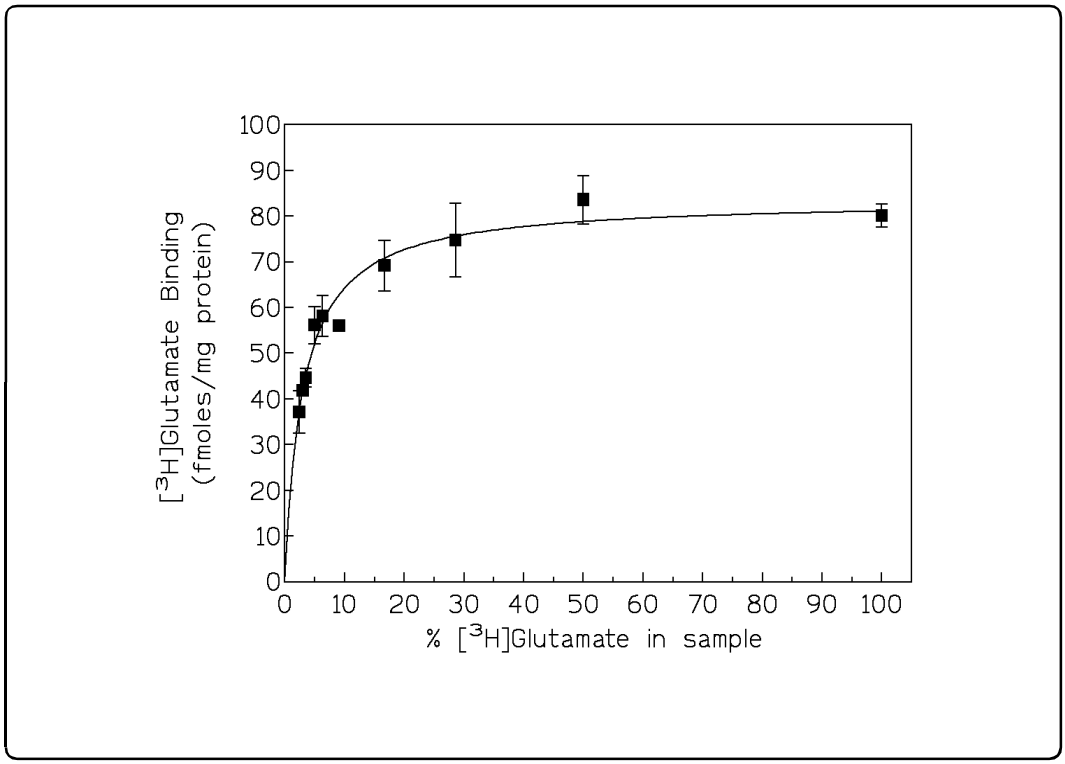


Figure 5.12: Saturation isotherm of ^{3}H-glutamate binding to hippocampal synaptic membranes from quinolinic acid and melatonin treated rats (mel (+)). (n=4)

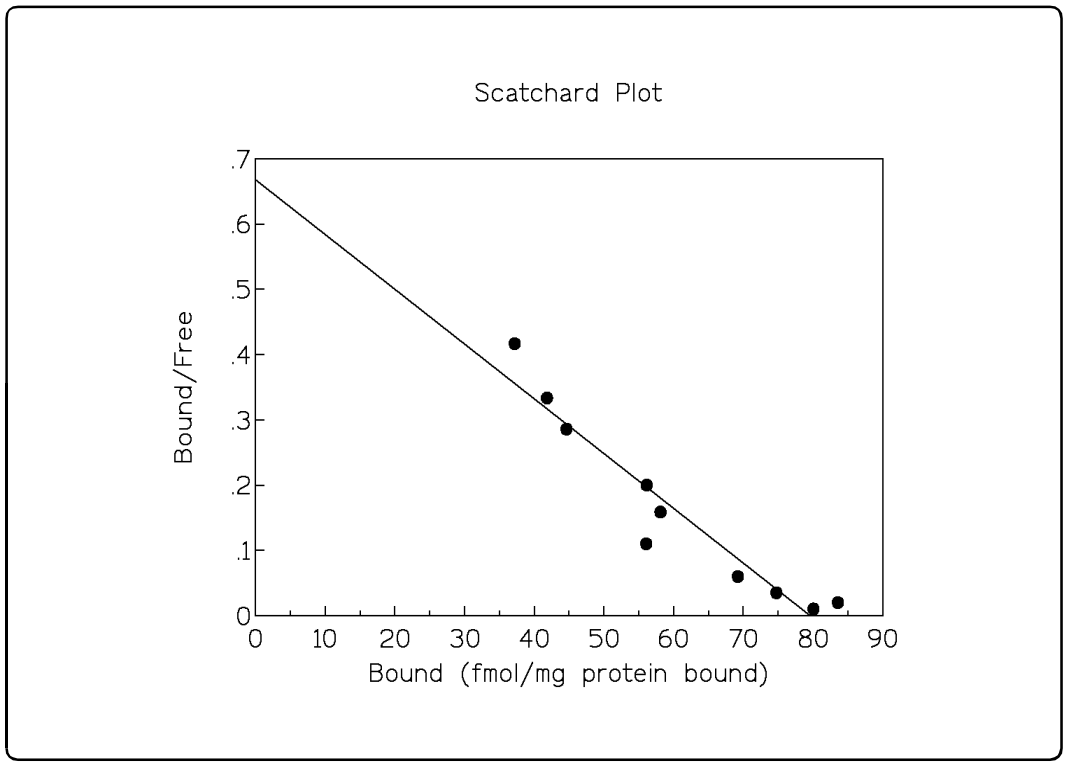


Figure 5.13: Scatchard plot of ^{3}H-glutamate binding to hippocampal synaptic membranes from quinolinic acid and melatonin treated rats (mel (+)).

5.3.4. Discussion

The results showed that QA treated animals that did not receive melatonin, had a very significant reduction in receptor numbers, while animals treated with melatonin did not exhibit the same sharp reduction in receptor numbers. QA treatment had also changed the nature of those receptors that were present on the neurons, in that they had a higher affinity for glutamate than did the control neurons. The receptors of animals that had been treated with both melatonin and glutamate had similar Kd values to control animals, and did not appear to have been affected by the QA treatment. These results demonstrate that melatonin treatment partially prevents the QA-induced reduction in glutamate receptor numbers. This could indicate that there are more functional neurons present in both control, and melatonin and QA co-treated animals, than in animals that only received QA. Melatonin thus appears to have protected the neurons.

5.4. CONCLUSION

The histological analysis of the hippocampus of animals treated with QA, showed that there was a very definite swelling of neurons in the CA1 region and degradation of neurons in the CA3 region. Neurons obtained from animals treated with 5 mg / kg melatonin, both before and for 5 days following the QA injection, did not appear to have been affected by the QA. These results appeared to show that melatonin offered neuroprotection against intrahippocampal injections of QA. However, these studies did not explain the method by which melatonin offers protection, nor the level of protection offered.

The binding studies attempted to quantify any neurodegeneration that may be induced by the QA. Results showed that there was a significant reduction in the Bmax values for both animals that did and those that did not receive melatonin prior to and following the QA injection. The reduction in the Bmax value was however considerably greater in those animals that did not receive melatonin.

In the QA treated animals that did not receive melatonin, the reduction in receptor numbers could be the result of a loss of neurons. For those animals that received both melatonin and quinolinic acid, melatonin treatment was not able to maintain receptor numbers at the same level as control animals. This could be because there was still some damage to the neurons, and melatonin was not able to completely protect them. However, the results obtained in section 2.2 show that melatonin clearly causes a down regulation in glutamate receptor numbers, while not affecting Kd values. The results obtained in this experiment could therefore indicate the natural decrease in glutamate receptor numbers that occurs from the administration of pharmacological doses of melatonin to rats.

The results from the *in vivo* studies thus show that melatonin appears to protect hippocampal neurons from degeneration caused by intrahippocampal injections of QA.

Together with the results obtained from the cell culture studies in Chapter 4, it could be argued that melatonin appears to be a very effective agent at protecting neurons from the glutamate receptor agents. It was therefore decided to investigate further the mode by which melatonin brings about this neuroprotection.

CHAPTER 6

EFFECT OF GLUTAMATE, N-METHYL-D-ASPARTATE, QUINOLINIC ACID, KAINIC ACID AND MELATONIN ON INTRACELLULAR CALCIUM LEVELS

6.1. INTRODUCTION

Calcium ions are the most abundant cations found in vertebrates, where they are used largely in a structural role in bones [312]. Ca^{2+} is also used widely in processes such as muscular contraction, stimulus secretion coupling, transduction of hormonal information and neuronal conduction [153,313]. More recently the discovery of calcium-dependent regulatory proteins [119] has suggested a role for Ca^{2+} in the modulation of various metabolic processes within the cell. The ability of Ca^{2+} to regulate metabolic processes within the cell, is believed to be due to the "sticky" nature of Ca^{2+} ions, which allows them to bind to a variety of ligands such as proteins and phosphate groups [314]. Ca^{2+} appears to damage cellular proteins and membranes by activating proteases such as the calpains [315], and by promoting free radical production via the activation of lipases [316] or nitric oxide synthetase [317]. Any changes in the regulation of Ca^{2+} will therefore cause a loss of control to many other intracellular processes.

EAA stimulated Ca^{2+} influx promotes free radical accumulation in neurons by several means. These include the activation of phospholipases that induce phospholipid hydrolysis, accumulation of arachidonic acid, and the subsequent oxidation by cyclo-oxygenase and lipoxygenase [316], conversion of xanthine-dehydrogenase to xanthine-oxidase which catalyses the oxidation of hypoxanthine to xanthine and generates the superoxide radical [143]; and activation of nitric oxide synthase [318] which generates nitric oxide, which reacts with $O^{-}_{2}\cdot$ to form the highly destructive peroxynitrite radical [317].

An investigation was made into the effect of agonist stimulation on intracellular calcium levels and whether melatonin had any effect on Ca^{2+} accumulation in neurons. Atomic absorption spectroscopy was used to determine the intracellular Ca^{2+} concentration in the primary neuronal cultures.

6.2. EFFECT OF THE GLUTAMATE RECEPTOR AGONISTS ON INTRACELLULAR CALCIUM ACCUMULATION

6.2.1. Introduction

The glutamate receptors allow the influx of Ca^{2+} into neurons. Under normal conditions this leads to a change in the cell membrane polarity, which allows neurons to transfer impulses from one to another. Excess stimulation by the EAA's can lead to intracellular Ca^{2+} levels rising. The normal mechanisms that remove Ca^{2+} from the neurons are not able to cope with this sudden influx. As Ca^{2+} is able to regulate many enzymatic reactions, the higher intracellular Ca^{2+} concentrations in neurons can initiate many neurotoxic reactions.

Both the NMDA and KA receptors [87,191] are reported to allow Ca^{2+} influx into cells. This investigation was conducted to determine changes in intracellular Ca^{2+} concentrations following neuronal stimulation by the various glutamate receptor agonists used in the previous experiments.

6.2.2. Materials and Methods

6.2.2.1. Chemical Reagents

Calcium Chloride ($CaCl_2$) standard was purchased from Unilab (South Africa). Glass Culture Tubes (10 x 75 mm) were purchased from Kimble (USA).

6.2.2.2. Glassware Preparation

All glassware was treated by the modified method of Kelly *et al* [319]. The procedure involved soaking the glassware in 25% (v / v) HNO_3 for 48 hours followed by another 48 hour soak in Milli-Q water containing 5 g / l of the chelating resin iminodiacetic acid.

6.2.2.3. Cell Culture Procedure

The neurons were treated with agonists as described in section 3.2.2.5.

The reactions were stopped at specific times to determine the intracellular Ca^{2+} concentration. The various groups are shown in Table 6.1.

Table 6.1: Experimental Groups

Group	Test
Control	Poured off MEM. No Agonist. Assayed immediately
0 min	Agonist Added. Immediately removed and assayed cells
20 min	Cells treated with agonist for 20 minutes. Cells assayed at the end of this time
60 min	Cells treated with agonist for 20 minutes, then allowed to recover in fresh MEM. Assayed 60 minutes after initiation of insult
18 hours	Cells treated exactly as in section 3.2.2.5. 18 hours after the insult, the cells were assayed

6.2.2.4. Calcium Assay Procedure

The existing media was poured out of the incubation flasks. A 2 ml aliquot of 50 mM Tris-HCl buffer (pH 7.4) was added to each incubation flask. The cells were scraped off the surface of the flasks with a cell scraper and the cell suspensions were poured into new glass culture tubes. The inside of the flask was rinsed with an additional 2 ml of Tris-HCl buffer to remove any cells, and this too was poured into culture tubes to give a final volume of 4 ml. The tubes were centrifuged at 3 500 x g for 10 minutes at 4^{o}C, before the supernatant was poured off. The pellets were resuspended in 1 ml of Tris-HCl buffer. A 100 µl fraction of the suspension was kept for cell enumeration using a haemacytometer. The tubes were then centrifuged at 4^{o}C at 3 500 x g for a further 10 minutes, before the supernatant was discarded. The pellets were resuspended in 0.2 ml of 68% nitric acid, before being boiled at 100^{o}C for ± 10 minutes, or until all the

solid material had been digested. An 800 µl aliquot of Milli-Q water was added to each tube to give a final volume of 1 ml. Ca^{2+} levels of the solution were determined by flame atomic absorption spectroscopy, using a GBC 909 Atomic Absorption Spectrophotometer. The fuel source was nitric oxide; acetylene; slit width was 0.5 nm; wavelengh set at 422.7 nm; and current 3 mA. The absorbance values were converted to µM Ca^{2+} concentrations for a standard curve (Appendix 2) generated from a standard $CaCl_2$ solution.

The data was expressed as µg calcium / 10^6 cells, and were analysed as described in section 2.2.2.8.

6.2.3. Results

The results are shown in Figures 6.1 - 6.4. Glutamate and QA caused a significant increase in intracellular Ca^{2+} levels at 0 minutes and 20 minutes, while KA and NMDA caused significant increases at 0, 20, and 60 minutes. None of the agonists caused a significant increase in intracellular Ca^{2+} when readings were taken 18 hours after the insult.

In all cases, the intracellular Ca^{2+} levels were highest at 0 minutes. KA caused the largest increase in intracellular Ca^{2+} concentration at 0 minutes, with a five fold increase, while glutamate caused the least with only a 3.4 fold increase.

6.2.4. Discussion

Application of the glutamate agonists caused a rapid increase in intracellular Ca^{2+} as measured by atomic absorption spectroscopy. All agonists caused at least a 3.4 fold increase in intracellular Ca^{2+} almost immediately. Ca^{2+} is known [72] to rapidly move into neurons following the opening of the Ca^{2+} channels by the glutamate receptor agonists. These results would indicate that this is indeed the case.

The agonists were applied for 20 minutes. At the end of this time, neurons were assayed and intracellular Ca^{2+} concentrations determined. Twenty minutes following the agonist insult, there was a significant increase in Ca^{2+} levels compared to controls. However Ca^{2+} levels were lower in all cases to those at 0 minutes. It therefore appeared that there was a general trend to remove Ca^{2+} from the neurons, even though the agonists would still have been stimulating the receptors. Active processes to remove Ca^{2+} from the neurons would have been in operation. These processes require ATP, which could result in the leakage of $O^{-}_{2}\cdot$ form the electron transport chain.

After 60 minutes Ca^{2+} levels were lower than the earlier two readings in all cases. Ca^{2+} levels were significantly higher than control cultures for KA and NMDA, while QA and glutamate treated neurons appeared to have already brought Ca^{2+} levels close to those of controls, just 1 hour following the insult. The agonists were no longer present in the incubation medium and the cells should have had time to reduce intracellular Ca^{2+} levels.

Eighteen hours following the agonist insult, all cultures had reduced Ca^{2+} levels so that they were no longer significantly different to control cultures. Intracellular processes had therefore been able to remove the excessive Ca^{2+} from the neurons, or neurons had died and so Ca^{2+} and other ions could move freely into and out of the dead cells, with their disrupted cell membranes.

This experiment has demonstrated that following an agonist insult, intracellular Ca^{2+} levels are significantly increased for at least one hour in the case of NMDA and KA. Levels for all agonists were significantly increased for the first 20 minutes. This increase in intracellular Ca^{2+} initiates a number of intracellular processes, that could be potentially neurotoxic to neurons.

