# Studies on the potential neurotoxicity of tetrahydroisoquinoline and nicotinamide derivatives.

Submitted in 1995 for the degree of Doctor of Philosophy to the University of Leicester by:

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**Studies on the potential neurotoxicity of tetrahydroisoquinoline and nicotinamide derivatives.**

**Jonathon M. Willets.**

**Abstract.**

The naturally occurring compounds N'-methyl nicotinamide (NMN), 6,7-dihydro-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) and 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ) are reported to interact with catecholaminergic systems in vivo and have similar structure to 1-methyl-4-phenylpyridinium ($MPP^+$), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Three cell lines, rat B65, human IMR32 and SH-SY5Y, were examined to find the most appropriate with which to study such toxins. These data indicate that both B65 and IMR32 cells do not take up catecholamines (<2 % after one hour), in monolayer culture. In contrast, SH-SY5Y cells take up significant ($P<0.05$) amounts of both noradrenaline (NA) and dopamine (DA), which can be blocked by $uptake_1$ inhibitors. Furthermore, SH-SY5Y cells store [$^3$H]NA in a releasable pool, which can be stimulated through receptor-occupancy and depolarization. Therefore, the human SH-SY5Y neuroblastoma cell line was selected for further study.

NMN induced cytotoxicity albeit at high concentrations (10mM), in SH-SY5Y cells. Lower concentrations had no significant ($P<0.05$) effect. Cytotoxicity was inhibited in a dose dependent manner by the addition of α-tocopherol. NMN had little effect upon catecholamine uptake and release.

Both salsolinol and TMIQ significantly ($P<0.05$) inhibited MTT reduction and increased LDH release, at concentrations within the range of $MPP^+$ cytotoxicity, in vitro. Cytotoxicity was not inhibited by the addition of monoamine oxidase inhibitors, antioxidants, or imipramine. TMIQ and salsolinol stimulated catecholamine uptake with $EC_{50}$ values of 7μM (NA) and 54μM (DA), 17μM (NA) and 11μM (DA), respectively. However, above 100μM, salsolinol inhibited uptake with $IC_{50}$ values of 411μM and 379μM for NA and DA, respectively. Inhibition of NA uptake by salsolinol, corresponded to the increased displacement of nisoxetine from the $uptake_1$ recognition site, since the $K_i$ (353μM) for displacement was similar to the $IC_{50}$ values (411 and 379μM) for uptake. TMIQ also displaced nisoxetine binding with $K_i$ of 71μM. TMIQ and salsolinol stimulated catecholamine uptake does not involve the uptake recognition site, and is not inhibited by elevation of cAMP or cGMP, or the inhibition of PKC. Salsolinol inhibited both carbachol (CCH) and potassium, whilst TMIQ inhibited only potassium evoked [$^3$H]NA release, from SH-SY5Y cells.

Thus both TMIQ and salsolinol are cytotoxic to SH-SY5Y cells, although the exact mechanism of toxicity requires further investigation, it appears not to involve bioactivation by MAO, and is not mediated through membrane based free radical damage. NMN is also cytotoxic to neurones, but only at high concentrations, and appears to be membrane directed. The high concentration of NMN required to induce cytotoxicity casts doubt over the relevance of NMN as an in vivo toxin. In contrast, both TMIQ and salsolinol are cytotoxic in vitro and may have a role in neurodegeneration.

TABLE OF CONTENTS. PAGE NUMBER.

CHAPTER 2. GENERAL MATERIAL AND METHODS



CHAPTER 3. SUITABILITY OF B65, SH-SY5Y AND IMR32 NEUROBLASTOMA CELLS AS MODELS FOR 'IN VITRO' NEUROTOXICITY TESTING



CHAPTER 4. STUDIES ON THE POTENTIAL NEUROTOXICITY OF THE NICOTINAMIDE DERIVATIVE N-METHYL NICOTINAMIDE

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**ABBREVIATIONS.**

**AA**: Ascorbic acid.; **AC**: Adenylate cyclase.; **ACE**: Acetylcholinesterase.; **ACh**: Acetylcholine.; **AD**: Alzheimer's disease.; **APP**: Amyloid precursor protein.; **ATP**: ; **BDNF**: Brain derived neurotrophic factor.; $[Ca^{2+}]_i$: Intracellular calcium concentration; **cAMP**: cyclic 1',5' adenosine monophosphate.; **CAT**: Choline acetyl transferase.; **CCH**: Carbachol.; **cGMP**: cyclic guanylate monophosphate.; **CNS**: Central nervous system.; **CN**: Caudate nucleus.; **COMT**: Catechol-ortho-methyl transferase.; **CSF**: Cerebral spinal fluid.; **DA**: Dopamine.; **DAG**: Diacylglycerol.; **DMSO**: Dimethyl sulphoxide.; **DOMA**: 3,4-Dihyroxymandelic acid.; **DOPA**: Dihydroxyphenylalanine.; **DOPAC**: 3,4-dihydroxyphenlacetate.; **DOPEG**: 3,4-Dihyroxyphenylgylcol.; **DPM**: Disintergrations per minute.; **EDTA**: Ethylenediamine-tetraacetic acid.; **GABA**: γ-amino butyrate.; **GC**: Guanylate cyclase.; **GSH**: Glutathione.; **HPLC**: High performance liquid chromatography.; **HD**: Huntington's disease.; **5HT**: 5-hydroxytryptamine.; $IP_3$: Inositol 1,4,5 triphosphate.; **ISF**: Interstitial fluid.; **LDH**: Lactate dehydrogenase.; **MAO**: Monoamine oxidase.; **MEM**: Minimal essential media.; **mV**: millivolts; **MORPEG**: methoxyphenylglycol.; **MPTP**: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.; $MPP^+$: 1-methyl-4-phenyl pyridinium.; **MTT**: 3[4,5-Dimethylyhiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue.; **NA**: Noradrenaline.; **NAD**: Nicotinamide adenine dinucleotide.; **NADH**: Nicotinamide adenine dinucleotide (reduced form).; **NIC**: Nicotinamide.; **NMDA**: N-methyl-D-aspartate.; **NMN**: N'-methyl nicotinamide.; **NMS**: N-methyl scopolamine.; **NSB**: Non-specific binding.; **NMTIQ**: N-methyl tetrahydroisoquinoline.; **PCA**: Perchloric

acid.; **PD**: Parkinson's disease.; **PHF**: Paired helical fragments.; **$PIP_2$**: Phosphatidyl-4,5-bisphosphate.; **PLC**: Phospholipase C.; **PKC**: Protein kinase C.; **RF**: Reticular formation.; **RN**: Red nucleus.; **ROS**: Reactive oxygen species.; **SAL**: Salsolinol; 6,7-dihydro-1,2,3,4-tetrahydroisoquinoline.; **SEM**: Standard error of the mean.; **SN**: Substantia nigra.; **ST**: Subthalamus.; **TB**: Trypan blue.; **TH**: Tyrosine hydroxylase.; **TIQ**: Tetrahydroisoquinoline.; **TMIQ**: 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol.; **VMA**: 3-methoxy-4-hydroxymandelic acid.; **VSCC**: Voltage sensitive calcium channel.; **XTT**: 2,3 Bis [2-methoxy-4-nitro-5-solfophenyl]-2H-tetrazolium-5-caroxanilelide inner salt.

# CHAPTER 1

# INTRODUCTION

### 1.1. Early Brain Development and Main Structures.

The brain is an extremely complex structure, consisting of over 200 billion neurones and approximately five times as many supporting cells known as neuroglia. Neuronal cells control all of the major acts involved in life, including breathing, learning and memory, heart rate, sexual function and emotional characteristics. The nervous system develops from the dorsal ectoderm of the early embryo (Ranson et al 1955, Brodal 1981). Further development occurs around the sixteenth day after fertilisation when the neural plate appears in the dorsal midline of the embryo. Two days later the neural plate develops into a groove with a fold along each side. This structure fuses at about the third week of development to form the neural tube, which is the precursor of the brain. Any neuroectodermal cells not incorporated into the neural tube, form neural crests that develop into the dorsal root ganglia of spinal nerves, comparable ganglia on cranial nerves, autonomic ganglia and the secretory cells of the adrenal medulla. After four weeks the whole structure develops into three primary brain vesicles, the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). During the next week, two of these primary vesicles divide to give five secondary vesicles, the telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. As the cells divide, the secondary vesicles develop into the commonly known regions of the brain, described below (table 1.1).