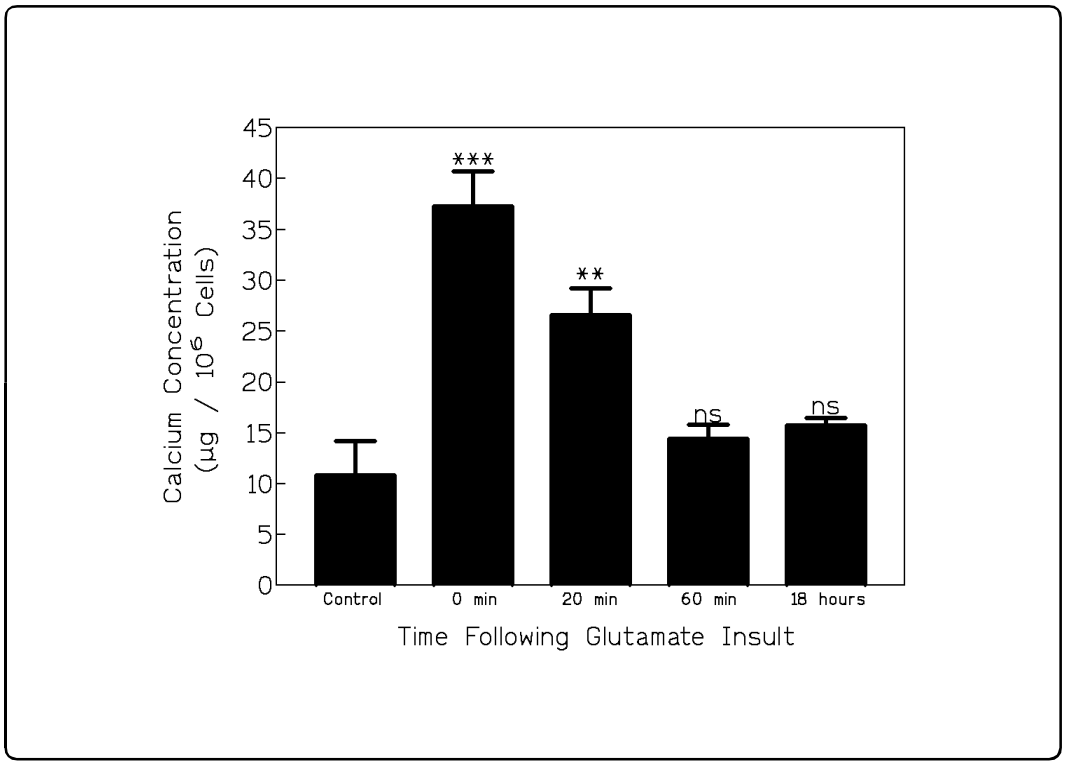


Figure 6.1: Effect of glutamate insult on intracellular calcium over time. Values represent the mean ± SEM (n=3) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls)

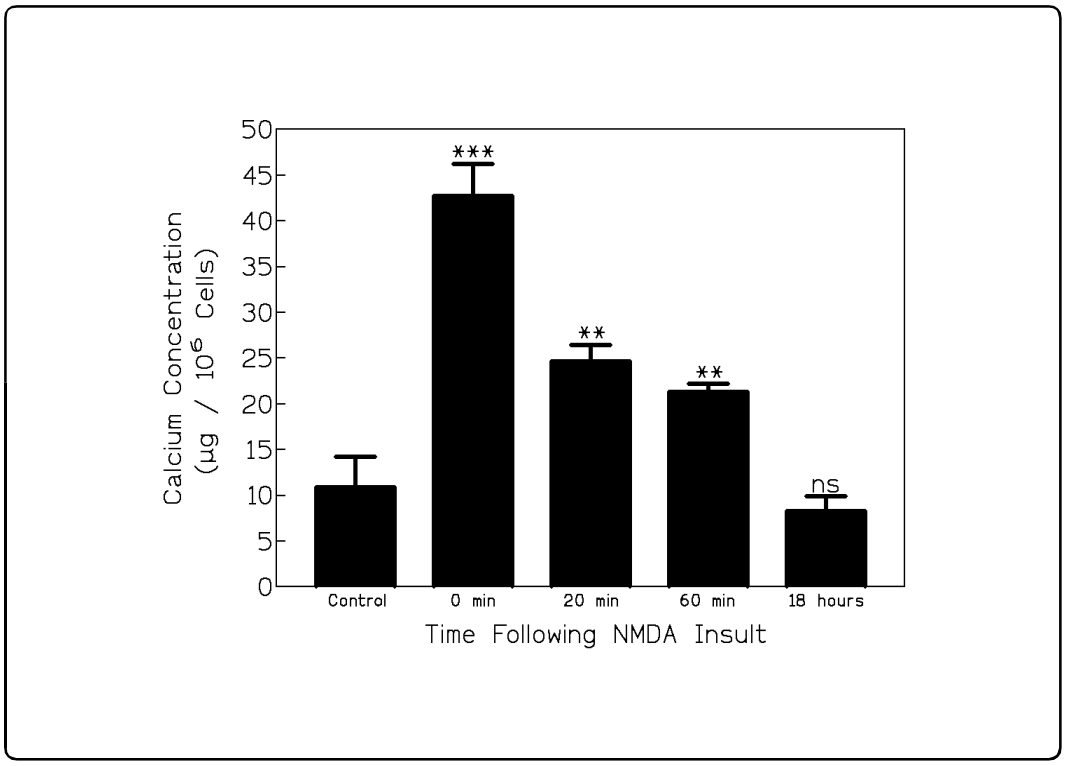


Figure 6.2: Effect of NMDA insult on intracellular calcium over time. Values represent the mean ± SEM (n=3) ($^{ns}P>0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to zero controls)

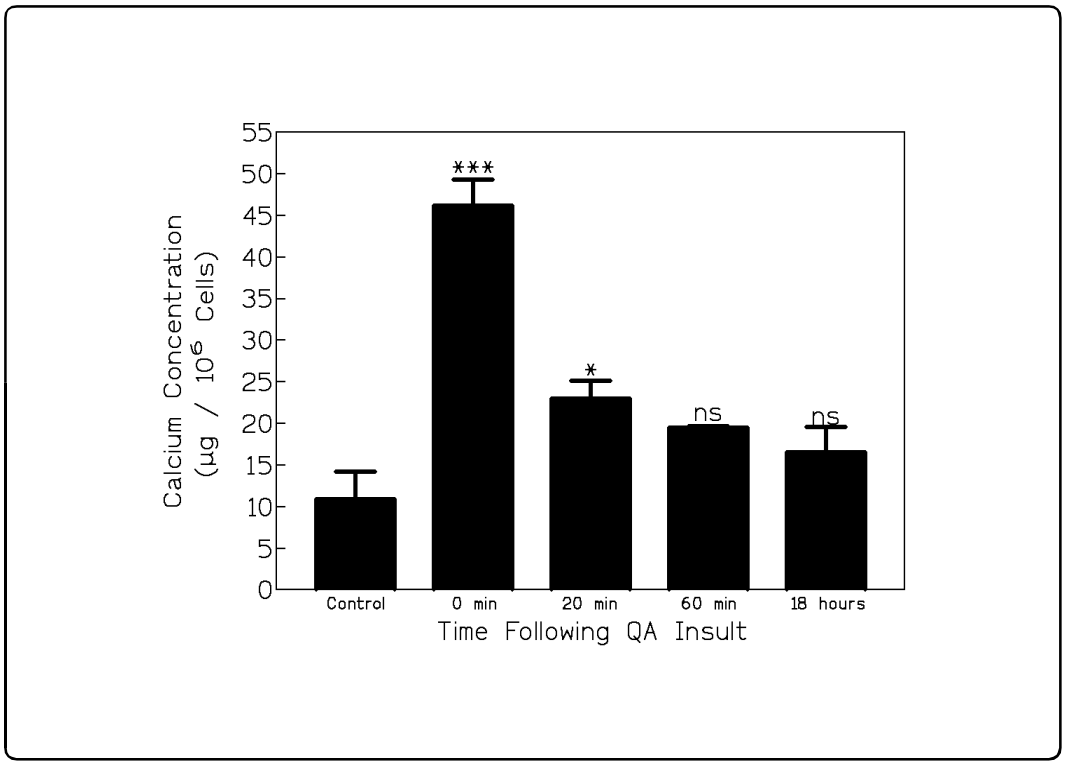


Figure 6.3: Effect of QA insult on intracellular calcium over time. Values represent the mean ± SEM (n=3) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{***}P<0.001$ in comparison to zero controls)

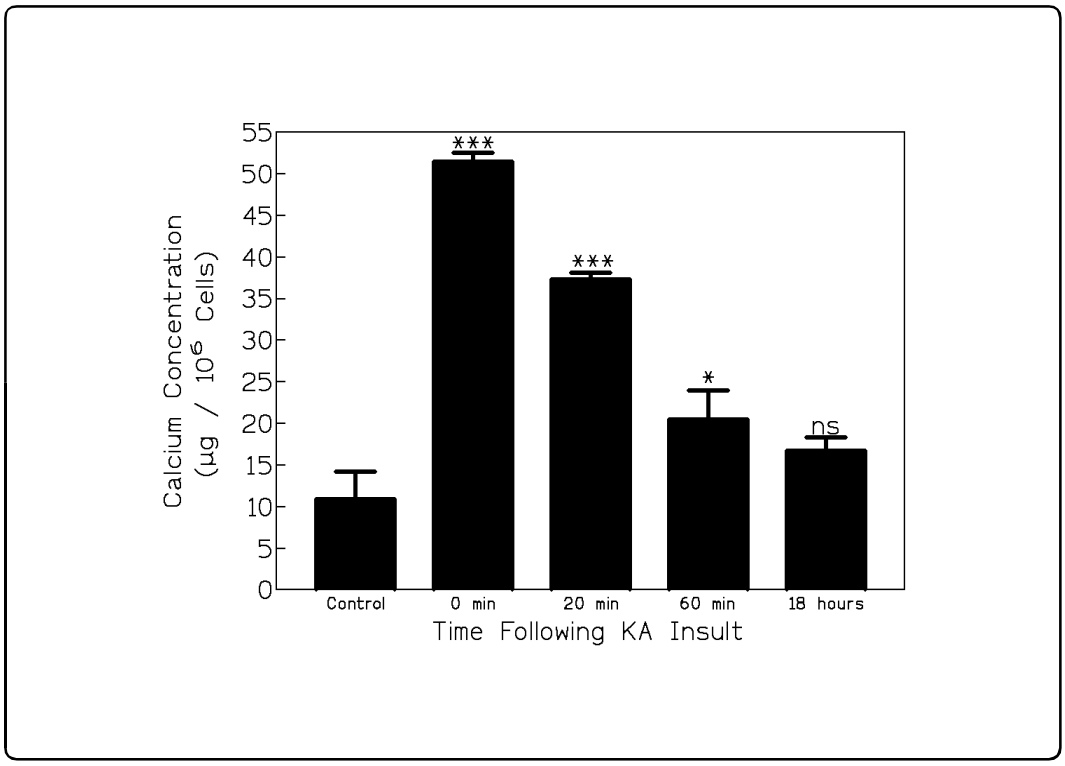


Figure 6.4: Effect of KA insult on intracellular calcium over time. Values represent the mean ± SEM (n=3) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{***}P<0.001$ in comparison to zero controls)

6.3. EFFECT OF MELATONIN ON INTRACELLULAR CALCIUM ACCUMULATION

6.3.1. Introduction

In Chapters 4 and 5 it had been demonstrated that melatonin was able to protect neurons from glutamate receptor agonist induced neurotoxicity. The method by which melatonin brought on this protection was however not known.

Intracellular Ca^{2+} is known to initiate a number of cellular processes that can prove neurotoxic to neurons. It was therefore decided to investigate whether the presence of melatonin during the 20 minute agonist incubation, and for 40 minutes after the application of the agonist, had any effect on intracellular Ca^{2+} levels.

6.3.2. Materials and Methods

6.3.2.1. Chemical Reagents

Melatonin (500 µM) was prepared as in 4.2.2.1. All other reagents were prepared as in 6.2.2.1.

6.3.2.2. Experimental Procedure

Glassware was prepared as per 6.2.2.2, while neuronal cultures were prepared as described in section 3.2.2.3.

Neurons were treated as in section 4.3.2.2, the sole difference being that melatonin (500 µM) was added to the MEM both during, and after, the agonist insult.

6.3.2.3. Calcium Assay Procedure

The method described in 6.2.2.5. was employed. Cultures were compared using a student t test to check for significance.

6.3.3. Results

Intracellular Ca^{2+} concentrations were compared between cultures that had received melatonin, and those that had not, one hour after the initiation of the agonist insult. In all cases the presence of melatonin did not cause a significant difference in intracellular Ca^{2+} levels compared to samples that had not received melatonin. Table 6.2. lists the results obtained.

Table 6.2: Effect of melatonin on Intracellular Ca^{2+} concentrations following treatment with glutamate receptor agonists (n = 5)

Agonist	Melatonin (μM)	Ca^{2+} (μg / 10^6 cells)	SEM	P value
Glutamate	0	14.36	1.418	0.3161
Glutamate	500	16.127	0.6095	
NMDA	0	21.289	1.807	0.5093
NMDA	500	17.856	4.387	
QA	0	19.511	0.2867	0.2607
QA	500	15.681	2.918	
KA	0	20.455	3.496	0.2634
KA	500	26.649	3.245	

6.3.4. Discussion

These results demonstrate that melatonin has no effect on intracellular Ca^{2+} accumulation within neurons. The effect of melatonin as a protective agent must be at some other level in neurons.

6.4. CONCLUSIONS

It has been demonstrated that application of the glutamate receptor agonists causes an increase in intracellular Ca^{2+} [59]. The increase occurs almost instantaneously as the increase in intracellular Ca^{2+} is used during neurotransmission under normal conditions. When neurons are overstimulated, rapid increases in intracellular Ca^{2+} concentrations result [320]. Neurons attempt to reduce the higher than normal intracellular Ca^{2+} concentrations but it can take hours before intracellular Ca^{2+} levels are retuned to levels that are no longer significantly higher than untreated cultures. Mitochondria are actually involved in the removal of Ca^{2+} from cells [128,129,321].

Ca^{2+} initiates a number of potentially neurotoxic processes within the neuron [322]. Many of these processes involve the production of $O^{-}_{2}\cdot$ ions. Another major area of $O^{-}_{2}\cdot$ production is the mitochondrion. Electron "leakage" occurs during the electron transport chain because the gradient has been disrupted by the Ca^{2+} accumulation within the mitochondria. This results in the production of $O^{-}_{2}\cdot$. The large influx of Ca^{2+} into the cell also requires active transport to remove the ions out of the neuron [323], which necessitates the production of ATP in the mitochondria. While the electron gradient is disrupted on the mitochondrial membrane, ATP cannot be produced very effectively [324]. This results in a drop in ATP levels and the further generation of $O^{-}_{2}\cdot$ by the mitochondria.

In Chapters 4 and 5, melatonin had been shown to protect neurons against neurotoxicity induced by certain glutamate receptor agonists. Melatonin does not act at the glutamate receptors, so an intracellular mode of action is envisaged. Ca^{2+} being the first neurotoxic step following excessive agonist stimulation has been investigated, but melatonin does not act at this level. An increase in intracellular Ca^{2+} concentrations is one of the first undesirable events that occur in a neuron following excessive stimulation by the EAA's [325]. Any method that could inhibit the rapid rise in intracellular Ca^{2+} accumulation would therefore be potentially protective to neurons. Melatonin does not appear to have

any influence on Ca^{2+} accumulation, so any neuroprotection afforded by melatonin must be at a further stage along the EAA-induced neurotoxic cascade. It was therefore decided to investigate whether melatonin was able to scavenge the potentially neurotoxic $O^{-}_{2}\cdot$ produced following Ca^{2+} stimulation.

CHAPTER 7

ROLE OF MELATONIN AS A SUPEROXIDE ANION SCAVENGER

7.1. INTRODUCTION

Reactive oxygen species are continually generated intracellularly in aerobic organisms. If allowed to react uncontrollably these are capable of causing extensive damage to DNA, carbohydrates, proteins and lipids within the cell [326].

Cells possess highly effective antioxidant defence mechanisms to protect neurons against free radical species [327]. These protective mechanisms can be classified into two main categories [146], namely the enzymatic defence mechanisms, which include superoxide dismutase, and the non-enzymatic cellular antioxidants, which include Vitamins A and E. Under normal conditions these defence 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 [139].

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 [143], and by activation of certain enzymes.

Overstimulation of EAA receptors results in the influx of Ca^{2+}, Na^{+}, and K^{+} into neuron [8]. 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. Enzymes such as xanthine dehydrogenase [324] and nitric oxide synthetase [167] are also activated by $Ca^{2+,}$ so the influx of Ca^{2+} into the neurons results in greater activity of these enzymes. One of the by-products of the activities of these enzymes is the production of $O^{-}_{2}\cdot$.