**Summary of Brain Regions.**

Spinal Cord

The spinal cord is the least differentiated component of the CNS with a segmented appearance. Pairs of spinal nerves are connected to the cord by a dorsal sensory root and sensory ventral root. The nerve bodies are situated in the centre of the spinal cord and are 'butterfly'-shaped, with connection both inside the cord running information to and from the brain and peripheral parts of the body. The periphery of the cord consists of the nerve fibres.

Medulla Oblongata

The fibre tracts of the spinal cord continue up to meet the medulla, which also contains clusters of nerve cells called nuclei. The most prominent of these, the inferior nuclei, send fibres to the cerebellum through the inferior cerebellar peduncles. Some of the smaller nuclei form components of the hypoglossal, accessory, vagus, glossopharyngeal and vestibulocohlear cranial nerves. The medulla contains the areas responsible for the control of respiration, cardiovascular and digestive function.

Pons

The pons consists of two separate regions with differing functions. Firstly, the dorsal area has shared features with the brain stem, consisting of sensory and motor tracts, together with the nuclei for several cranial nerves. The basal area of the pons provides extensive connections between the cortex of the cerebral hemisphere and the contralateral cerebellar hemisphere. These connections are important in motor control. In addition the pons contains the inhibitory control centres for respiration.

Cerebellum

The cerebellum, is situated below the tentorium cerebelli in the posterior fossa of the skull, in close relationship to the pons and medulla. Its chief functions are to control muscle tone, posture, movement and co-ordination at a subconscious level.

Diencephalon

This forms the largest part of the cerebrum, of which the largest component is the thalamus. The thalamus receives data from all sensory systems, except the olfactory, and projects to the sensory areas of the cerebral cortex. Areas of the thalamus are thought to be involved in emotional responses, whilst other areas are incorporated into pathways from the cerebellum and corpus striatum to the cerebral cortex motor areas. When areas of the corpus striatum are affected in neurodegenerative diseases, such as Parkinson's disease (PD) (Young and Penney 1993), the motor control circuit including the thalamus is disrupted. The subthalamus includes sensory tracts that link the thalamus to cerebellum, and the corpus striatum, which are also affected in PD (section 1.7.*i* for further explanation). The hypothalamus is the principal autonomic centre of the brain, and influences both sympathetic and parasympathetic systems. In addition neurosecretory cells in the hypothalamus release hormones, which reach the blood stream by way of the neurohypophysis or influence the hormonal output of the adenohypophysis by way of portal blood vessels.

Telencephalon

This area, also known as the cerebral hemisphere is very large in the human, especially due the large areas of neocortex. The neocortex is responsible for the control of most conscious movement, sensation, and memory and learnt behaviour combined with other higher levels of neural function. Loss of cellular functioning in certain areas of

the neocortex through neurodegeneration can induce dementia, such as Alzheimer's disease (AD) (Reisberg 1983, Katzman and Jackson 1991, Gibson et al 1991). The neurodegeneration seen in AD begins in the trans-entorhinal region of the temporal isocortex and spreads to involve the adjacent entorhinal, and gradually many diverse areas of the brain (see section 1.5 on AD, and Braak and Braak 1991). Each hemisphere is divided into four lobes. The posterior areas of the frontal lobes are specialised for motor functions, including language, whilst the anterior areas are involved in learning and planning. The temporal lobe consists of structures related to hearing and some speech areas. The occipital lobe is involved mainly in visual control. In addition, the medullary centre contains fibres connecting the cortical areas of the same hemisphere, fibres passing in both directions from the cortex and subcortical areas, and fibres that cross the midline between the two cortical hemispheres. Another area of the cerebrum is the basal ganglia, consisting of the globus pallidus, substantia nigra, putamen and caudate nucleus. These centres are all interconnected and are related to motor control. Furthermore, they are connected to both the cerebellum and frontal cortex motor centres, to enable the planning and co-ordination of gross movements. The substantia nigra is the primary site of neurodegeneration in the parkinsonian brain. The loss of dopamine from this area leads to the removal of inhibitory effects upon the other basal ganglia nuclei, and leads to the clinical symptoms seen in PD.

Table 1.1. The development of the mature brain from early vesicles.

| Secondary Brain Vesicles | Mature Brain |
|---|---|
| Myelencephalon | Medulla Oblongata |
| Metencephalon | Pons and cerebellum |
| Mesencephalon | Midbrain |
| Diencephalon | Thalamus, epithalamus, hypothalamus, and subthalamus |
| Telencephalon | Cerebral hemispheres, i.e. olfactory system, corpus striatum, cortex, and medullary centre. |

## 1.2. Cells of the Nervous System.

The nervous system consists of many diverse cell types. Large and small neurones are known as Golgi type I and Golgi type II neurones, respectively (Ranson et al 1955). An example of large Golgi type I neurones are pyramidal motor cells (Nathan 1982). These neurones are very large, with a pyramidal nucleus. They extend from the cortical motor areas to connect with spinal neurones. There are many more Golgi type II cells than type I. Type II neurones are much smaller in size and are often called interneurones as they are usually situated between other neurones (Barr 1979). They act to establish circuits which can have great complexity. These interneurones are abundant in the cortex and are thought to be involved in learning, memory and other higher functions. Examples of interneurones in the cortex are stellate cells, the cells of Martinotti and the horizontal cells of Cajal (Barr 1979). Peripheral nerve cells consist of sensory cells that relay information to the spinal cord from all areas of the body. This information is passed to the brain by ascending spinal nerves, and responses passed to effector areas in the body by means of descending neurones, and spinal nerves.

In addition to neuronal cells the nervous system consists of many supporting cells, called neuroglia. There are several types of neuroglia; astrocytes, oligodendrocytes, ependyma and microglia. Astrocytes are primarily involved as connective tissue supporting neurones, but play an important role in the elimination of neurotoxins, glutamate and production of neurotrophic factors (Fedoroff and Verndakis 1986, Kimelberg and Norenberg 1989, Barres 1991, Eddleston and Mucke 1993, Tomac et al 1995). Furthermore, astrocytes may regulate immune responses and may help protect

the brain from oxidative damage after haemorrhage (Halliwell 1992).

Oligodendrocytes not only support neurones but can produce myelin, which wraps around several neurones, to provide insulation of neural action potentials. Microglial cells display phagocytic properties when neuronal cells become damaged (Graeber et al 1993).

## 1.3. Neuronal Structure, Function, and the Control of Neurotransmission.

### *1.3.i. Neuronal Structure and Function.*

The specific shape and size of nerve cells varies greatly in relation to their individual function. However, there are many common features (Brodal 1981). The neurone has an enlarged cell body containing the nucleus, surrounded by short branching projections called dendrites, which improves the capacity of the cell to receive information from other neurones. This is achieved by the close inter-twining of the dendrites and other neuronal processes to form synapses. These are small gaps between cells, where, through a process of neurotransmission, information is passed from one cell to another (Ganong 1987). Most neurones have one process, called an axon, which conducts impulses away from the nucleus. The axon varies a great deal between types of neurone and carries information from the nucleus to effector organs (e.g. muscle fibres) or other neuronal cells. The size and structure of individual neurones depend upon their role in the nervous system. Pyramidal cells, named after their pyramid-shaped cell body, are involved in the control of motor function (Barr 1979). Thus they have several branching dendrites that connect them to the higher control areas of the neocortex, and a long axon that may reach to the lower spinal cord.

Conversely, interneurones such as the cells of Martinotti, are small multi-dendritic cells that form many connections between other neurones in areas such as the cerebral cortex (Barr 1979).

The neuronal cell contains all the normal cellular components, but unlike most cells the distance between the nucleus and parts of the cell, such as the axonal synapse is much greater. Therefore, a system is required to transport cellular proteins, such as those associated with neurotransmitter synthesis, from the nucleus to the extremities of the cell (Weiss and Gorio 1982). This is undertaken by means of a transport system called anterograde transport. This system has both fast and slow components, and may depend upon microtubules. The fast transport system is thought to be energy dependent and independent of nerve impulses. In addition to anterograde transport, there is a system that carries components back to the nucleus and is known as retrograde transport. Some neurones are surrounded by a myelin sheath, produced by a Schwann cell (from neuroglial origin). This is an insulating layer of lipid and protein that forms segments around some neurones. There are gaps in the sheath called the nodes of Ranvier (Ganong 1987). These structures allow faster conduction of nerve impulses as the electrical resistance is reduced between the nodes.

Neurones contain large numbers of mitochondria scattered throughout the cell body, axon, and dendrites. They are very important as neuronal cells are reliant upon glucose metabolism for their energy requirements. Furthermore, mitochondria contain monoamine oxidase, one of the important enzymes of catecholamine metabolism (Tipton et al 1984, Kopin 1993).

The primary function of the neurone is to transfer information to other neurones. Neuronal cells collect information relating to many external and internal stimuli. This

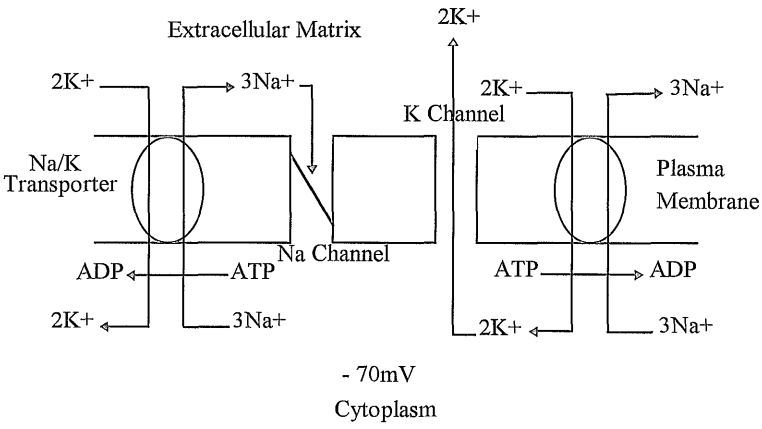
information is passed to other neuronal cells, which in turn elicit an appropriate response. For example in the simplest system, sensory nerve cells receive information from the environment (e.g. painful stimuli), and transfer this information to the neurones of the spinal tract. The sensory neurone is linked to an effector neurone, which responds to the painful stimulus by rapidly activating the skeletal muscles required to remove the affected area away from the source of the pain. In addition, the information is passed to the brain via the ascending tracts.

*1.3.ii. Membrane Potential and Generation of Action Potential.*

All excitatory neurones have complex systems which allow the selective extrusion of sodium ions (Armstrong 1981). Sodium ($Na^+$) ions are actively extruded from neuronal cells in return for the counter transport of potassium ($K^+$) ions in the ratio of 3 $Na^+$ to 2 $K^+$ (Skou 1988). This process involves a transporter protein, a $Na^+$ / $K^+$ ATPase, and requires the hydrolysis of ATP to drive the transport system (Nicholls et al 1992). In addition, there are ion channels (gates) that traverse the cell membrane, which are selectively permeable to either $Na^+$ or $K^+$. When the cell is at rest the $K^+$ channels are more permeable than the $Na^+$ channels. Therefore, $K^+$ ions diffuse back out of the cell at a greater rate than the influx of $Na^+$ ions into the cell, down their respective concentration gradients. Subsequently, the nerve cell has a negative internal potential of approximately - 70 millivolts (mV) (figure 1.1). This electrical difference is the basis of membrane potential, and is exploited to produce an action potential. The action potential carries an input signal from one area of the neurone to another and is the basis of axonal conductance. Action potentials are the product of alterations of membrane potential and not the change of ionic concentrations of $Na^+$ and $K^+$ in the

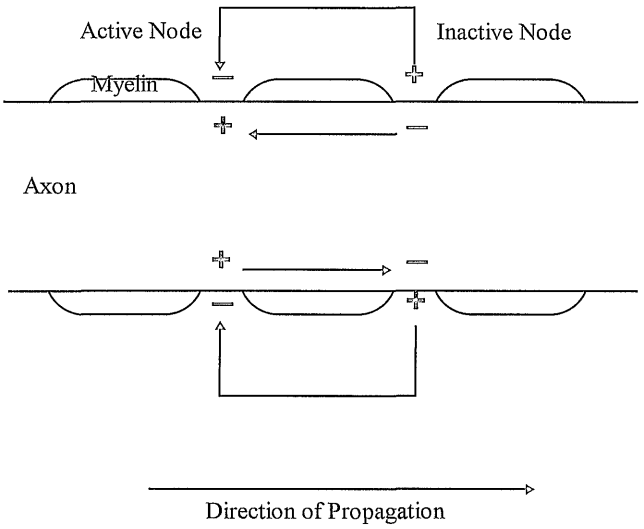
cytoplasm. Action potentials can be generated through mechanical, electrical, or receptor-mediated stimuli. The incoming stimulation must exceed a threshold level before it increases the permeability of $Na^+$ channels sufficiently to reverse the resting membrane potential to approximately + 35mV. The stimulus gradually increases the influx of $Na^+$ ions, and depolarization is exacerbated as a small change in the membrane potential of around 7mV rapidly induces the increased permeability of $Na^+$ channels. The depolarization of the membrane produces a local change in membrane potential, producing a negative external potential. Consequently, positive ions flow towards this area reducing the potential difference at other areas of the nerve fibre (Ganong 1987) (figure 1.2). Through this process the action potential travels from one area to another until the fibre ends at a synapse where neurotransmission may occur.

*Figure 1.1. Diagrammatic representation of the generation of resting potential.*




*The active exchange of 3 $Na^+$ ions for 2 $K^+$ ions, together with the selective permeability of the respective ion channels, creates a negative intracellular potential of around - 70mV whilst the neurone is at rest.*

*Figure 1.2. Schematic representation of the changes in membrane potential that occur to allow movement of the action potential along the neurone.*




*As one area of the neurone depolarizes the local electrochemical potential becomes more negative, which in turn, attracts positive ions from further along the neurone. Consequently, the potential difference is decreased and the action potential travels towards the area of lower potential difference, and hence along the fibre.*

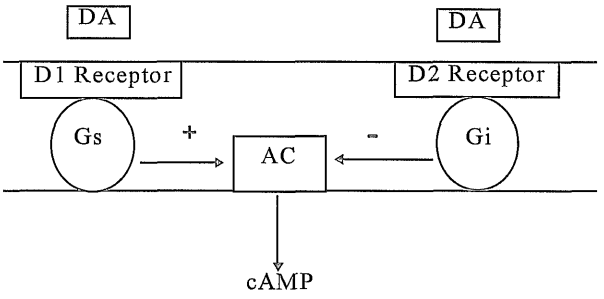
*1.3.iii. Neurotransmission and Signal Transduction.*

Neurotransmission is the transfer of an action potential from one neurone to another, across the synaptic cleft. This process forms the basis of neuronal cell communication allowing the transfer of sensory information collected from all parts of the body, and the subsequent induction of appropriate motor responses. In most cases the neurones must find another mechanism besides the action potential to bridge the gap between

cells. This is achieved by the release of neurotransmitters from vesicular stores at nerve terminals. The action potential induces depolarization of the presynaptic membrane, which facilitates the influx of calcium ions, through voltage sensitive calcium channels (Miller 1990, Jessel and Kandel 1993). The influx of $Ca^{2+}$ ions is thought to cause the fusion of several submembranal transmitter vesicles with the cell membrane and subsequent release of their contents into the synaptic cleft. $Ca^{2+}$ ions are normally kept at high extracellular and low intracellular concentration by the action of two separate mechanisms. Firstly, there is a high affinity $Ca^{2+}$ / $Mg^{2+}$ ATPase pump. This system requires energy from the hydrolysis of ATP and has low capacity pumping one $Ca^{2+}$ ion for one $Na^{+}$ ion per ATP expended (Blaustein 1988, Carafoli 1988). The $Ca^{2+}$ / $Mg^{2+}$ ATPase is sparsely distributed throughout the cell membrane but under normal conditions maintains a low $Ca^{2+}$ concentration within the cell. Secondly, there is a $Na^{+}$ / $Ca^{2+}$ ion exchanger, that has lower affinity for $Ca^{+}$ ions but a greater capacity. In most cells 3 $Na^{+}$ ions are exchanged for 1 $Ca^{2+}$ (Caputo et al 1989). The reduction of extracellular $Ca^{2+}$ concentration reduces the quantity of neurotransmitter released. Postsynaptic cells have receptors for specific neurotransmitters, which when occupied transfer the nerve impulse to the next cell. However, the response can be either excitatory or inhibitory depending upon the particular neurotransmitter and receptor type. For example excitatory responses may be initiated by binding of the neurotransmitter to the receptor that triggers the opening of $Na^{+}$ channels. Thus the postsynaptic membrane becomes depolarized and an action potential is propagated. However, if the receptor or transmitter is of an inhibitory nature, then the binding of transmitter to receptor may initiate the opening of chloride ($Cl^{-}$) channels, resulting in hyperpolarization.