Superoxide anions can be very reactive and can lead to neurodegeneration [328]. It has been proposed [310] that the EAA's are neurotoxic because they lead to increased production of $O^{-}_{2}\cdot$. Melatonin has been shown to reduce neurodegeneration under a number of different conditions that involve the production of $O^{-}_{2}\cdot$ [234]. As melatonin did not affect intracellular Ca^{2+} accumulation in neurons following an EAA insult, it was decided to investigate whether melatonin could affect the production of the potentially neurotoxic $O^{-}_{2}\cdot$ ions in neurons.

7.2. EFFECTS OF GLUTAMATE, N-METHYL-D-ASPARTATE, QUINOLINIC ACID AND KAINIC ACID ON SUPEROXIDE ANION FORMATION IN RAT FOREBRAIN HOMOGENATE

7.2.1. Introduction

Previous experiments (see Chapter 3) had demonstrated that the glutamate receptor agonists were able to cause neurodegeneration. One mechanism that had been proposed for these neurotoxic effects was the production of $O^{-}_{2}\cdot$. It was therefore decided to investigate whether incubating homogenised rat forebrain with increasing concentrations of the glutamate receptor agonists caused an increase in $O^{-}_{2}\cdot$ production.

The nitro-blue tetrazolium (NBT) assay, which is generally accepted [304] 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.

7.2.2. Materials and Methods

7.2.2.1. Chemical Reagents

Nitro-blue tetrazolium and Nitro-blue diformazan (NBD) were purchased from the Sigma Chemical Company (USA). Glacial acetic acid was purchased from Holpro Chemical Company (South Africa). All other chemicals used were of the highest quality available from commercial sources.

A 0.1% NBT solution was made by dissolving the NBT in ethanol before making up to the required volume with Milli-Q water. The final ethanol concentration in the incubation flasks was less than 1.5%.

7.2.2.2. Preparation of Standards

NBD was used as a standard. A series of reaction tubes, each containing appropriate aliquots of NBD dissolved in acetic acid were prepared. A standard curve (Appendix 3) was determined by measuring the absorbance at 20µM intervals. The absorbance was read at 560 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer.

7.2.2.3. Preparation of Rat Forebrain Homogenate

Adult, male Wistar rats were used for the experiment. These were cared for as explained in section 2.2.2.2.

On the morning of the experiment, the brains were removed as described in section 2.2.2.4. The forebrains were weighed before being homogenised in ice cold 50 mM Tris-HCl buffer (pH 7.4) in a glass homogeniser to produce a 10% (w / v) homogenate.

7.2.2.4. The Nitro-Blue Tetrazolium Assay

A modified method of Ottino and Duncan [304] was used for this assay. To 1 ml samples of brain homogenate, 0.4 ml of a 0.1% NBT solution and 100 µl of the relevant agonist being tested was added and allowed to incubate for 90 minutes at 37^{o}C. On termination of the incubation, the suspensions were centrifuged at 3000 x g for 10 minutes. The supernatant was poured off and the pellet was dissolved in 2.5 ml glacial acetic acid.

The relative absorbance values were measured at 560 nm. The amount of NBD formed was determined from the standard curve in Appendix 3. Final results were expressed as µM diformazan produced / mg protein.

Protein assays were carried out as described in section 2.2.2.6, and all results were analysed as described in section 2.2.2.8.

7.2.2.5. Exposure of Brain Homogenate to the Different Agonists

Stock solutions were prepared so that on addition of 100 µl of agonist dissolved in Milli-Q water, the stock solution would be diluted down to the correct incubation concentration. The following agonists were tested; glutamate, NMDA, QA, and KA. Agonists were tested at the following concentrations; 0, 0.25, 0.5, 0.75 and 1 mM.

7.2.3. Results

The results are shown in Table 7.1. In all cases the addition of agonists to the brain homogenate was not able to cause significant changes ($P>0.05$) in the production of $O^{-}_{2}\cdot$.

7.2.4. Discussion

The production of $O^{-}_{2}\cdot$ in rat forebrain homogenate was not significantly changed by exposure of any of the agonists.

Although it would have been expected that the production of $O^{-}_{2}\cdot$ would increase in response to the agonist insult, this did not occur. The most probable reason for this is that rat forebrain homogenate was used. If glutamate, or any of the other agonists, were to cause an increase in $O^{-}_{2}\cdot$ production, the process would be initiated by an increase in intracellular Ca^{2+} levels. By using homogenate, any Ca^{2+} control was effectively destroyed, and so the binding of the agonists to the receptors would have had no regulating effects. This would explain why similar results were obtained for all of the different concentrations of agonists used.

Because Ca^{2+} would have been present to initiate many of the $O^{-}_{2}\cdot$ producing pathways, there would still have been production of superoxide anions. It would therefore still be possible to investigate whether melatonin removes superoxide anions from rat forebrain homogenate.

Table 7.1: Diformazan produced at different agonist concentration (n = 5)

Agonist	Concentration (mM)	Diformazan (μM/mg Protein)	SEM
Glutamate	0	75.748	1.455
Glutamate	0.25	82.534	3.511
Glutamate	0.5	84.916	4.097
Glutamate	0.75	83.756	3.669
Glutamate	1	81.636	1.956
NMDA	0	87.624	6.024
NMDA	0.25	89	6.529
NMDA	0.5	87.644	6.366
NMDA	0.75	85.996	6.507
NMDA	1	86.108	6.307
QA	0	91.778	6.788
QA	0.25	85.478	5.623
QA	0.5	83.618	6.891
QA	0.75	85.602	4.257
QA	1	83.938	3.11
KA	0	79.418	2.957
KA	0.25	75.9	4.737
KA	0.5	77.534	5.964
KA	0.75	80.674	3.806
KA	1	78.816	2.606

7.3. MELATONIN AS A SUPEROXIDE ANION SCAVENGER IN RAT FOREBRAIN HOMOGENATE

7.3.1. Introduction

In the previous experiment it had been demonstrated that although the glutamate receptor agonists did not appear to have any effect on $O^{-}_{2}\cdot$ production, the production of $O^{-}_{2}\cdot$ never the less took place in the bain homogenate.

Mitochondrial dysfunction and free radical-induced oxidative damage have been implicated in the pathogenesis of several neurodegenerative diseases, such as Alzheimer's Disease and Huntington's Disease [234]. Melatonin has been suggested to be a very powerful anti-oxidant that could protect against neurotoxicity. It was thus decided to investigate whether melatonin was able to act as an $O^{-}_{2}\cdot$ scavenger in rat forebrain homogenate.

7.3.2. Materials and Methods

7.3.2.1. Chemical Reagents

Melatonin was purchased from Sigma Chemical Company (USA). It was dissolved in absolute ethanol and then made up with water so that the final solution was a 50% ethanol solution.

7.3.2.2. Experimental Protocol

Rat forebrain homogenate was prepared as in section 7.2.2.3, while the NBT assay was performed as in section 7.2.2.4. The only alteration was that melatonin was added instead of 100 µl agonist. The homogenate was treated with two different concentrations of melatonin, viz. 100 µM and 500 µM.

7.3.3. Results

The results are shown in Figure 7.1. Melatonin at 100 µM was unable to cause a significant reduction in $O^{-}_{2}\cdot$ concentration as measured by the NBT assay. Treatment of the homogenate with 500 µM melatonin caused a significant reduction in the amount of NBD produced.

7.3.4. Discussion

These results demonstrate that at high concentrations, melatonin was able to act as a superoxide anion scavenger. The fact that melatonin was only able to protect when used at high concentrations, demonstrates that melatonin was not a potent $O^{-}_{2}\cdot$ scavenger. Melatonin (500 µM) was only able to reduce the amount of diformazan produced by 13.2% when compared to untreated controls.

These results show that although melatonin is able to act as an antioxidant in forebrain homogenate, is has only limited neuroprotective effects, and must be present in high concentrations.

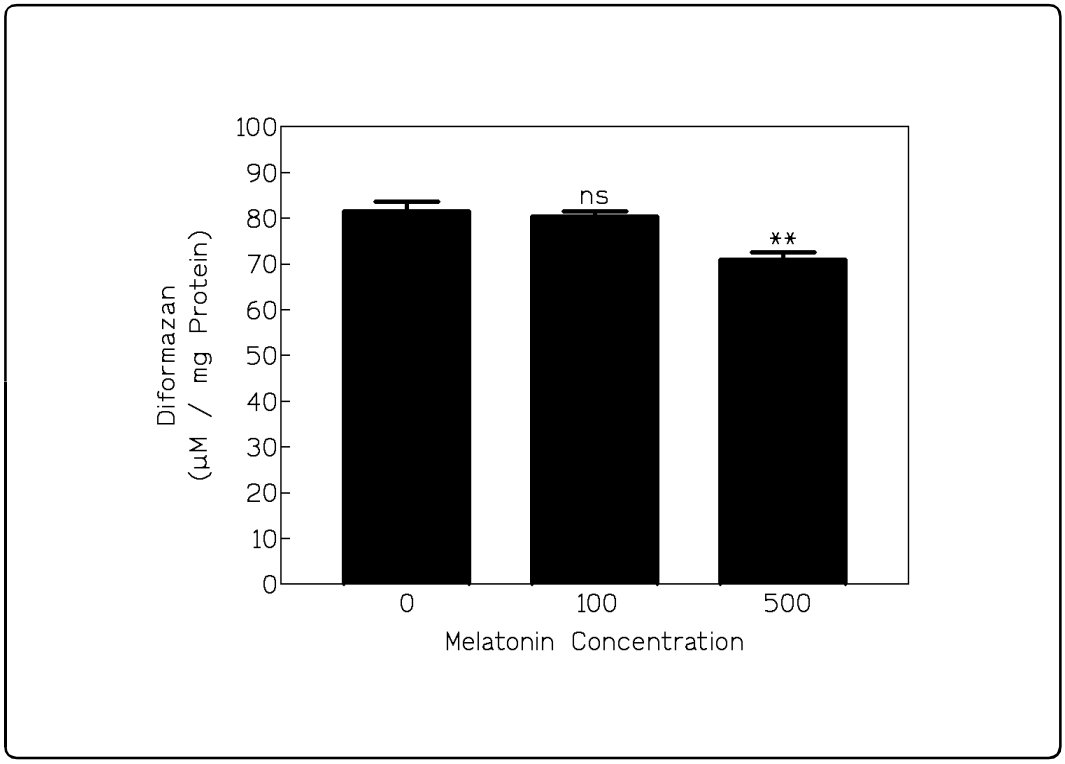


Figure 7.1: Effect of melatonin on production of superoxide anions in rat brain homogenate. Values represent the mean ± SEM (n = 5) (^{ns} P>0.05 , ** P<0.01 in comparison to zero control)

7.4. SUPEROXIDE ANION SCAVENGING PROPERTIES OF MELATONIN IN NEURONAL CELL CULTURES

7.4.1. Introduction

Previous experiments using rat forebrain homogenate had demonstrated that melatonin was able to scavenge $O^{-}_{2}\cdot$ when applied in high doses, and that the glutamate receptor agonists had no effect on $O^{-}_{2}\cdot$ production. It was thought that the reason that the agonists had no effect was because the integrity of the neurons had been disrupted by homogenisation.

It was therefore decided to investigate the effects of co-treatment of neuronal cell cultures with the same glutamate receptor agonists and melatonin. It was hoped that this method would give an indication of the degree to which $O^{-}_{2}\cdot$ production was affected by the various agonists, and also demonstrate whether melatonin had any $O^{-}_{2}\cdot$ scavenging properties.

7.4.2. Methods and Materials

7.4.2.1. Chemical Reagents

Cell culture media preparation was carried out as explained in section 3.2.2.2. The agonists were prepared in sterile Milli-Q water as described in 7.2.2.5, and melatonin was prepared as described in 7.3.2.1.

7.4.2.2. Preparation of Neuronal Cultures

Primary neuronal cultures were established as explained in section 3.2.2.3.

7.4.2.3. Experimental Procedure

A modified method of Ottino and Duncan [304] was used to measure $O^{-}_{2}\cdot$ production. Ten day old cultures were washed with 3 ml of HBSS, after which 3 ml of fresh MEM was placed in each culture flask. A 1.2 ml aliquot of NBT was added to each flask, after which the cultures were treated as shown in Table 7.2.

After a 3 hour incubation at 37°C, the cells were scraped off the surface of the culture flasks using a cell scraper. The media containing the suspended cells was poured into centrifugation tubes. Five hundred microlitres of the cell suspension was kept for enumeration using a haemacytometer, while the rest was centrifuged for 10 minutes at 4000 x g. The pellets were resuspended in 1ml of glacial acetic acid.

The absorbance of the extracts was measured at 560 nm, and converted to µM diformazan using the NBD standard curve (Appendix 3). Final results were expressed as µM diformazan / 10^4 cells. All results were analysed as described in section 2.2.2.8.

7.4.3. Results

The effect of various glutamate receptor agonists on superoxide anion production was first investigated. All of the neuronal cultures incubated with the agonists showed significant increases in the production of $O^{-}_{2}\cdot$ (see Figure 7.2). QA caused the greatest increase, with $O^{-}_{2}\cdot$ production being increased 14.6 fold. Glutamate caused the least increase in $O^{-}_{2}\cdot$ production, but it still increased production 8.1 fold.

The effectiveness of melatonin as a $O^{-}_{2}\cdot$ scavenger varied depending on the agonist used. Melatonin was able to protect against glutamate (Figure 7.3) and NMDA (Figure 7.4) induced $O^{-}_{2}\cdot$ production when used at 500 µM. Low doses of melatonin offered no significant protection against $O^{-}_{2}\cdot$. Melatonin was able to offer significant protection against QA (Figure 7.5) at both high and low concentrations. Melatonin did not appear

to offer any reduction in KA (Figure 7.6) induced $O^{-}_{2}\cdot$ production with either of the doses used.

Table 7.2: Experimental groups used to measure superoxide anion production in primary neuronal cell cultures.

Group	Agonist (350 μM)	Melatonin Concentration (μM)
1	Control (Zero)	0
2	Glutamate	0
3	Glutamate	100
4	Glutamate	500
5	NMDA	0
6	NMDA	100
7	NMDA	500
8	QA	0
9	QA	100
10	QA	500
11	KA	0
12	KA	100
13	KA	500

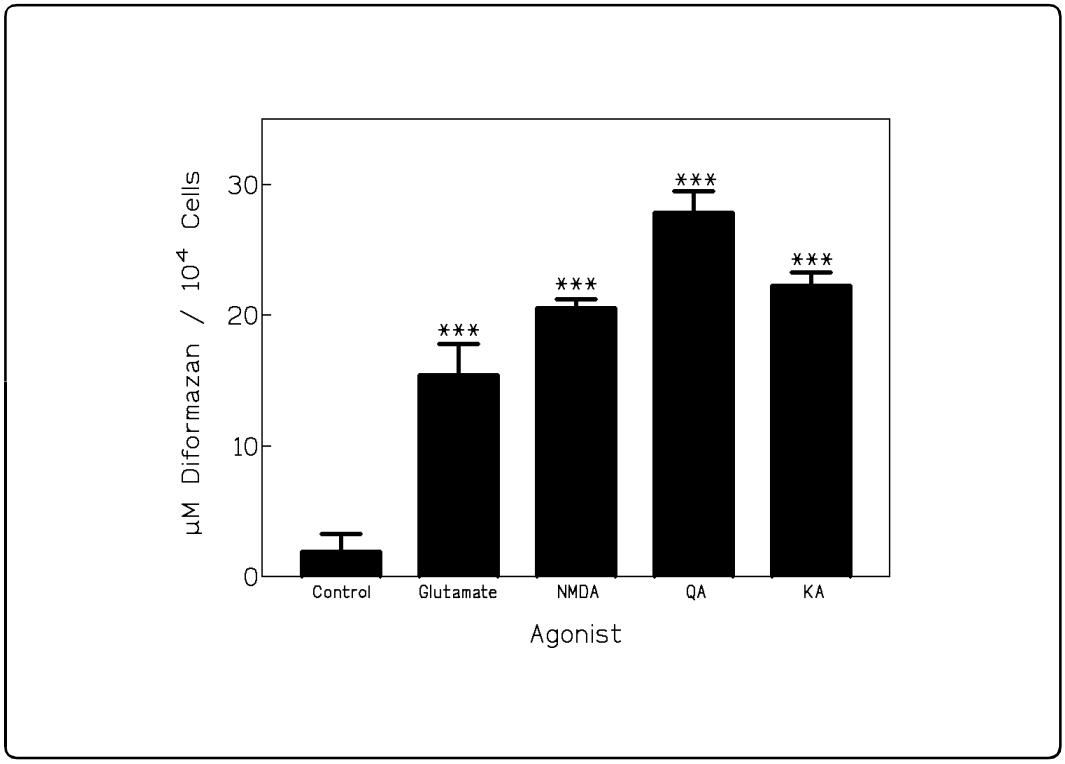


Figure 7.2: Effect of various glutamate receptor agonists on superoxide anion production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (*** P<0.001 in comparison to zero control)