Postsynaptic receptors exist that are not directly linked to voltage sensitive channels. These receptors instigate their actions through the process of signal transduction. This process usually takes much longer than other receptor mediated ion channel opening, however, once initiated the time course of the response is greatly increased. Receptor based neurotransmission requires a process of signal transduction to pass the signal (ligand binding to receptor) across the membrane, and produce an intracellular response. This process involves the production of second messengers inside the cell, such as cyclic 1',5' adenosine monophosphate (cAMP) or inositol-1,4,5-triphosphate ($IP_3$), as described below (figures 1.3. and 1.4).

*Figure 1.3. Diagrammatic Representation of the Regulation of Second Messenger Production Due to Different Receptor Occupation.*

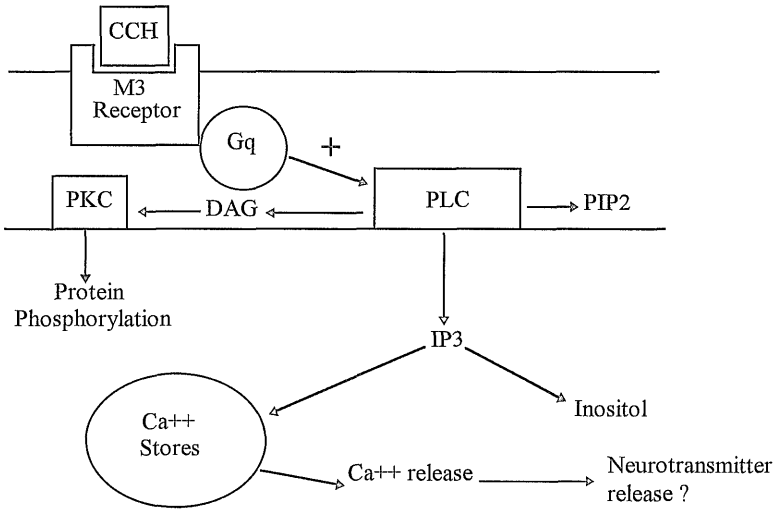



Both $D_1$ and $D_2$ dopamine receptors are linked to G-proteins. However, the G-proteins have opposite actions upon adenylate cyclase (AC), with $G_i$ inhibiting AC, and $G_s$ stimulating AC (Rodbell 1980). Therefore, the $D_1$ receptor stimulates cAMP formation, whilst the $D_2$ receptor inhibits cAMP formation (Birnbaumer et al 1990). Thus, through this system the concentration of cAMP is regulated. Therefore, the

degree of receptor occupancy, and types of G-protein linked to that receptor, determine the overall cAMP concentration, and hence regulate the further cellular responses. In this example the $D_2$ receptor mediated inhibition of cAMP formation is linked to the opening of $K^+$ channels which allows the efflux of $K^+$. Thus the neuronal membrane becomes hyperpolarized and more resistant to excitatory stimuli. In addition decreased cAMP formation may close $Ca^{2+}$ channels further decreasing the excitability of the neurone. Examples of $D_1$ and $D_2$ receptors can be found in the caudate nucleus and pituitary lactotroph cells, respectively.

G-proteins also link receptors to phospholipase C (PLC, Berridge 1993). This membrane bound enzyme hydrolyses phosphatidyl 4,5-bisphosphate ($PIP_2$), to generate two second messengers, inositol-1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG). The cleaved $IP_3$ is released from the membrane and releases $Ca^{2+}$ from intracellular stores, whilst DAG is known to activate protein kinase C (PKC, Niskizuka 1986, Allgaier 1986, Berridge 1993). The increased intracellular $Ca^{2+}$ will alone or in combination with calmodulin alter the phosphorylation of target proteins, thus modulating their activity (Erulkar 1983). A similar effect is generated through the action of PKC. $IP_3$ is repeatedly dephosphorylated to inositol, which is recycled back into the cell membrane as $PIP_2$. This system is activated by carbachol (CCH), a non-specific cholinergic agonist, in SH-SY5Y cells, leading to release of $Ca^{2+}$ from intracellular stores (Atcheson et al 1994a) (figure 1.4). Subsequently, the raised intracellular $Ca^{2+}$ concentration may induce noradrenaline release in SH-SY5Y cells.

*Figure 1.4. Diagrammatic Representation of Receptor-Mediated Neurotransmitter Release From SH-SY5Y Neuroblastoma Cells.*




*For a full explanation of this figure see above text.*

The removal of the particular neurotransmitter from the synapse produces a cessation of the postsynaptic stimulus, and subsequently termination of neurotransmission. In all cases, with the exception of acetylcholine, the removal of neurotransmitter is achieved through rapid uptake, facilitated by a high affinity transporter (Amara and Pacholczyk 1991). Acetylcholine is deactivated prior to uptake by membrane bound acetylcholinesterase (Silver 1974) to form choline and acetate. Choline is then taken into the cell via the choline specific uptake transporter (Kuhar and Murrin 1978, Tucek 1978, Griffiths and Norman 1993). The dopamine, GABA, noradrenaline and serotonin (5HT) transporters are all sodium dependent (Kanner and Schuldiner 1987).

In all cases re-uptake transporters are driven by active uptake through the action of a sodium dependent ATPase. Once inside the neurone the relevant neurotransmitters are concentrated into vesicles by transport systems driven by electrochemical gradients generated by vesicular $H^+$ ATPases. There is much homology between these sodium-dependent uptake transporters. The GABA transporter exchanges two $Na^+$ ions and one $Cl^-$ for each GABA molecule (Radian and Kanner 1983, Keynan and Kanner 1988, Worrall and Williams 1994). The 5HT transporter co-transports one $Na^+$ and one $Cl^-$ for each 5HT molecule, whilst counter-transporting one $K^+$ ion (Rudnick 1977). There are two forms of the NA uptake transporter. The $uptake_1$ transporter has high affinity, but low maximum rate of uptake, whilst the $uptake_2$ system has high maximum uptake but low affinity. $Uptake_1$ is found on neuronal cells and $uptake_2$ on non-neuronal cells. All the transporter proteins of this family have 12 regions that span the membrane. Each transporter is inhibited by different compounds. For example NA uptake can be inhibited by antidepressants such as imipramine. The dopamine transporter enables the selective uptake of the 1-methyl-4-phenylpyridine, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Jarvitch et al 1985, Del Zompo 1993). Once inside the neurone both catecholamines (DA and NA) and indolamines (5HT) are either stored in vesicles prior to re-release, or metabolised by the action of monoamine oxidase.