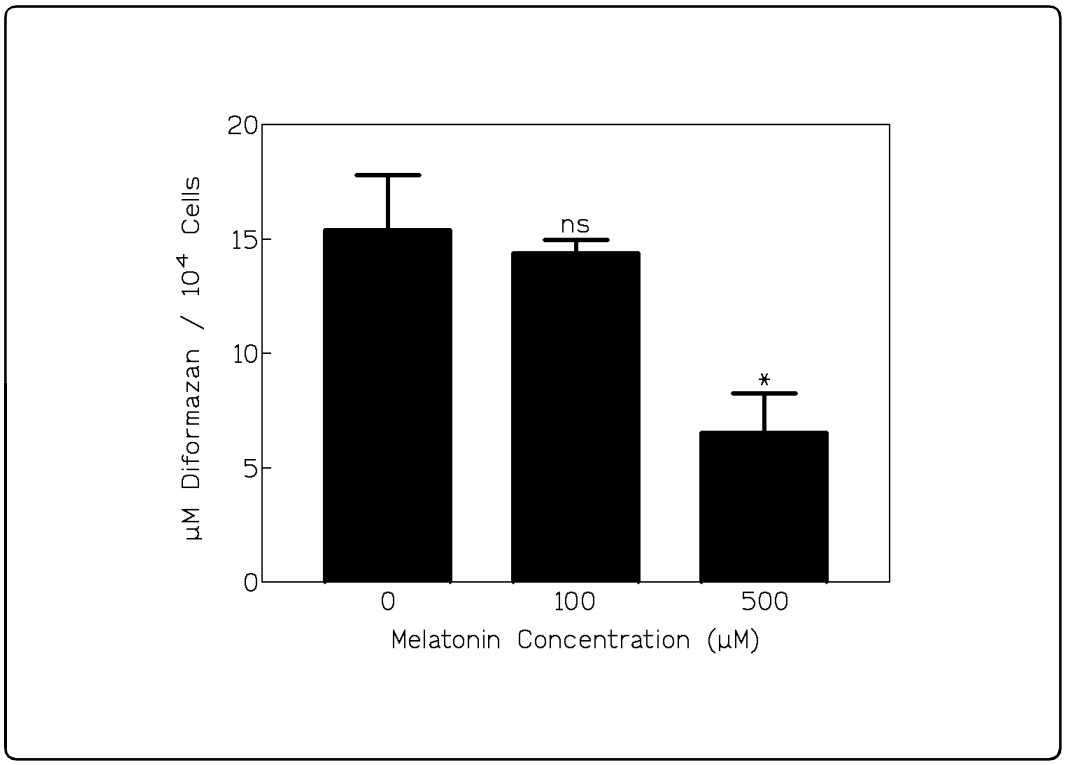


Figure 7.3: Effect of various concentrations of melatonin on glutamate-induced superoxide anion production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (^{ns} P> 0.05; * P< 0.05 in comparison to zero control)

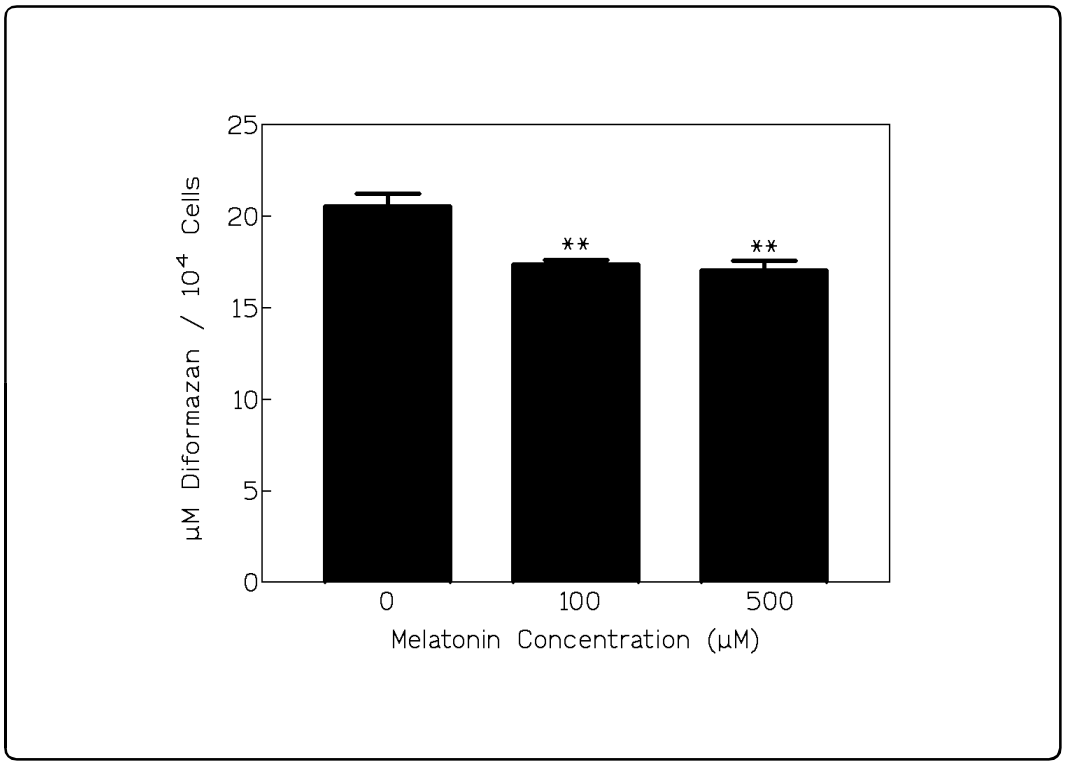


Figure 7.4: Effect of various concentrations of melatonin on NMDA-induced superoxide anion production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (** P< 0.01 in comparison to zero control)

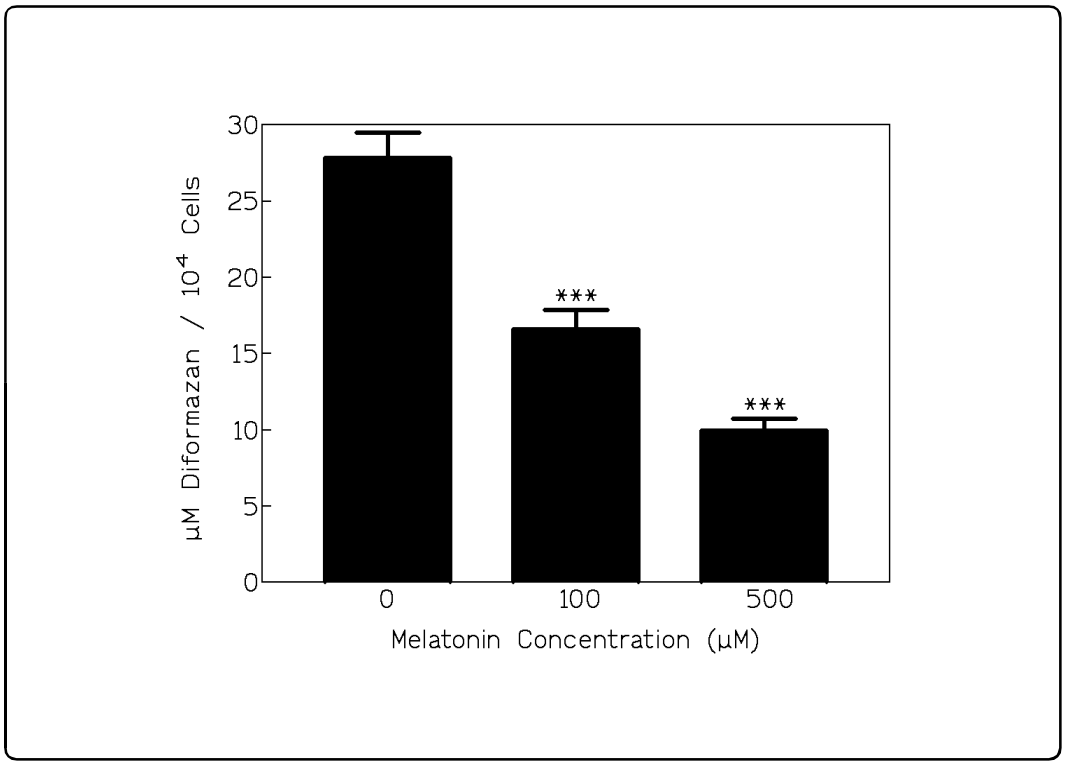


Figure 7.5: Effect of various concentrations of melatonin on QA-induced superoxide anion production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (*** P< 0.001 in comparison to zero control)

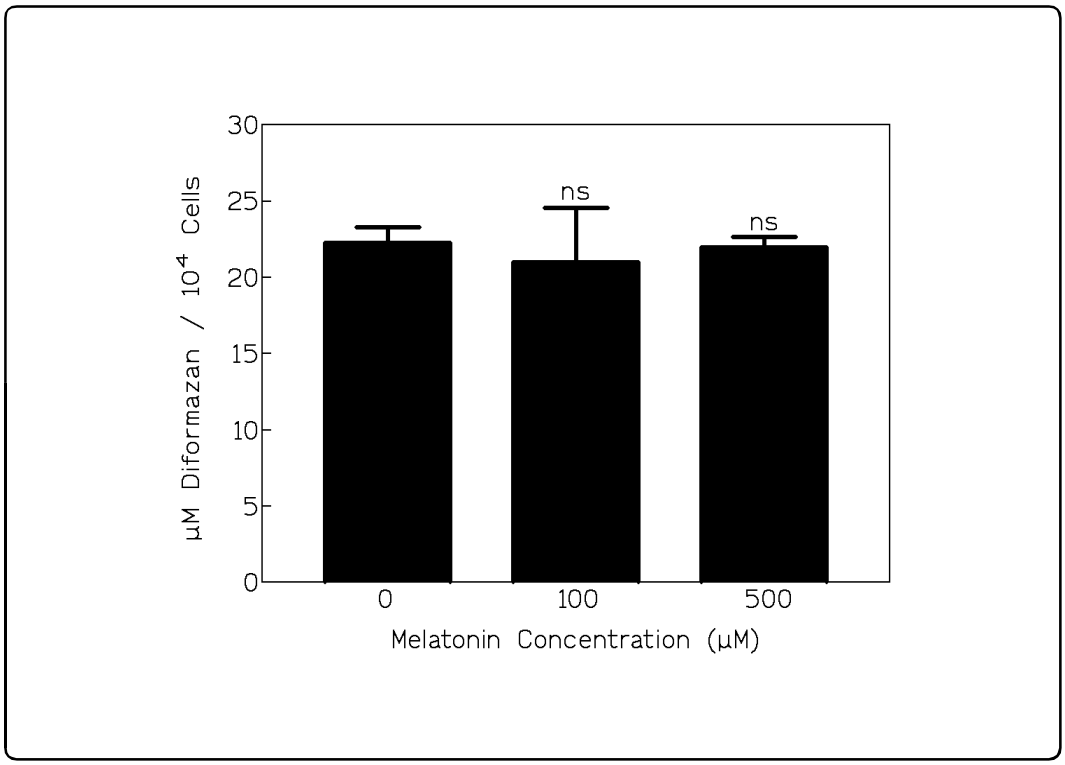


Figure 7.6: Effect of various concentrations of melatonin on KA-induced superoxide anion production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (^{ns} P> 0.05 in comparison to zero control)

7.4.4. Discussion

All of the agonists increased $O^{-}_{2}\cdot$ production in neuronal cells. The increase in $O^{-}_{2}\cdot$ production could be caused by the increased intracellular Ca^{2-} brought on by overstimulation of the glutamate receptors. $O^{-}_{2}\cdot$ is toxic to neurons [310] and could lead to neuronal death.

Melatonin has been reported [234] to be an effective $O^{-}_{2}\cdot$ scavenger. This experiment investigated the $O^{-}_{2}\cdot$ scavenging properties of melatonin brought about by the application of agonists. Melatonin was able to significantly scavenge $O^{-}_{2}\cdot$ when applied at 500 µM to neurons treated with glutamate. Low doses (100 µM) of melatonin caused no significant reduction in $O^{-}_{2}\cdot$ levels. Melatonin was also able to significantly reduce the amount of $O^{-}_{2}\cdot$ able to react with NBT in neurons treated with NMDA and QA. Both of these agonists act at the NMDA receptor. Melatonin was particularly effective at scavenging $O^{-}_{2}\cdot$ from neurons treated with QA. This is significant as QA caused the greatest increase in $O^{-}_{2}\cdot$ levels when compared to controls. Melatonin did not appear to offer protection against KA-induced $O^{-}_{2}\cdot$ production.

In no case did melatonin bring $O^{-}_{2}\cdot$ levels down to those of control cultures that did not receive treatment. The fact that melatonin was able to reduce $O^{-}_{2}\cdot$ levels in neurons treated with three of the agonists, and not in cultures treated with KA, is also of interest. More studies would need to be carried out to investigate the exact mechanism that was occurring.

7.5. CONCLUSION

These results demonstrated that melatonin was able to reduce $O^{-}_{2}\cdot$ levels in rat brain homogenate. High doses of melatonin (500 µM) were able to significantly reduce $O^{-}_{2}\cdot$ levels, although the effect appeared to be rather limited.

Because the neurons had been disrupted when working with rat brain homogenate, it was found that addition of the agonist had no effect on $O^{-}_{2}\cdot$ production. It was therefore decided to investigate the effect of the agonists and melatonin on primary neuronal cultures.

The agonists tested, all caused a significant increase in $O^{-}_{2}\cdot$ production. QA caused the largest increase in $O^{-}_{2}\cdot$ production. As $O^{-}_{2}\cdot$ is a very neurotoxic agent, this could explain the reason why QA was also the most neurotoxic agonist in the tests carried out in Chapter 3.

Melatonin was able to offer some protection against $O^{-}_{2}\cdot$ production by scavenging the $O^{-}_{2}\cdot$ in most cultures. High doses (500 µM) of melatonin were much more effective than the low (100 µM) doses at scavenging $O^{-}_{2}\cdot$. With glutamate treated neurons, 500 µM melatonin was able to more than halve the amount of $O^{-}_{2}\cdot$ able to interact with NBT, while in the QA treated neurons, 500 µM melatonin cut the amount of $O^{-}_{2}\cdot$ that reacted with NBT down to a third. Melatonin was however not able to offer any significant $O^{-}_{2}\cdot$ scavenging activity with KA treated neurons. The reasons for this are not clear, and more investigations are necessary to determine why melatonin was ineffective against one agonist, but effective against the others.

In conclusion, it was shown that the glutamate receptor agonists cause a large increase in $O^{-}_{2}\cdot$ production. Melatonin was able to scavenge $O^{-}_{2}\cdot$ and was able to significantly lower $O^{-}_{2}\cdot$ concentrations in rat brain homogenate. In primary neuronal cultures, 500 µM melatonin was able to significantly reduce the amount of free $O^{-}_{2}\cdot$ able to react with NBT

in cultures treated with glutamate, NMDA and QA. Unfortunately, melatonin did not appear to be able to reduce the amount of $O^{-}_{2}\cdot$ that was able to interact with NBT in KA treated cultures.

CHAPTER 8

EFFECT OF GLUTAMATE RECEPTOR AGONISTS AND MELATONIN ON LIPID PEROXIDATION

8.1. INTRODUCTION

In all living cells, the cell membrane separates the cell interior from the external environment, and also functions to compartmentalise the internal structures of the cell. Membranes are not static boundaries that segregate regions, but are dynamic systems responsible for among other things; the production of ATP, the selective transport of substances into and out of the cells, the binding of regulatory agents (such as hormones and growth factors), and the binding of neurotransmitters that mediate the transmission of nerve impulses [329].

Membranes are composed primarily of protein and lipid molecules. The fluid mosaic model of membrane structure proposed by Singer and Nicholson [174], is the accepted model of membrane structure. Membranes are believed to consist of a lipid bi-layer containing a mixture of proteins and carbohydrates.