## 1.4. Synthesis and Metabolism of Neurotransmitters.

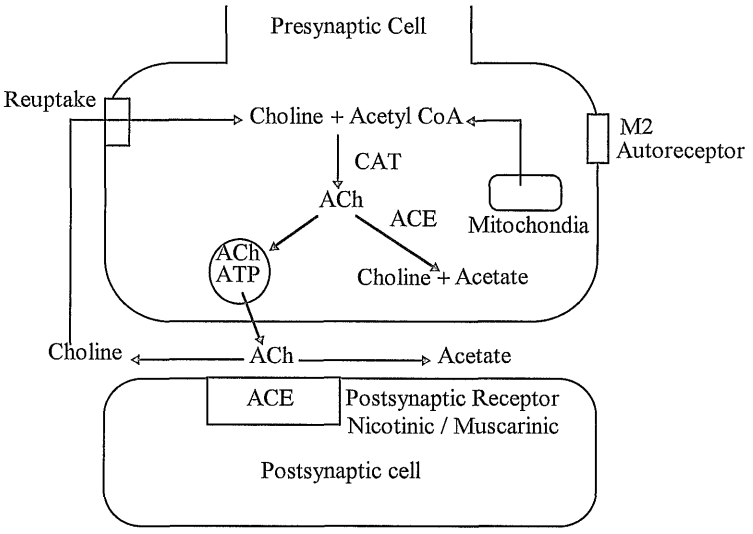
There are many neurotransmitters found in the central nervous system and describing the synthesis and catabolism of all would be excessive. Therefore, only the neurotransmitters affected in the neurodegenerative diseases discussed later will be addressed. For example there is overlap in the loss of acetylcholine (ACh), and the catecholamines, dopamine (DA), noradrenaline (NA) and serotonin (5HT) in Alzheimer's, Parkinson's and Huntington's Diseases.

### *1.4.i. Acetylcholine.*

Acetylcholine (ACh) is released from many neurones, such as the neurones of the basal forebrain. Synthesis of ACh occurs through the combination of acetyl CoA and choline by the enzyme, choline acetyl transferase (CAT) in the nerve terminals (figure 1.5). Once ACh is synthesised it is available for immediate metabolism to acetate and choline, by acetylcholinesterase (ACE). ACh is stored in vesicles with ATP prior to release to prevent degradation. After release ACh is metabolised by postsynaptic membrane bound acetylcholinesterase, to form choline and acetate. Finally choline is recycled via a presynaptic re-uptake system. The control of synthesis depends primarily upon the concentrations of choline and acetyl CoA present in the cell. The concentration of acetyl CoA, formed in the mitochondria as an intermediate of glucose metabolism, depends upon the energy status of the cell and mitochondrial functional integrity. Periods of mild hypoxia and hypoglycemia result in the reduction of acetylcholine synthesis (Gibson et al 1975, Gibson and Blass 1976, Gibson et al 1991). In addition, ACh synthesis is dependent upon the availability of choline. Therefore, an

equilibrium exists between the synthesis and metabolism within ACh neurones, which is controlled by the rate of enzymic action and availability of substrates.

*Figure 1.5. Diagrammatic scheme of acetylcholine synthesis and catabolism at a cholinergic nerve terminal.*




*1.4.ii. Catecholamines.*

Dopamine (DA) and noradrenaline (NA) are synthesised from the same substrate, tyrosine. Furthermore, noradrenaline is produced by the enzymatic conversion of dopamine, as described below (figure 1.6). Initially, the amino acid tyrosine is taken into neurones, followed by conversion to dihydroxyphenylalanine (DOPA), via the enzyme tyrosine hydroxylase. This first step in the synthetic pathway is rate limiting

for the production of both DA and NA. Tyrosine hydroxylase (TH) requires tetrahydrobiopterin as a cofactor, which donates a hydroxyl group, and is subsequently converted to dihydrobiopterin during the reaction. The overall activity of this enzyme is controlled by a negative feedback process; i.e. the greater the intracellular concentration of DA or NA, the greater the inhibition of tyrosine hydroxylase. Therefore, the concentration of DA or NA will remain relatively constant. The next stage of synthesis converts DOPA to DA by the action of DOPA decarboxylase. The absence of the next enzyme in the pathway, dopamine-β-hydroxylase, in the neurones of the substantia nigra, means that they produce and store DA as their neurotransmitter. In all noradrenergic neurones, dopamine-β-hydroxylase converts DA to noradrenaline . NA and DA are stored with ATP and chromogranins in vesicles at nerve terminals prior to release. The major control of over stimulation of postsynaptic cells is the rapid re-uptake of both dopamine and noradrenaline from the synaptic cleft. This is undertaken by the presence of an active re-uptake transporter situated in the presynaptic membrane (figure 1.8).

Both NA and DA have similar catabolic pathways, involving two separate but complementary enzymes. Firstly, monoamine oxidase (MAO) a flavin containing enzyme, found in the mitochondrial outer membrane, deaminates catecholamines. There are three types of MAO, A, B and C. MAO-A is thought to preferentially deaminate NA, whilst DA is thought to be a substrate for both types A and B (Tipton et al 1984, Kopin 1993). Deamination of NA produces 3,4-dihydroxymandelic acid (DOMA) or the corresponding glycol 3,4-dihydroxyphenylgylcol (DOPEG). Both DOMA and DOPEG enter the circulation where they may be converted to 3 methoxy-4-hydroxymandelic acid (VMA) or methoxyphenylglycol (MORPEG) respectively, by

the action of catecholamine-ortho-methyltransferase (COMT) (figure 1.7). Similarly, DA is deaminated by MAO to produce 3,4-dihydroxyphenylacetic acid (DOPAC), followed by formation of homovanillic acid by COMT. The sequential action of MAO and COMT can be reversed, but the final excretory product is homovanillic acid (HVA) (figure 1.7).