Changes in membrane fluidity through physical and chemical disturbances, e.g. oxidative stress, can cause changes to neuronal characteristics, and the activities of the transport proteins. Neuronal membranes are especially vulnerable to free radical mediated oxidative stress, since the brain consumes 20% of the total body oxygen, is relatively deficient in protection mechanisms such as glutathione and vitamin E, and contains large amounts of polyunsaturated lipids [330]. In Chapter 7, the glutamate receptor agonists were shown to greatly increase $O^{-}_{2}\cdot$ production. The oxidative destruction of polyunsaturated fatty acids is known as lipid peroxidation, and is extremely damaging as a result of the self-perpetuating chain reactions they cause [310]. Since some free radical production is inevitable in cells, several enzymatic and non-enzymatic

defence mechanisms have evolved to protect cells. Any alterations though, in either the defence mechanisms or the production of free radicals, could upset this balance and lead to neurotoxicity.

This series of experiments were conducted to investigate the effect of the glutamate receptor agonists; glutamate, NMDA, QA and KA, on lipid peroxidation levels. The use of melatonin as a free radical scavenger was also investigated. The thiobarbaturic acid (TBA) assay was used to determine lipid peroxidation. The principal of the experiment involves the reaction of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) with TBA to yield a pink complex which can be extracted with butanol and read at 532 nm. MDA and 4-HDA are degraded lipid products from cell membranes, and are taken as reliable indicators of oxidative stress [140,331].

8.2. EFFECT OF GLUTAMATE, NMDA, QA AND KA ON LIPID PEROXIDATION IN RAT FOREBRAIN HOMOGENATE

8.2.1. Introduction

The $O^{-}_{2}\cdot$ can react with membranes to set off membrane destroying reactions [8]. As membranes are vital for proper cell function, any damage to membranes could be toxic to neurons. Among the products of the membrane destroying reactions are MDA and 4-HDA [140].

The following experiment was conducted to investigate the effects of the various glutamate receptor agonists on lipid peroxidation in rat forebrain homogenate.

8.2.2. Materials and Methods

8.2.2.1. Chemical Reagents

Butylated Hydroxytoluene (BHT) and 2-Thiobarbaturic acid (TBA) were purchased from the Sigma Chemical Company (USA). 1,1,3,3-Tetramethoxypropane was obtained from Fluka AG (Switzerland). Trichloroacetic Acid (TCA) was obtained from Saarchem (Pty) Ltd (South Africa), while butanol was supplied by Holpro Chemical Company (South Africa).

BHT was dissolved in absolute ethanol, while all the other chemicals were dissolved in Milli-Q water.

All agonists were prepared as described in 7.2.2.5.

8.2.2.2. Preparation of Standard Curve

1,1,3,3-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 1 ml. A calibration curve was generated by measuring the absorbance at 5 nmole intervals. The absorbance was read at 532 nm using a Shimadzu UV-160A UV-visible recording spectrophotometer and plotted against the molar equivalent weight of MDA in the complex assayed (see Appendix 4).

8.2.2.3. Incubation of Rat Brain Homogenate with the Glutamate Receptor Agonists

Rat forebrain homogenate was prepared as explained in section 7.2.2.3. The homogenate was frozen in liquid nitrogen and stored at -70^{o}C until used in the assay. All samples were used within 14 days of the homogenate preparation. Test samples revealed that storage of the homogenate did not alter lipid peroxidation levels compared to fresh brains.

A modified method of the TBA assay as described by Ottino [288] was used to determine lipid peroxidation.

Rat brain homogenate (1 ml) contained varying concentrations of the glutamate agonists (0 - 1 mM) was incubated in a shaking water bath for 1 hour at 37^{o}C. At the end of the incubation, 0.5ml BHT (0.5g / l in absolute ethanol) and 1 ml 25% TCA were added to the mixture. The samples were centrifuged at 2000 x g for 20 minutes at 4^{o}C to remove insoluble proteins. Following centrifugation, 2 ml of protein free supernatant was removed from each tube, and a 0.5 ml aliquot of 0.33% TBA was added to this fraction. All tubes were heated for an hour at 95^{o}C in a water bath. After cooling, the TBA-MDA complexes were extracted with 2 ml butanol. The absorbance was read at 532 nm and MDA levels were determined from the standard curve generated from 1,1,3,3-tetramethoxypropane. Final results were expressed as nmoles MDA / mg protein.

Protein estimation was performed as described in section 2.2.2.6, and all results were analysed as described in section 2.2.2.8.

8.2.3. Results

The results obtained are shown in Table 8.1. QA was the only agonist that caused a significant increase ($P<0.001$) in lipid peroxidation, when compared to controls.

8.2.4. Discussion

Increasing concentrations of glutamate, KA, and NMDA up to 1 mM had no significant effect on MDA production. QA was the only one of the agonists that caused a significant increase in MDA production.

As in Chapter 7, where increasing concentrations of the agonists had no effect on the production of $O^{-}_{2}\cdot$ in rat brain homogenate, most of the agonists had no effect on MDA production. This could be because the experiment used disrupted cells, where receptor mediated control of intracellular processes would no longer have been active. An increase in lipid peroxidation would have been expected from the agonists, because the previous experiment had demonstrated that the glutamate agonist caused an increase in $O^{-}_{2}\cdot$ production.

These results are similar to those of Rios and Santamaria [332] who found that glutamate and KA were not able to cause significant increases in lipid peroxidation using rat brain homogenate but, QA was found to be a potent lipid peroxidant in rat brain homogenate. Melchiorri *et al* [191] were able to get KA to induce lipid peroxidation in rat brain homogenate. They were however, using far higher KA concentrations than were used in either the present experiment, or by Rios and Santamaria [332].

QA caused lipid peroxidation in a dose dependent manner. The reason for this is not clear, but it is possible that QA was acting as a pro-oxidant, thus initiating lipid

peroxidation. Štipek *et al* [333] have however shown that the effect of QA on lipid peroxidation in the rat brain depends on iron. QA is able to chelate ferrous (but not ferric) ions to form a complex, and it is this complex that stimulates lipid peroxidation. The results suggest that QA does not have a direct peroxidative effect, but that it modulates lipid peroxidation via its interaction with Fe^{2+}. This would explain why only QA was able to stimulate lipid peroxidation when using brain homogenate.

Table 8.1: Amount of MDA Produced At Various Agonist Concentrations

Agonist	Concentration (mM)	MDA Produced (nmoles / mg Protein)	SEM
Glutamate	0	1.285	0.031
Glutamate	0.25	1.293	0.078
Glutamate	0.5	1.226	0.066
Glutamate	0.75	1.034	0.038
Glutamate	1.0	1.263	0.053
NMDA	0	0.92	0.002
NMDA	0.25	1.039	0.017
NMDA	0.5	0.856	0.051
NMDA	0.75	0.964	0.043
NMDA	1.0	0.882	0.08
QA	0	1.176	0.037
QA	0.25	2.15	0.049
QA	0.5	3.147	0.113
QA	0.75	3.514	0.11
QA	1.0	4.245	0.047
KA	0	0.851	0.027
KA	0.25	0.911	0.045
KA	0.5	0.938	0.036
KA	0.75	0.948	0.035
KA	1.0	1.027	0.031

8.3. EFFECT OF MELATONIN ON QUINOLINIC ACID INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE

8.3.1. Introduction

Experiment 8.2. demonstrated that QA was the only one of the agonists tested that induced lipid peroxidation in rat homogenate. Although neither the exact mechanism, nor why QA exclusively caused an increase in lipid peroxidation was known, it was speculated that the induction of lipid peroxidation must involve free radicals.

Melatonin is a very effective free radical scavenger, and so it was decided to investigate whether melatonin could reduce QA induced lipid peroxidation in rat brain homogenate.

8.3.2. Materials and Methods

Experiments were conducted exactly as in experiment 8.2, except that only QA was used. In addition, melatonin was added at various concentrations (0 - 1mM) at each QA concentration. Melatonin was prepared by dissolving it in absolute ethanol, and subsequently diluting it with Milli-Q water so that the final ethanol concentration in the brain homogenate was 0.5%.

8.3.3. Results

Co-treatment of brain homogenate with QA and increasing melatonin concentrations resulted in an overall decline in MDA production. Melatonin at a concentration of 1 mM reduced the MDA formed to ±2.25 nmoles / mg protein, irrespective of how high the MDA concentration had reached in the absence of melatonin in each of the QA treated samples. These results suggest that melatonin significantly decreases the basal level of lipid peroxidation compared, to controls in a concentration dependent manner (see Figure 8.1).

8.3.4. Discussion

The results demonstrate that QA-lipid peroxidation of rat whole brain homogenate is markedly inhibited by melatonin in a concentration dependent manner.

Melatonin was not however able to decrease MDA production to levels attained in samples without QA. Thus, although melatonin is effective at lowering lipid peroxidation, it is not able to protect completely against QA induced neurotoxicity.

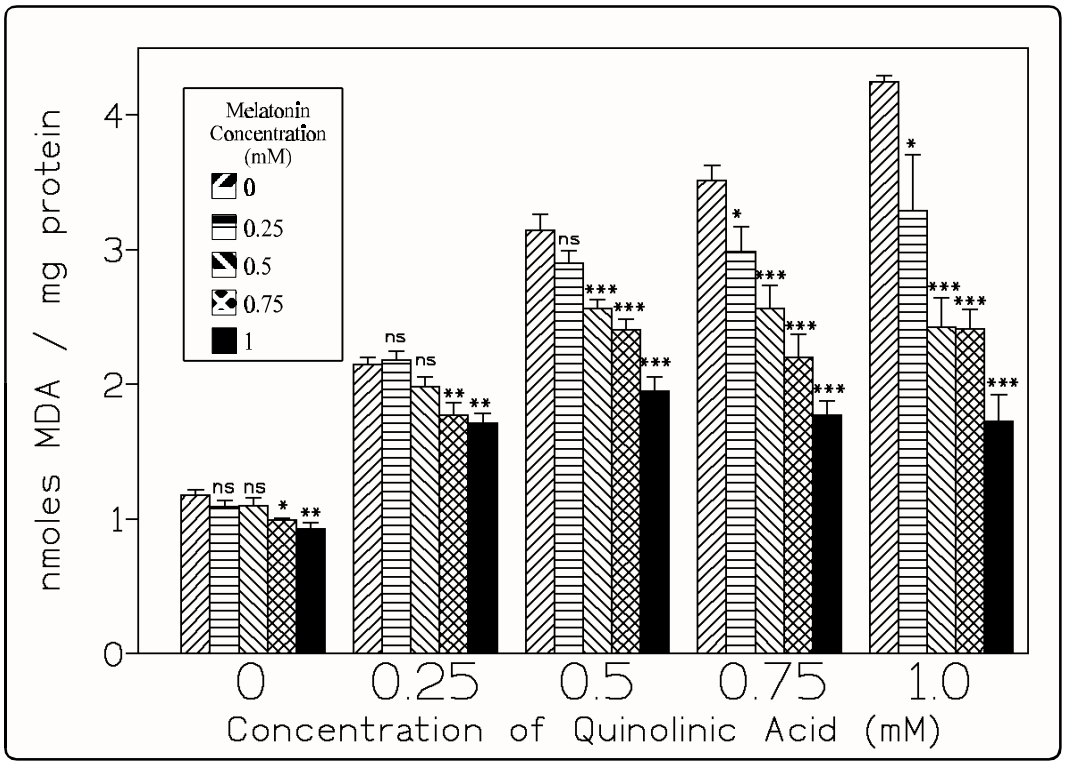


Figure 8.1: Effect of melatonin on quinolinic acid (0-1mM)-induced increase in MDA concentration in homogenate of whole rat brain. Values represent the mean ±SEM (n=6) ($^{ns}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ in comparison to 0 melatonin control)

8.4. EFFECT OF MELATONIN ON GLUTAMATE AGONIST INDUCED LIPID PEROXIDATION IN NEURONAL CELL CULTURES

8.4.1. Introduction

In the previous two experiments, rat forebrain homogenate had been used to test the effect of melatonin and the various agonists on lipid peroxidation. As in Chapter 7, it was found that rat forebrain homogenate was not the most effective medium to investigate the effect of agonists. The neurons had been disrupted, and so any effect caused by the agonist binding to membrane receptors would not cause physiological changes in the cell.

It was therefore decided to investigate the effect of the various glutamate agonists on neuronal cell cultures. Neuronal cultures offer an opportunity to work with uniform whole cells. Any intracellular changes that result from the binding of the agonist to the receptors could therefore be investigated. The effect of melatonin on lipid peroxidation was also investigated.

8.4.2. Materials and Methods

8.4.2.1. Chemical Reagents

Cell culture reagents were prepared as explained in section 3.2.2.2. The glutamate agonists were prepared in Milli-Q water, and melatonin was prepared as described in 7.3.2.1.

Chemicals used for the TBA assay were prepared as described in 8.2.2.1.

8.4.2.2. Preparation of Primary Neuronal Cultures

Primary neuronal cultures were established as explained in section 3.2.2.3.

8.4.2.3. Experimental Procedure

A modified method of Ottino [288] was used to measure lipid peroxidation. Seven to ten day old cultures were washed with 4 ml of HBSS, after which 3 ml of fresh MEM containing the agonists and melatonin (see Table 8.2) was placed in each culture flask.

Cultures were returned to the 37^{o}C incubator for 20 minutes. These were then washed twice with HBSS before the addition of 3 ml of fresh MEM. The neurons were incubated at 37^{o}C for a further 18 hours. At the end of this time, the neurons were scraped off the surface of the culture flasks using a cell scraper. The media containing the suspended cells was poured into centrifugation tubes and centrifuged at 4000 x g for 10 minutes. The cells were then resuspended in 1.1 ml of 50mM Tris HCl (pH 7.4). A 100 µl aliquot was removed for later enumeration using a haemacytometer. To the 1 ml of sample that remained, 0.5 ml BHT and 1 ml of 0.25% TCA were added. The suspension was vortexed and then centrifuged at 2000 x g for 20 minutes at 4^{o}C. A 2 ml aliquot of the protein free supernatant was removed, and 0.5 ml of 0.33% TBA was added. The solution was heated for 1 hour at 95^{o}C. After cooling, the TBA-MDA complexes were extracted with 2 ml butanol. The absorbance was read at 532 nm, and the MDA levels were determined from the standard curve generated from 1,1,3,3,-tetramethoxypropane. Final results were expressed as nmoles MDA / 10^{6} cells. All results were analysed as described in section 2.2.2.8.

Table 8.2: Experimental groups used to measure lipid peroxidation in cell culture.

Group	Agonist (500 μM)	Melatonin Concentration (μM)
1	Control (Zero)	0
2	Glutamate	0
3	Glutamate	100
4	Glutamate	1000
5	NMDA	0
6	NMDA	100
7	NMDA	1000
8	QA	0
9	QA	100
10	QA	1000
11	KA	0
12	KA	100
13	KA	1000

8.4.3. Results

The results demonstrate that all the agonists except glutamate caused a significant increase in lipid peroxidation (see Figure 8.2) when compared to untreated cultures. QA and NMDA caused the most significant increases in lipid peroxidation, with MDA production increasing more than 3.5 fold in QA treated cultures when compared to controls. KA also caused a significant increase in lipid peroxidation, with just less than a 2 fold increase occurring.

In all cases, melatonin was able to significantly reduce lipid peroxidation (see Figures 8.3 - 8.6). Co-treatment with 1 mM melatonin at the same time as 500 mM glutamate or KA resulted in MDA concentrations even lower than those for control cultures. NMDA and QA, which caused the largest increase in lipid peroxidation, were not able to induce lipid peroxidation to the same levels in the presence of melatonin. A 1 mM dose of melatonin reduced MDA levels to slightly above those of untreated control cultures.

8.4.4. Discussion

Each of the agonists investigated was able to increase MDA levels in neuronal cell cultures, although glutamate did not cause a significant increase. KA was able to almost double the production of MDA, while NMDA and QA caused the most significant increases in lipid peroxidation. Both of these agonists act at the NMDA receptor. The fact that both of these agonists act at the same receptor demonstrates, that overstimulation of the NMDA receptor in particular could make neurons more susceptible to neuronal degeneration caused by lipid peroxidation.

Melatonin is a powerful free radical scavenger. Lipid peroxidation results from the destruction of cell membranes by free radicals. By removing free radicals, melatonin could offer protection to the neurons. In this set of experiments, it has been clearly shown that the presence of melatonin in the culture flasks, while the neurons were being subjected to the agonist attack, significantly reduces the amount of lipid peroxidation that occurs. In all cases, except for KA, doses as low as 100 µM melatonin significantly reduced MDA levels when compared to cultures untreated with melatonin. At a dose of 1 mM melatonin, lipid peroxidation had been brought down to levels below that of control cultures for glutamate and KA treated cultures, while MDA levels were only slightly higher than control values for NMDA and QA treated cultures.