*Figure 1.6. The Synthesis of Dopamine and Noradrenaline.*

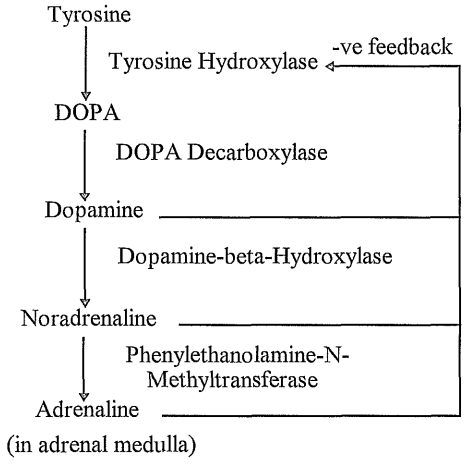
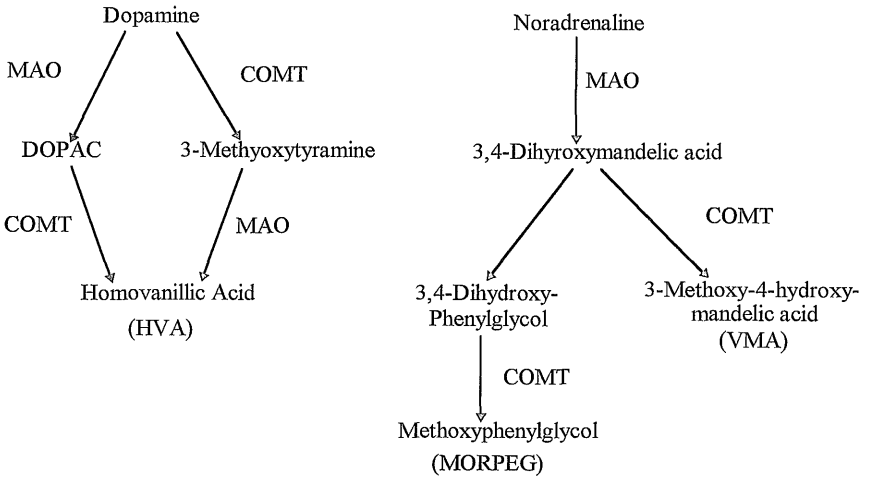
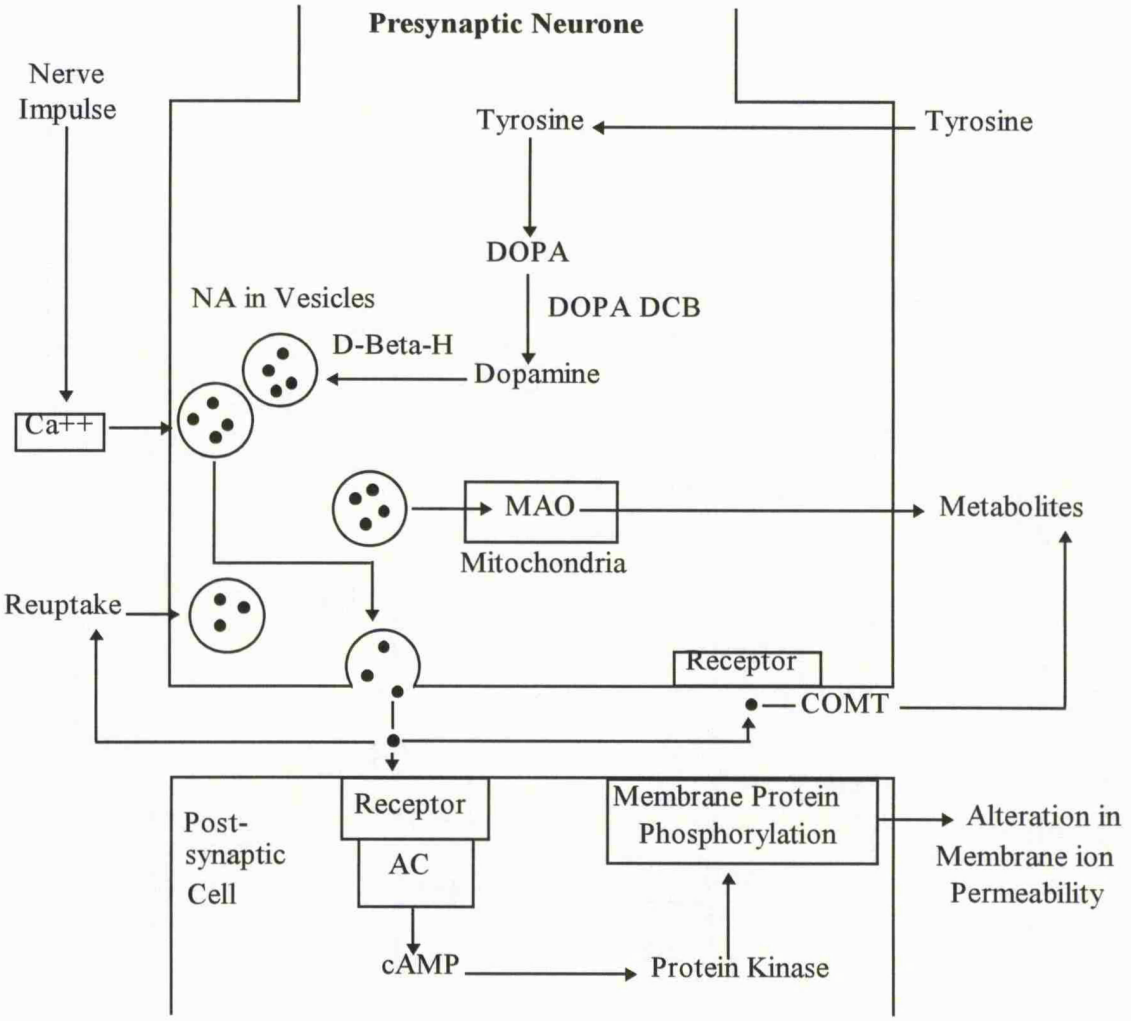


*Figure 1.7. The Catabolism of Dopamine and Noradrenaline.*




*Both dopamine and noradrenaline are catabolised by monoamine oxidase (MAO) in the mitochondria of nerve terminals. Additionally, both DA and NA can be catabolised by catechol-ortho-methyl transferase (COMT). Finally, the resulting compounds homovanillic acid, methoxyphenylglycol and methoxyhydroxymandelic acid are excreted in the urine.*

*Figure 1.8. Schematic representation of the mechanism of neurotransmission at a catecholaminergic synapse.*



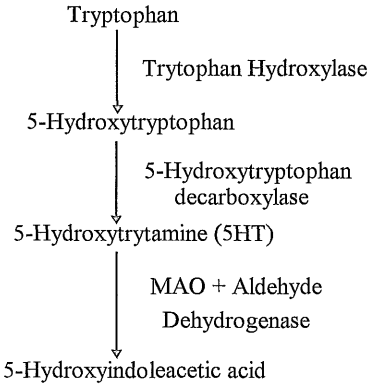

*Tyrosine is taken into neurones prior to conversion into noradrenaline, or dopamine in dopaminergic cells. Conversion of DA to NA occurs in vesicles via the action of dopamine-β-hydroxylase (D-beta-H). NA, or DA in dopaminergic cells, is released upon $Ca^{2+}$ entry, induced by either depolarization or receptor-mediated mechanisms. DA or NA can act upon presynaptic receptors, which when occupied may inhibited DA or NA release through hyperpolarization of the membrane. DA and NA can also*

*inhibit TH activity. Once released DA or NA can activate postsynaptic receptors, which may excite or inhibit the cell through, adenylate cyclase, cAMP and protein kinase activity. Neurotransmission is terminated by inactivation of DA and NA by monoamine oxidase (MAO), or catechol-ortho-methyl transferase (COMT), and also by re-uptake.*

*1.4.iii. Serotonin.*

Serotonin, or 5-hydroxytryptamine is formed from the hydroxylation of tryptophan by the enzyme tryptophan hydroxylase, followed by decarboxylation by 5-hydroxytrytophan decarboxylase (figure 1.9). 5HT is thought to be involved in the regulation of mood, since elevation of 5HT can relieve depression. Metabolism of 5HT is similar to NA and DA, as 5HT is a substrate for MAO. Deamination followed by dehydrogenation produces 5-hydroxyindoleacetic acid, which is the excretion product. 5HT can also be converted into the pigment melatonin.

*Figure 1.9. The Biosynthesis and Catabolism of Serotonin 5HT.*



In summary the central nervous system controls all basic, and higher functions involved in life, through the interconnection of neuronal cells. Furthermore, neuronal cells are terminally differentiated and thus are unable to replicate. Consequently, if any damage occurs, irreversible loss of neurones can seriously affect the normal functioning of the brain. Neuronal cells have some capacity to regenerate but this can only occur if the damage is peripheral and not to the cell body. Therefore, as nerve cells are terminally differentiated in vivo, no replacement of damaged neurones is possible. Subsequently, many debilitating neurodegenerative diseases exist that involve the death of neurones. The aetiologies of many are poorly understood, and the presentation of symptoms depends upon the area(s) of brain affected. Presenting symptoms may overlap between different degenerative diseases. For example, damage to cholinergic neurones may occur in several disorders, including Alzheimer's and Parkinson's diseases. Consequently, dementia may be present in both PD and AD. Despite intensive epidemiological, in vivo and in vitro studies, the causal factors of these and other neurodegenerative diseases are unknown. However, several theories exist which will be discussed further in the following sections.

**Neurodegenerative Diseases: are toxins involved in their aetiology ?**

There is a growing body of evidence to suggest that various neurodegenerative diseases may be induced by toxic damage to neurones. In addition, there is evidence that both endogenous and exogenous compounds may be involved in the initiation or progression of neurodegeneration. Moreover, increasing age of the population is also a major factor in the predisposition to neurodegenerative disease. Therefore as the population increasingly lives longer the problem of neurodegeneration becomes more important. The aetiologies of three of the most common and most studied neurodegenerative diseases (Alzheimer's disease, Parkinson's disease and Huntington's Chorea), will be discussed further.

**1.5. Alzheimer's Disease (AD).**

The neurodegeneration seen in AD was first described by Alois Alzheimer in 1907, and is related to the progressive loss of neurones from the cerebral cortex. The destruction of these cells results in the gradual loss of memory, learning and ability to undertake or manipulate information during tasks involving concentration and intellect (Todorov et al 1975, Adolfsson et al 1981, Roth 1978, Reisberg 1983, Katzman and Jackson 1991, Tariot et al 1993, Lamour 1994). Further progressive cell death leaves the patient incontinent, mute and with many neurological abnormalities including seizures (Freidman 1988).