The glutamate agonists caused a significant increase in MDA production in all cultures tested for lipid peroxidation. The presence of melatonin in the culture flasks, together with the agonists, during the incubation, was able to cause a significant reduction in lipid peroxidation. Melatonin is therefore a very potent inhibitor of neuronal lipid peroxidation.

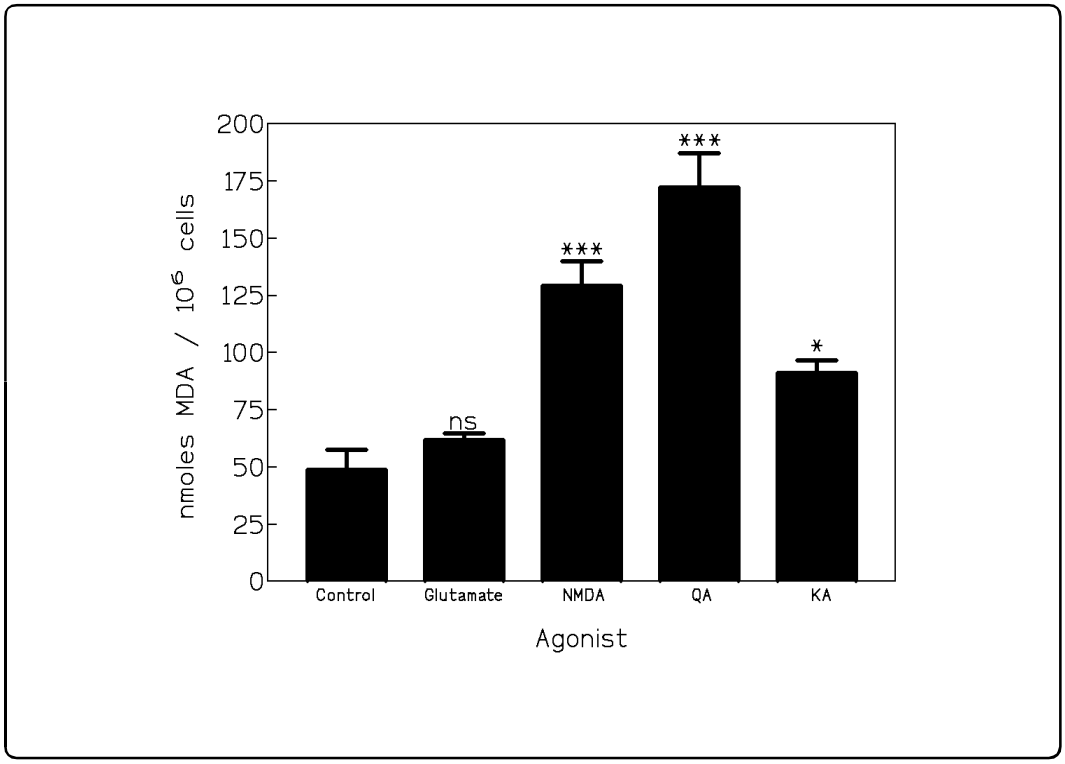


Figure 8.2: Effect of various glutamate receptor agonists (500nM) on MDA production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) ($^{ns}P>0.05$; * $P<0.05$; *** $P<0.001$ in comparison to zero control)

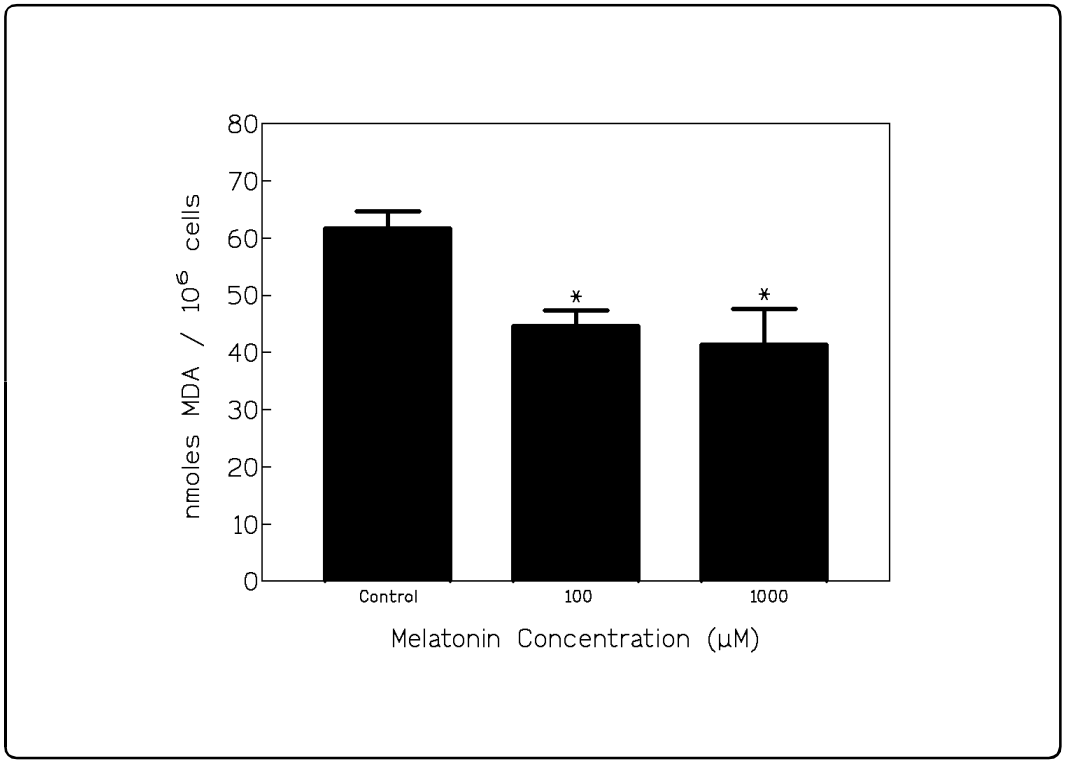


Figure 8.3: Effect of various concentrations of melatonin on glutamate-induced MDA production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (* P< 0.05 in comparison to zero control)

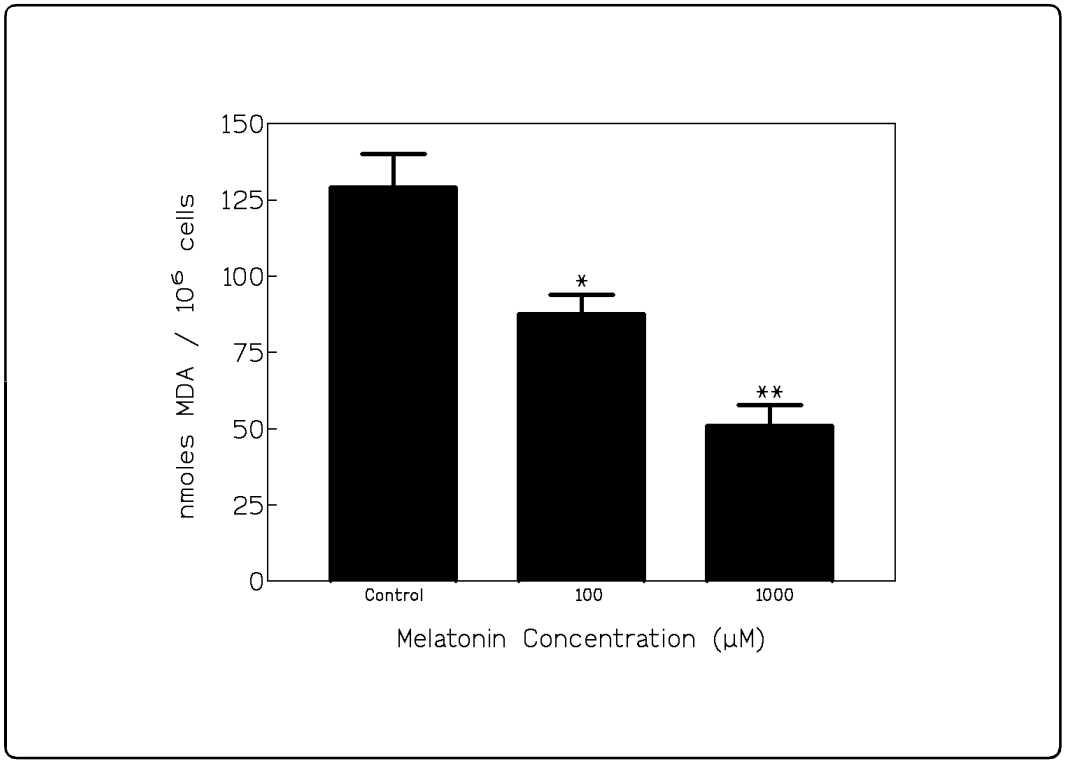


Figure 8.4: Effect of various concentrations of melatonin on NMDA-induced MDA production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (* P< 0.05; ** P< 0.01 in comparison to zero control)

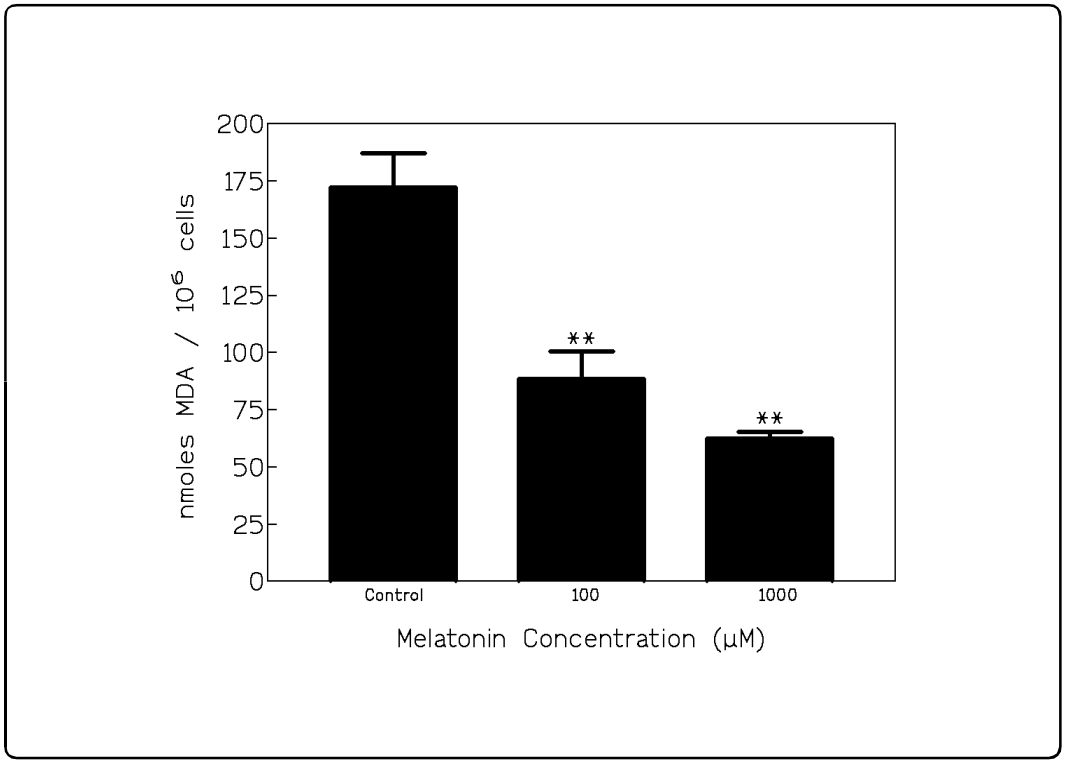


Figure 8.5: Effect of various concentrations of melatonin on QA-induced MDA production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3)(** P< 0.01 in comparison to zero control)

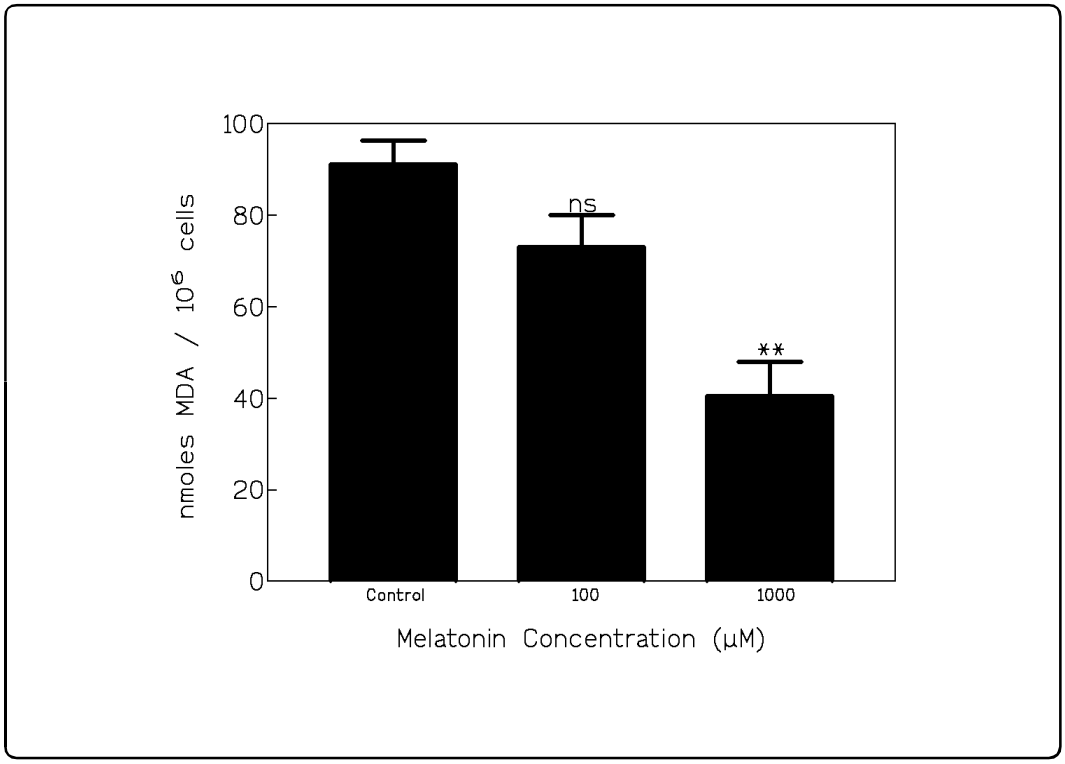


Figure 8.6: Effect of various concentrations of melatonin on KA-induced MDA production in primary neuronal cell cultures. Values represent the mean ± SEM. (n = 3) (^{ns} P> 0.05; ** P< 0.01 in comparison to zero control)

8.5. CONCLUSION

These results demonstrate that QA was the only agonist able to induce lipid peroxidation in rat forebrain homogenate. The reason that lipid peroxidation may not have been induced by the other agonists, is that disrupted cells were used. Lipid peroxidation occurs as a result of a free radical attack on a lipid membrane. By disrupting the neurons there may not have been receptor based control over free radical production. Another reason why there may not have been an increase in lipid peroxidation is that agonists were only used up to a concentration of 1 mM. Melchiorri *et al* [191] have demonstrated that melatonin is able to protect against KA-induced lipid peroxidation in a concentration dependent manner. These workers used a KA concentration of 12 mM. QA is a more potent excitotoxin than KA, and is thus able to produce marked increases in lipid peroxidation at concentrations as low as 100 µM.

Even in the presence of the potent excitotoxin, QA, melatonin was able to protect against lipid peroxidation. This was demonstrated by the significant reduction in the production of MDA in QA treated brain homogenate. Melatonin was however not able to decrease MDA production to levels attained in samples without QA.

It was also demonstrated that all of the agonists except glutamate caused a significant increase in lipid peroxidation in primary neuronal cell cultures. When incubated in the presence of 500 µM agonist and 1 mM melatonin, the agonists had little to no effect on lipid peroxidation levels. In fact, lipid peroxidation was lower in glutamate and KA-induced samples that were co-treated with 1 mM melatonin, than in untreated controls.

Melatonin has been shown to be a very potent free radical scavenger in previous experiments. Tan *et al* [3] showed that melatonin is a more efficient hydroxyl radical scavenger than either glutathione or mannitol, within an artificial hydroxyl radical generating system. Pieri *et al* [299] showed that melatonin was the most effective agent

at detoxifying the peroxyl radical when compared to trolox (water soluble vitamin E), ascorbic acid and glutathione. Subsequently, Tan *et al* [183] demonstrated that melatonin is able to protect against the DNA damage *in vivo* caused by the carcinogen safrole.

Melatonin has been shown to be protective against a number of other toxins including; the prooxidative agent carbon tetrachloride [189], the excitatory amino acid glutamate [295], the apoptosis promoting agent dexamethasone [334], and nitric oxide synthase [6]. This data demonstrates that there is a definite role for melatonin in the antioxidative defence system of an organism.

It is therefore proposed that melatonin reduces lipid peroxidation in both primary neuronal cultures and forebrain homogenate by removing free radicals from the solution. By removing free radicals, melatonin would be protecting the neuronal membranes, which are very susceptible to free radical attack.

CHAPTER 9

EFFECT OF MELATONIN AND VARIOUS GLUTAMATE RECEPTOR AGONISTS ON NITRIC OXIDE SYNTHASE ACTIVITY

9.1. INTRODUCTION

Nitric oxide is a unique molecule that is involved in many biological processes. NO is able to interact with the epithelium of blood vessels to mediate blood flow [164], is used by macrophages as an immune defence mechanism, and is a neurotransmitter of central and peripheral nervous systems [161]. Neuroscientists regard NO as a highly reactive molecule, owing to its short half-life (3 - 6 seconds) [161].

NO influences neurotransmitter release [335], by augmenting the phosphorylation of synaptic vesicle proteins associated with neurotransmitter release. This is thought to occur through cGMP activation of cGMP-dependent-protein kinases. NO is known to play a role in NMDA-receptor mediated neurotransmitter release, as NOS inhibitors block NMDA-receptor mediated neurotransmitter release [336]. After activation of the NMDA receptor [92], L-arginine is converted to NO by nitric oxide synthase. This enzyme has to interact with a number of co-factors to be effective. NOS must first bind to calmodulin, while FAD, Ca^{2+}, $NADP^{+}$ and tetrahydrobiopterin must also be present for the enzyme to be active in neuronal cells [167].

NOS can induce neurotoxicity in one of two ways. When NO is present in large concentrations, it can initiate a neurotoxic cascade. NO mainly kills neurons via its conversion to peroxynitrite [5,142]. NO interacts with the superoxide ion to form the peroxynitrite radical, which is highly toxic to neurons.

Apart from this, in the absence of the L-arginine substrate, NOS is able to produce $O^{-}_{2}\cdot$

and hydrogen peroxide in substantial quantities [161]. This is especially relevant to neuronal cells as the rate of NADPH oxidation in the neuronal isoform is primarily influenced by the binding of a Ca^{2+}-calmodulin complex to the NOS, and not by L-arginine concentrations. An increase in intracellular Ca^{2+} would allow more Ca^{2+}-calmodulin complexes to form, and so more NOS would be induced into action. This could result in greater NO and $O^{-}_{2}\cdot$ production, both of which are highly toxic compounds to neurons.

Melatonin has been shown to be highly effective in removing $O^{-}_{2}\cdot$ from brain homogenate, and so could offer neuroprotection. Melatonin has also been shown to be a peroxynitrite scavenger [173]. Recently, Pozo *et al* [6] demonstrated that melatonin was able to inhibit NOS activity. It was therefore decided to investigate this on rat forebrain homogenate.

9.2. EFFECT OF MELATONIN ON NITRIC OXIDE SYNTHASE ACTIVITY

9.2.1. Introduction

The enzyme NOS converts L-arginine to L-citrulline. NO is the main product of the reaction, although $O^{-}_{2}\cdot$ and other free radicals may also be produced. The rate of the reaction is controlled by a Ca^{2+} / calmodulin complex that interacts with NOS to cause a conformational change that results in NOS becoming active.

The experiment was conducted to determine whether the presence of melatonin in rat forebrain homogenate had any effect on NOS activity.

9.2.2. Materials and Methods

9.2.2.1. Chemical Reagents

L-citrulline, DL-dithiothreitol, (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, hypoxanthine-9-β-D-arabinofuranoside, BSA, Dowex -50W (50X8-100) and L-arginine were purchased from Sigma Chemical Company (USA). L-[U-^{14}C] Arginine monochloride (50 µCi / ml) was purchased from Amersham International (England). Flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Boehringer Mannheim (Germany). N-[2-hydroxyethyl] piperazine-N'-2-hydroxypropanesulfonic acid (HEPES), EDTA and Tris were obtained from Unilab (South Africa). All other chemicals were purchased from normal commercial sources and, were of the highest quality available.

Homogenisation buffer consisted of 25 mM Tris-HCl buffer (pH 7.4) containing 1 mM DL-dithiothreitol.

Incubation buffer (pH 7.6) consisted of the following (concentration is final once added to homogenate and NADP); 25 mM Tris-HCl Buffer, 1 mM DL-dithiothreitol, 30 µM (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride, 10 µM FAD, 0.5 mM hypoxanthine-9-β-D-arabinofuranoside, 0.5 mg / ml BSA, 0.1 mM $CaCl_2$, 10 µM L-arginine and 40 nM ^{3}H-arginine.

HEPES buffer consisted of 0.1 M HEPES, 10 mM EDTA, and 0.175 mg / ml L-citruline.

9.2.2.2. Preparation of Brain Homogenate

Adult, male Wistar Rats that had been housed as described in section 2.2.2.2. were used in this study. On the day of the experiment, the animals were sacrificed and their brains were removed using the method described in section 2.2.2.4.

The brains were placed in ice-cold homogenisation buffer. All subsequent procedures were carried out at 0 - 4^{o}C. The forebrain was separated from the cerebellum, and placed in a vial. Each forebrain was finely chopped and 2 ml of homogenisation buffer was added, before the homogenate was sonicated (6 x 10 seconds) using a Vibracell™ sonicator. The crude homogenate was centrifuged for 5 minutes at 1000 x g, and the aliquots of supernatant were either stored at -20^{o}C for protein determination (see section 2.2.2.6), or used immediately to measure NOS activity.

9.2.2.3. NOS Activity Assay

Total NOS activity (soluble and membrane bound isoforms) was measured using a modified method of Bredt and Snyder [337]. The final incubation volume was 100 µl, and consisted of 10 µl crude homogenate, 80 µl of pre-warmed incubation buffer and 10 µl of (1 mM final) NADP to initiate the reaction. The reaction vial was incubated at 37^{o}C for 30 minutes. Control incubations were performed by the omission of NADP from the solution. The reaction was terminated by the addition of 400µl of ice-cold HEPES buffer.

The reaction mixture was poured into a disposable beaker containing 1.5 ml of Dowex - 50W ion exchange resin at pH 7.0. To this was added 1 ml of Milli-Q water. The cocktail was mixed together and the Dowex was allowed to settle at the bottom of the beaker. An 850 µl aliquot of solution, containing no Dowex, was removed and added to 3 ml of liquid scintillation cocktail. After 24 hours the radioactivity in the vial was measured using a Beckman Liquid Scintillation Counter. All radioactivity was expressed as pmoles L-^{14}C-citrulline produced / mg protein / minute.

Where melatonin or the glutamate receptor agonists were used, these were mixed into the incubation buffer at the required concentrations.

Results were analysed as explained in section 2.2.2.8.

9.2.3. Results

The results obtained (see Figure 9.1) demonstrate that melatonin causes a significant reduction in NOS activity. A 100 µM dose of melatonin caused a 13.3 % decrease in NOS activity.

In experiments conducted to see if the presence of 350 µM of each of the glutamate agonists caused (see Table 9.1) any increase in NOS activity, no significant difference ($P = 0.2226$) was found. There was also no significant difference in NOS activity between the different agonists.

Table 9.1: Effect of the various glutamate receptor agonists on L-^{14}C-citrulline production

Agonist	**L-^{14}C-Citrulline produced (pmol / mg protein / min)**	**SEM**
Control	5.214	0.1336
Glutamate	5.005	0.1772
NMDA	5.396	0.2612
QA	5.204	0.1332
KA	5.548	0.3682

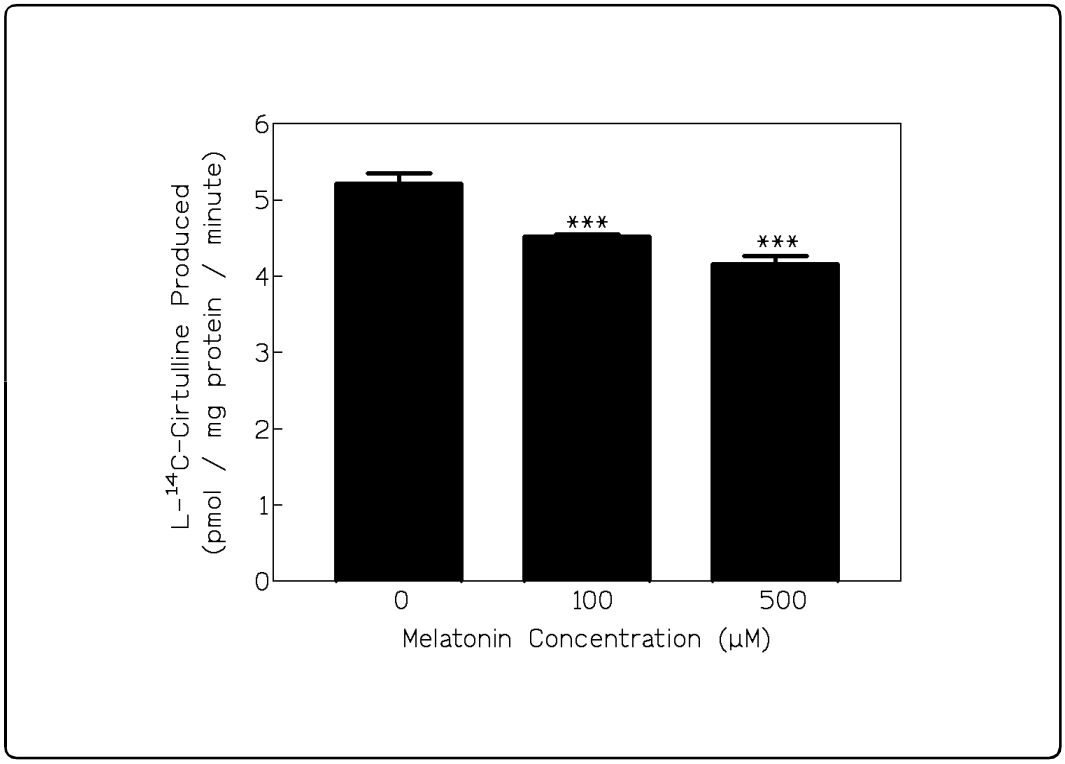


Figure 9.1: Rate of L-^{14}C-Citrulline production by NOS while being treated with various concentrations of melatonin in rat forebrain homogenate. Values represent the mean (± SEM) (n=5) (*** P< 0.001 in comparison to zero control)

9.2.4. Discussion

These results demonstrate that melatonin reduces NOS activity in a dose dependent manner. This would be beneficial to the neuron, because increased NOS activity brought on by excessive NMDA stimulation can produce NO and $O^{-}_{2}\cdot$, both of which are neurotoxic.

The glutamate agonists did not cause an increase in NOS activity. It is accepted in the literature [161,167] that NMDA, and the agonists that act at the NMDA receptor, are the main instigators of increased NOS activity. The most probable reason for not getting any significant changes in this experiment is that brain homogenate was used. As in experiments 7 and 8, brain homogenate was not the ideal substrate for testing the effects of the receptor agonists on intracellular processes. This is because the integrity of the neurons had been disrupted. It is believed that the same process is taking place here.

9.3. CONCLUSION

These results support the findings of Pozo *et al* [6], who found that melatonin inhibits NOS activity. The results demonstrate that melatonin significantly decreases NOS activity with as low a dose as 100 µM.

It was not possible to show that agonists that act at the NMDA receptor increase NOS activity, however it is well documented that [161,167] this is the case. Brain homogenate has been found to be an unsuitable method for investigating intracellular activities that are regulated by membrane bound receptors. This is probably because the cell integrity had been disrupted by homogenisation. Although the use of cell culture techniques may have remedied this problem, the techniques used for brain homogenate were not adaptable for cell culture work.

After determining that melatonin inhibited NOS activity, Pozo *et al* [6] determined the possible mechanism for this action. As mentioned in the introduction, the binding of Ca^{2+} to calmodulin to form a Ca^{2+}-calmodulin complex is the first step in initiating NOS activity. This is because it is the Ca^{2+}-calmodulin complex that interacts with NOS to cause a conformational change, that results in NOS becoming active. In neurons then, it is the presence of the Ca^{2+}-calmodulin complex and not the concentration of the arginine substrate that determines NOS activity.

Pozo *et al* [6] also determined that melatonin inhibited both NOS and cyclic GMP production. The authors further discovered that melatonin brought about this action by a calmodulin-mediated mechanism. Melatonin appears to bind to calmodulin, thus preventing the Ca^{2+}-calmodulin complex from being able to bind to NOS. This effectively prevents activation of NOS.

This is a whole new mode of action for melatonin. The Pozo *et al* [6] found that normal physiological concentrations of melatonin were able to inhibit NOS activity. The

experiments conducted in previous chapters had demonstrated the free radical scavenging properties of melatonin while, results in this experiment show that melatonin is possibly acting via another mechanism as a neuroprotector.

Melatonin could therefore be playing a very significant role in neuroprotection within the CNS. Not only is it able to rapidly move into neurons, but it acts as a very powerful free radical scavenger, and now it has been shown that melatonin is even able to partially inhibit NOS activity. The role of melatonin as a naturally occurring neuroprotector within the CNS, therefore needs to be considered very seriously.

CHAPTER 10

SUMMARY

10.1. CHAPTER 1

A general review of melatonin and glutamatergic neuronal transmission was presented. Special attention was paid to the concept of excitotoxicity and melatonin's role in protecting against these neurotoxic insults. Finally there were brief discussions on glucocorticoids, and neuronal disorders in which excitotoxicity plays a part in the pathology of the disorder.

10.2. CHAPTER 2

The effects of stress and melatonin treatment on glutamatergic receptors were investigated in this chapter. Both pharmacologically administered corticosterone, and the application of physical stresses, were used to bring about the stress response.

The results obtained demonstrate that corticosterone causes an increase in glutamate receptor numbers, while decreasing the affinity of the receptor for its ligand. Melatonin caused a decrease in glutamate receptor numbers, both for animals with elevated corticosterone levels and for unstressed animals.

10.3. CHAPTER 3

This chapter was concerned with the effect of glutamate, NMDA, QA, and KA on neuronal cell viability. Primary neuronal cultures were used to test the effects of the various glutamate agonists.

The results obtained demonstrated that all of the glutamate receptor agonists tested

were neurotoxic, as cell viability was significantly decreased in a dose dependent manner.

10.4. CHAPTER 4

The effect of treatment of primary neuronal cultures with melatonin and the glutamate receptor agonists was investigated.

Melatonin was found to have no neurotoxic properties when applied to primary neuronal cultures in an ethanol vehicle. Later, melatonin was shown to protect against glutamate agonist-induced neurotoxicity in a dose dependent manner. Melatonin was only found to be neuroprotective when administered either, during or after the agonist insult. Pre-treatment of neurons with melatonin had no effect on neuronal viability. These results demonstrated that melatonin was a potential neuroprotective agent against glutamate agonist-induced neurotoxicity, and that the mechanism of action needed to be further investigated.

10.5. CHAPTER 5

An investigation was conducted to determine whether melatonin was able to protect neurons in an *in vivo* situation. Intrahippocampal injections of QA were used to cause neurodegeneration.

Micrographs taken of the neurons in the hippocampus of rats killed five days after the QA injection showed signs of neurodegeneration. Melatonin administration appeared to protect against the neurodegeneration, as the neurons appeared healthy. QA treatment also reduced glutamate receptor numbers. Animals co-treated with QA and melatonin had reduced glutamate receptor numbers, but the decrease was not as pronounced as for animals that did not receive melatonin. These results demonstrated that melatonin appeared to offer neuroprotection under *in vivo* conditions, which further strengthened the argument that melatonin showed potential as a neuroprotective agent.

10.6. CHAPTER 6

A rise in intracellular Ca^{2+} concentrations has been proposed as one of the initial events in the excitotoxic process. These experiments were concerned with investigating whether the glutamate receptor agonists had any effect on intracellular Ca^{2+} concentrations, and whether melatonin was able to inhibit intracellular Ca^{2+} accumulation.

All of the glutamate agonists caused a rapid increase in intracellular Ca^{2+}, as measured by atomic absorption spectroscopy. The highest intracellular Ca^{2+} concentrations occurred immediately following the agonist insult. Eighteen hours after the initiation of the agonist insult, all intracellular Ca^{2+} concentrations had returned to those of untreated controls. Melatonin had no effect on intracellular Ca^{2+} accumulation within neurons. These results demonstrated that the glutamate agonists cause a rapid increase in intracellular Ca^{2+} concentrations, but that melatonin must act at a different level to afford neuroprotection.