The disease is characterised by the appearance of protein dense masses, known as neurofibrillary tangles and amyloid plaques (Iqbal and Wisniewski 1983, Wisniewski

1983). They appear in six stages (Braak and Braak 1991). The first cells to be affected are in the trans-entorhinal region. Stage II sees the further involvement of entorhinal area (Jones 1993). During stage III, extracellular neurofibrillary tangles start to appear, and mild cognitive impairment is apparent. Additionally, severe neurofibrillary tangles appear in the pre-alpha regions of entorhinal and trans-entorhinal areas of the temporal isocortex. Stages IV, V and VI are characterised by further progressive neurofibrillary tangle formation in the hippocampus, subcortical nuclei and isocortical areas.

*1.5.i. Morphological Changes in AD.*

The classical morphological change in AD is the production of firstly intracellular, followed by extracellular neurofibrillary tangles and plaques (Katzman 1986). These tangles are formed from a combination of proteins. The extracellular plaques have a central core of amyloid protein surrounded by distended abnormal dendrites and axons. Amyloid is a group term for fibrillary proteins that contain β-pleated sheet structure, formed from a precursor protein (amyloid precursor protein, APP, Kang et al 1987). The precise role of APP is unclear. However, APP is increased in the foetal brain, and has a trophic role in the culturing of some cells (Saitoh et al 1989, Whitson et al 1989, Yankner 1990). Intracellular lesions that form during neuronal degeneration contain paired helical fragments (PHF) as their main constituents. PHF's are composed of the protein tau (Joachim et al 1987, Goedart 1993), which is a microtubule associated protein (Weingarten et al 1975, Drechsel 1992). However, in PHF's tau is abnormally phosphorylated, giving rise to problems transporting components from nucleus to nerve terminal. Recent studies have shown that the degree of synaptic loss between the

cerebral cortex and hippocampus is directly linked to clinical dementia (Terry and Katzman 1983, Hamos et al 1989, Masliah et al 1989, and DeKosky and Scheff 1990, Tariot et al 1993).

The main group of cells to degenerate during AD are the large cholinergic cells of the basal nucleus of the forebrain (Hohmann et al 1988). Subsequently there is a gradual decrease in the neurotransmitter acetylcholine in areas related to the cerebral cortex, such as the locus ceruleus projection to the hippocampus and cerebral cortex (Coyle et al 1983, Hubbard and Anderson 1985, Tariot et al 1993). Areas of the dorsal raphe that project to the cortex are also affected during the progression of the diseases, and all are related to memory.

*1.5.ii. Genetic Predisposition.*

There is conflicting evidence relating to the importance of genetic factors in AD, however the primary risk factor for AD is increasing age (Heston 1982, Schoenborg et al 1987, Mortimer 1990, Zhang et al 1990). Analysis of families with high incidence of AD has shown that in some families with early onset AD there is a locus present on chromosome 21, that is absent in other groups (St. George-Hyslop et al 1987, Roses et al 1990). The frequency of AD occurring in both identical twins has never reached 100 % (Jarvik et al 1971). Furthermore, the numbers of families with familial AD is very small. There appears to be a bias to female susceptibility for the development of AD (Gaillard 1984, Constantinidis 1984, Akesson 1969, Molsa et al 1982, Kaneko 1975), which is independent of age. There also seems to be no difference between the incidence of AD between different racial groups. Therefore, a genetic predisposition

may play a role in increased susceptibility but appears not to be, in the majority of cases the cause of AD.

*1.5.iii. Involvement of Toxic Agents in the Aetiology of AD.*

Environmental Toxins.

Aluminium intoxication can induce neurological damage presenting with memory loss, tremor, jerky movements and impaired co-ordination (Spofforth 1921, Alfrey et al 1972, Winship 1993, Doll 1993). Therefore, aluminium poisoning has been suggested as a potential toxin involved in the aetiology of AD (Yates et al 1980, Crapper et al 1976, Delaney 1979, Foncin 1987, Perl and Brody 1980, Shore et al 1980, Wascher and Cohn 1985, Exley and Birchall 1992). Aluminium is known to increase the permeability of the blood brain barrier, and as such may gain access to the central nervous system (Banks and Kastin 1983). The accumulation of aluminium has been demonstrated previously in PHF's (Crapper et al 1973), and also in plaques (Edwarson et al 1986, Candy 1986). In addition, intracranial injection of aluminium is known to produce similar lesions to those seen in AD (Crapper et al 1973). Intracellularly, aluminium is attracted to the negatively charged phosphates of the nucleus (Crapper et al 1980, Crapper et al 1978, Wen and Wisniewski 1985, Wedrychowski et al 1986), where interference with nuclear function can occur (Lukiw et al 1989, Tarkka et al 1993), thereby affecting cell viability. However, the accumulation of aluminium may be coincidental to the disease process, as it may be deposited in degenerating neurones (Murray et al 1991, Lord Walton 1992). Therefore, it appears unclear how far aluminium can be implicated as a causal factor in AD.

Some fragments of the amyloid precursor protein appear to be cytotoxic to neuronal cells in vitro (Yanker et al 1989, Cotman and Anderson 1995, Grammas et al 1995, Mattson and Goodman 1995). Furthermore, one study reported a high frequency of typical Alzheimer-type changes in patients who were regular users of the analgesic phenacetin (Murray et al 1971).

Toxins and Metabolic Dysfunction.

There is some evidence that an as yet unidentified mechanism or toxin may be involved in the cellular degeneration, involving metabolic dysfunction. Evidence indicates reduced glucose metabolism in the temporoparietal area, which occurs prior to clinical symptoms of non-memory cognitive deficits at an early stage of AD (Duara et al 1986, Haxby et al 1986). Furthermore, examination of glucose metabolism in the frontal cortex indicated reduced pyruvate dehydrogenase activity, consistent with reduced cell number, but no alteration in succinate dehydrogenase activity (Yates et al 1990). Therefore, severe defects in mitochondrial metabolism can occur in AD (Parker et al 1994, Kumar et al 1994). In addition, the synthesis of acetylcholine is exquisitely sensitive to alterations in the rate of mitochondrial activity (Gibson et al 1975, Gibson and Blass 1976, Gibson et al 1991). Reduction of oxidative phosphorylation may also produce a parallel decrease in the synthesis of acetylcholine as seen during the ageing process (Gibson et al 1975, Gibson et al 1991).

*1.5.iv. Head Injury.*

There is a positive correlation between boxing and the subsequent development of dementia (Wisniewski et al 1976, Corsellis 1978, Henderson 1988, Mayeux et al 1995,

Kondo et al 1995). In addition, a link exists between head trauma and development of AD in later life (Mortimer et al 1985, Heyman et al 1984). The damage induced by head injury may accelerate the normal ageing process by producing ischemic damage to regions of the brain involved in AD, thus lowering the threshold for clinical symptom of AD to be observed. Ischemic damage is usually exacerbated by reperfusion injury. After, ischemia cells may die, or sustain damage resulting in the leakage of transition metals (e.g. iron). These ions (e.g. $Fe^{2+}$) can induce peroxidative damage, via lipid peroxidation and interact with compounds such as hydrogen peroxide to produce oxygen free radicals (Halliwell and Gutteridge 1985a, Halliwell and Gutteridge 1985b, Halliwell 1992). The increased oxygen concentration in the blood after reperfusion potentiates these reactions, which do not proceed without oxygen (McCord 1985).

### 1.6. Huntington's Chorea.

Huntington's Chorea (Huntington's disease, HD), is an autosomal dominant, neuropsychiatric disorder. The disease presents with increasing random and involuntary movements of muscles. This leads to the chorea, or dancing movements, which are exacerbated by emotional stress and voluntary movement, but often stop during sleep (Martin 1984, Folstein 1986). The range of age at onset is very varied, however a family history must always be present. The early symptoms may include impulsiveness, moodiness, and irritability. Physical symptoms of jerkiness, clumsiness and mild unco-ordination often are first to develop. Moreover, as the disease progresses the excessive movements increase and affect more muscle systems of the

body, such as facial muscles. In addition cognitive loss and dystonia can occur (Myers et al 1988, Folstein 1989). In some patients rigidity, similar to that in PD is observed (Hamilton 1908, Bittenbender and Quadfsael 1962).