10.7. CHAPTER 7

These experiments were conducted to determine, firstly, if glutamate agonists induced superoxide production, and secondly, to determine whether melatonin could act as a superoxide radical scavenger. Superoxide production is known to be induced by elevated intracellular Ca^{2+} concentrations [8]. As melatonin had been unable to protect against increasing Ca^{2+} concentrations, it was theorised that melatonin was offering neuroprotection as an antioxidant.

The glutamate agonists were not able to induce $O^{-}_{2}\cdot$ production in rat brain homogenate. Using primary neuronal cell cultures, all of the agonists-induced $O^{-}_{2}\cdot$ production. Under both experimental conditions, melatonin was able to reduce $O^{-}_{2}\cdot$ production. It was therefore concluded that melatonin was acting as an antioxidant, and that this was the mechanism by which neuroprotection was being offered.

10.8. CHAPTER 8

These experiments investigated the effect of the glutamate agonists and melatonin on lipid peroxidation. Lipid peroxidation involves the destruction of the lipid membranes by a process initiated by ROS. Melatonin had exhibited antioxidant properties in Chapter 7, and these were further investigated in this experiment.

As in the previous experiment, the glutamate receptor agonists were not able to promote lipid peroxidation using rat brain homogenate. The exception was QA, which was very efficient at promoting lipid peroxidation. Using primary neuronal cultures, all of the agonists except glutamate, induced lipid peroxidation. Melatonin was very effective at reducing lipid peroxidation under all conditions. Using rat brain homogenate, melatonin protected against QA-induced lipid peroxidation in a dose dependent manner. Melatonin thus appeared to be deriving most of it's neuroprotective effects by acting as an antioxidant and free radical scavenger.

10.9. CHAPTER 9

It had been reported [6] that melatonin was able to inhibit NOS activity, thus preventing the production of NO and $O^{-}_{2}\cdot$. The experiment in this chapter was conducted to investigate this novel neuroprotective role for melatonin.

Melatonin was found to decrease NOS activity in a dose dependent manner. The glutamate receptor agonists were not able to promote NOS activity, most probably because disrupted neurons in the form of rat brain homogenate was used. Melatonin causes a decrease in NOS activity, by inhibiting the formation of the NOS enzyme.

CHAPTER 11

GENERAL CONCLUSIONS

The experiments conducted in this study have demonstrated conclusively that melatonin acts as a neuroprotective agent. Initial studies were conducted to investigate whether melatonin could offer any protection against neurotoxicity induced by elevated glucocorticoid concentrations caused by stress. Although direct evidence of neuroprotection was not obtained, melatonin did decrease the number of glutamate receptors on synaptic membranes. When it is considered that melatonin production is increased at times of stress [220,221,338], and that pinealectomised rats produce more corticosterone [222], it becomes apparent that melatonin must have a role to play following the stress response. Melatonin appears to be modulating corticosterone secretion and helping to decrease some of the deleterious effects of corticosterone release, such as increased glutamate receptor numbers. These factors together could help to protect neurons.

The neuroprotective effects of melatonin against EAA-induced neurotoxicity were investigated next. The glutamate receptor agonists were shown to increase intracellular Ca^{2+} concentrations, increase $O^{-}_{2}\cdot$ production and increase lipid peroxidation in primary neuronal cultures. These results provided evidence that it was the excitotoxic cascade that was giving rise to neuronal cell death, as neuronal viability was also markedly decreased following exposure to high concentrations of the glutamate receptor agonists.

Melatonin appeared to be offering neuroprotection against excitotoxic neuronal death in primary neuronal cultures. This was evident by the increase in cell viability when neurons were co-incubated with melatonin, while begin exposed to the glutamate receptor agonists. The method by which melatonin was offering this neuroprotection was therefore investigated.

Melatonin did not have any effect on intracellular Ca^{2+} accumulation in the neurons. It

was however found to be effective at scavenging $O^{-}_{2}\cdot$ and inhibiting lipid peroxidation. This was consistent with work done by other authors [179,340] who had also found melatonin to be a powerful antioxidant. It was further found that, similar to Pozo's results [6], melatonin was also able to inhibit NOS activity. Melatonin therefore appeared to be a very effective neuroprotectant against glutamate agonist-induced neurotoxicity. The melatonin molecule was able to obstruct the excitotoxic cascade at a number of points. Firstly, it was able to scavenge ROS from the neurons, while secondly, melatonin decreased lipid peroxidation. Finally, melatonin was able to inhibit the activity of NOS, an enzyme responsible for the production of NO and $O^{-}_{2}\cdot$.

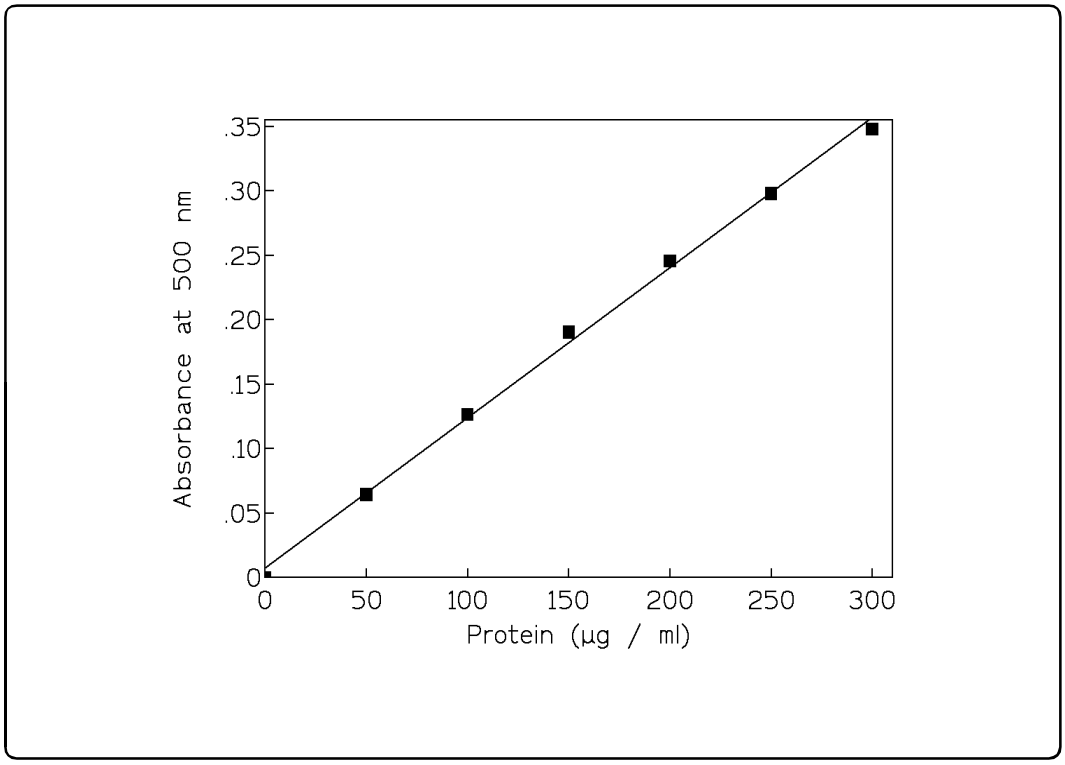
These results were not just limited to *in vitro* primary neuronal cultures. Melatonin also protected hippocampal neurons against QA-induced neurotoxicity under *in vivo* conditions. Melatonin was able to decrease lipid peroxidation and superoxide anion production in rat brain homogenate too. These results were consistent with those of other researchers, who had found melatonin to be a powerful antioxidant and free radical scavenger [330,339]. Melatonin had also been shown to protect against KA-induced neuronal death [340], safrole-induced DNA damage [183], CCl_4-induced lipid peroxidation [189], and peroxynitrite damage [173].

The results obtained thus serve to confirm that melatonin is a powerful antioxidant. It is particularly effective at protecting against excitotoxicity induced by agonists acting on the ionic glutamate receptors. Melatonin achieves this neuroprotection by interrupting many of the steps in the excitotoxic cascade.

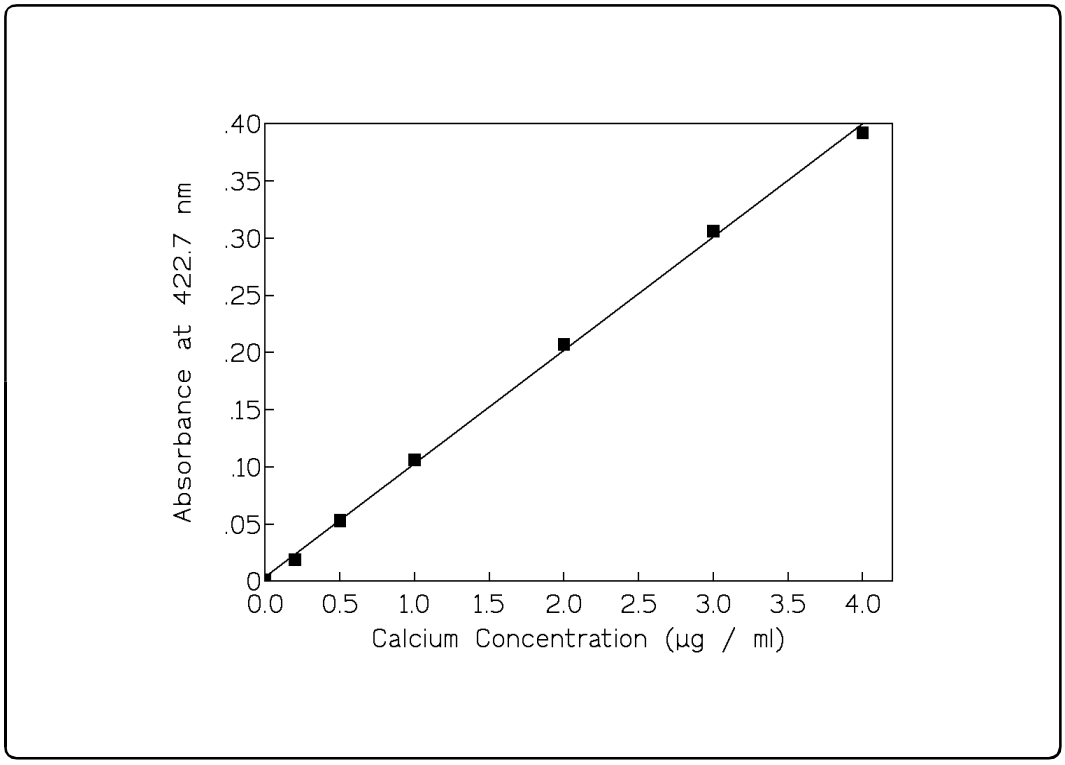
The neuroprotective effects outlined above offer many therapeutic opportunities. Diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease are all thought to involve excitotoxicity at some stage of their pathologies. Superoxide anions are also know to play a role in the pathology of aging. Melatonin production decreases with age, while ROS production increases [233]. Chronic stress also accelerates the aging process. The administration of melatonin supplements to aged

persons, or persons suffering from stress, may therefore have therapeutic benefits. The only side effect of melatonin administration is that it causes drowsiness in humans [46]. This can however be easily countered, by taking the supplements just before going to sleep. In the future, melatonin may therefore come to be regarded as an accepted neuroprotective and therapeutic agent.

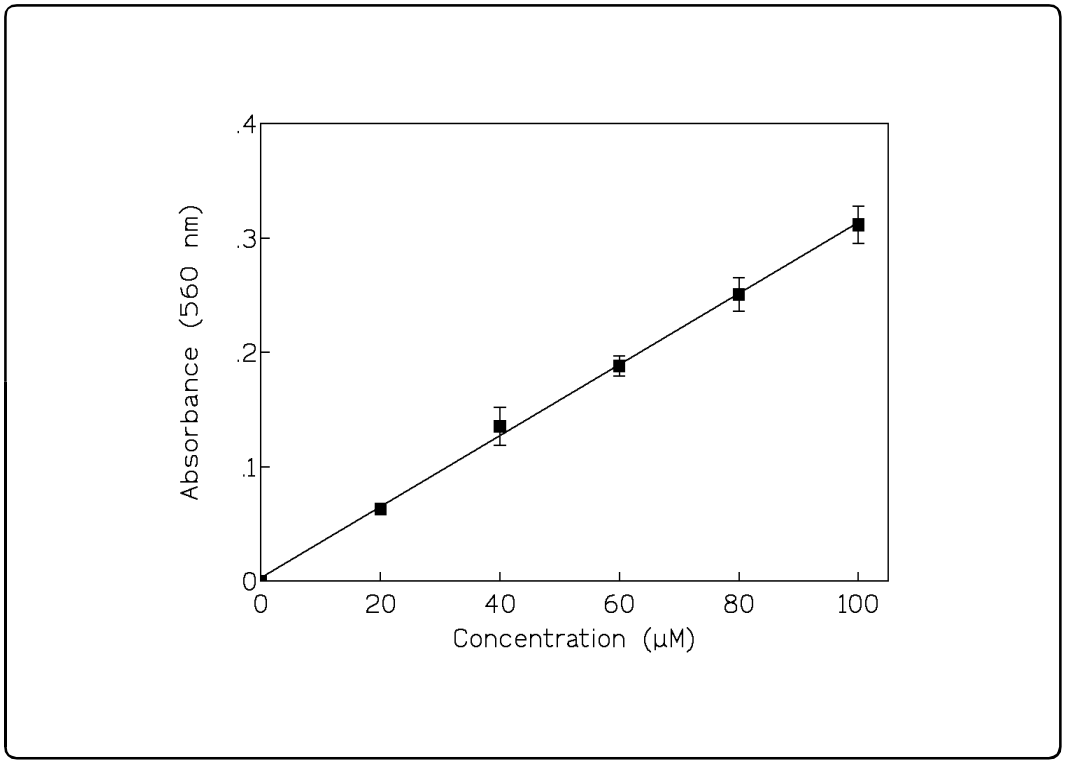
APPENDICES



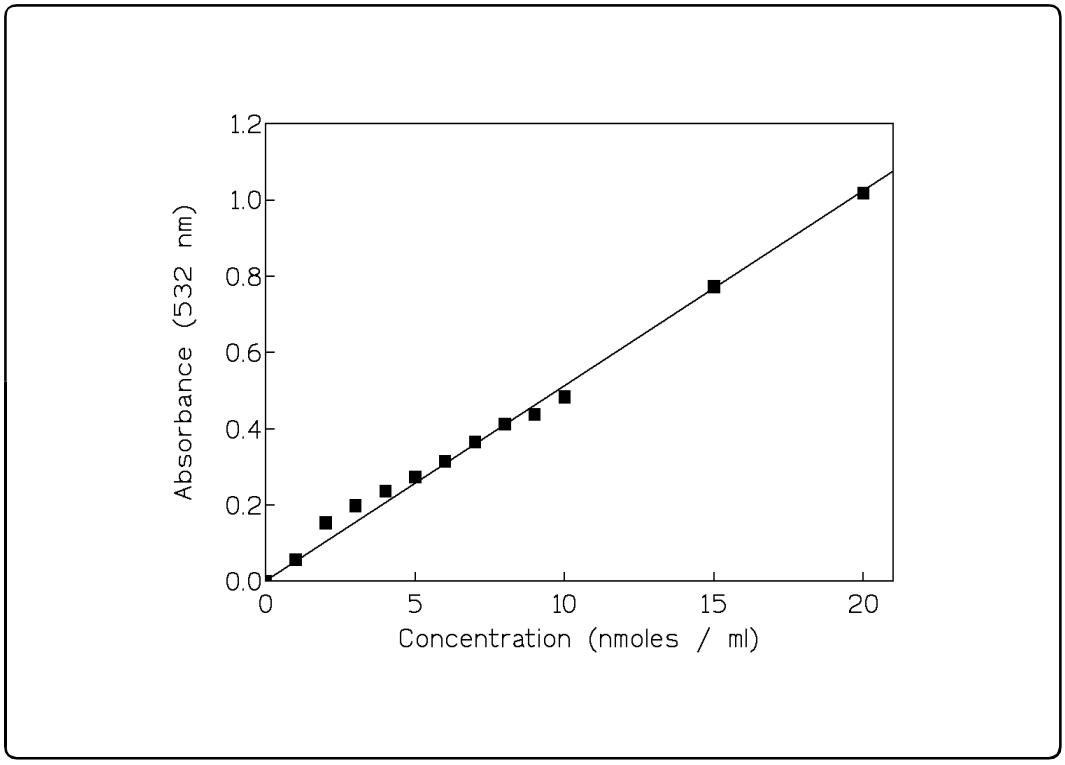
Appendix 1: Protein Standard Curve



Appendix 2: Calcium Standard Curve



Appendix 3: Diformazan Standard Curve



Appendix 4: Malondialdehyde Standard Curve

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