*1.6.i. Morphological, Biochemical and Pharmacological Changes.*

Post-mortem examination of HD brain reveals a reduction of brain mass, particularly within the cerebral cortex, where cell shrinkage and demyelination is observed. The neurones most affected in this disorder are the γ-amino butyrate (GABA) secreting cells of the caudate nucleus and putamen. These neurones send fibres to the substantia nigra and globus pallidus. Normally, through the action of GABA, an inhibitory effect is maintained upon the substantia nigra and globus pallidus. In HD this is gradually eroded with increasing cell loss in the caudate nucleus and putamen. The excessive involuntary movement is thought to occur through the reduced inhibition of the globus pallidus. Therefore, an imbalance in the regulation of voluntary movement control occurs, which appears to be the reverse of that found in PD. In addition to the loss of GABA and cholinergic neurones (Kowall et al 1987), several studies indicate the loss of dopamine receptors in the putamen and caudate nucleus in HD (Reisine et al 1977, Seeman et al 1987, Joyce et al 1988).

Despite being an autosomal inherited disorder, the exact progression of the disease is unclear, especially the degenerative process involved in neuronal loss. Therefore, the genetic defect may be the primary cause or may predispose the individual to be more susceptible to other stresses. An example of the latter scenario could involve the disturbance of metabolism in neuronal cells. Studies using positron emission tomography have shown reduced glucose metabolism in cells of the basal ganglia cells

(Kuhl et al 1982, Hayden et al 1986, Mazziotta et al 1987, Young et al 1987, Hayden et al 1987, Grafton et al 1990, Beal 1992). Kuwert et al (1990), found reduced glucose metabolism in the cortex of HD patients. Alterations in the activity of pyruvate dehydrogenase have been localised to the hippocampus and basal ganglia in HD patients (Sorbi et al 1983, Butterworth et al 1985). Furthermore, mitochondrial enzyme activity can be decreased in the caudate nucleus of HD patients (Mann et al 1990), including mitochondrial complex I (Parker et al 1990). Finally, many HD patients are known to exhibit great weight loss despite high calorific intake. The metabolic insufficiencies described here could lead to wide spread cellular damage. One such example is that the intracellular $Ca^{2+}$ concentration is kept low through active extrusion. This process occurs to prevent unregulated activation of cellular enzymes, which if unchecked could irreversibly damage the cell. If the energy balance of the neurone is depleted far enough, then it is unable to maintain $Ca^{2+}$ homeostasis. An example of the consequences of the disturbance of $Ca^{2+}$ homeostasis is glutamate excitotoxicity (Meldrum and Garthwaite 1990). Some of the first neurones affected by HD are thought to possess the highest number of excitotoxic receptors, such as N-methyl-D-aspartate (Albin et al 1990, Young et al 1988, Dure et al 1991, Beal 1992). However, these studies cannot determine whether the effects upon glucose related metabolism are the primary cause or secondary to the initiation of the disease process.

## 1.7. Parkinson's Disease.

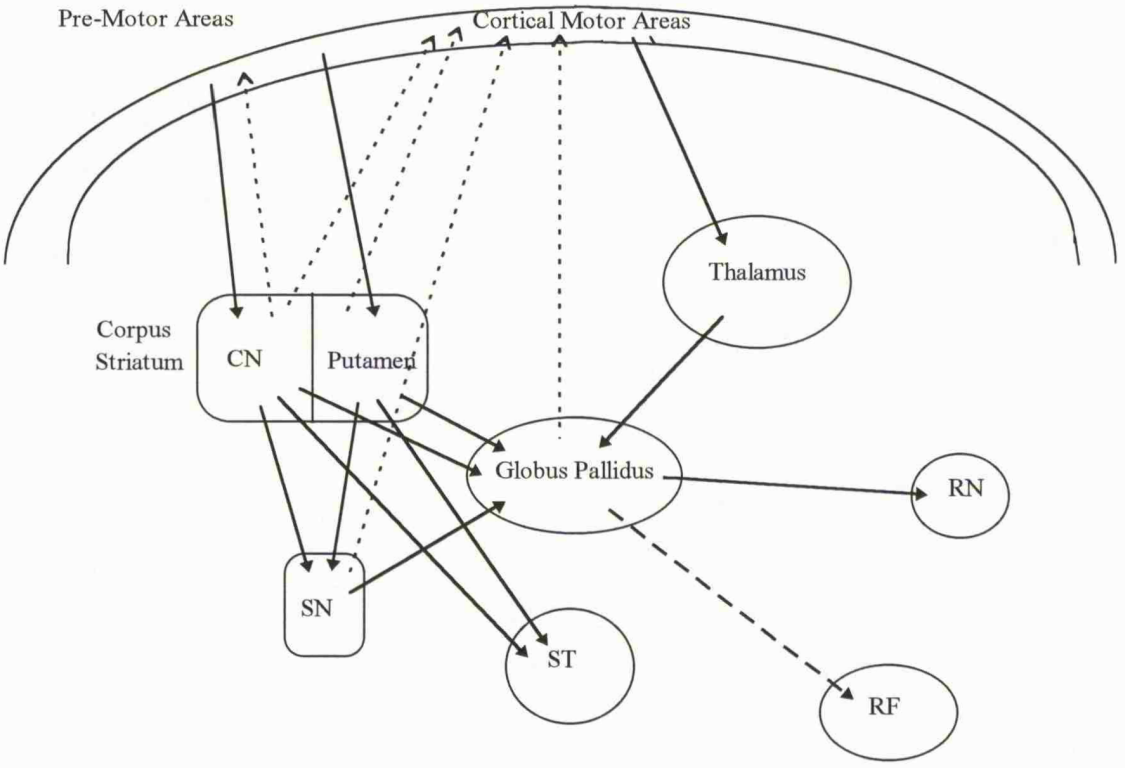
### *1.7.i. Structure, Function and Control of Neural Motor Activity.*

To understand the symptomatic dysfunction related to Parkinson's disease (PD) one must understand the basic functioning of the areas of the brain that are damaged in this syndrome. There are two main neuronal pathways that control the motor co-ordination and function of skeletal muscle. They are known as the pyramidal and extrapyramidal systems. The pyramidal system is responsible, in conjunction with the thalamus, for certain aspects of the planned voluntary motor control. The system links regions of the cortical motor areas to the motor areas of the brain stem, through the thalamus. The extrapyramidal systems consist generally of all motor pathways not related to the pyramidal tracts. This system involves, neuronal connection from the frontal and parietal lobes of the cortex (i.e. the premotor area), to the basal ganglia. The basal ganglia consist of the following groups of nuclei, the corpus striatum, putamen, globus pallidus, substantia nigra and subthalamic nucleus. There are three interconnecting pathways. Firstly, nerve fibres from the premotor areas are connected to the caudate nucleus and putamen. The caudate nucleus and putamen send fibres to the globus pallidus, substantia nigra and subthalamus. To complete a circular pathway nerve fibres return from these regions to the motor areas of the motor cortex (figure 1.10). Secondly, the basal ganglia are interconnected by the following pathway; the caudate nucleus and putamen pass fibres to the globus pallidus and substantia nigra. This pathway is reciprocally connected and both routes are thought to be inhibitory, the descending pathway releasing GABA, and the ascending pathway releasing dopamine. Finally, the basal ganglia have multiple small connections between themselves, and the

globus pallidus and substantia nigra have connections with the reticular nuclei. Through this pathway the basal ganglia can directly affect motor control (Evered and O'Connor 1984, Carlsson and Carlsson 1990, Young and Penney 1993).

The caudate nucleus and putamen act in concert to initiate and regulate large scale intentional body movement. This is accomplished though the connections from the globus pallidus and substantia nigra, to the thalamus, cerebral motor cortex and finally the spinal motor fibres. In addition, another pathway can be used through the globus pallidus and substantia nigra to the reticular motor areas. The globus pallidus is thought to be responsible for the control of background muscle tone during conscious movement, independently from the site of initiation. This is achieved though connections to the reticular formation (a motor area) in the brain stem, and through feedback connection with the thalamus and cerbral cortex. The substantia nigra, through its connections with the other nuclei of the basal ganglia, is inhibitory via the release of dopamine. Therefore, any interruption of one or more components of this intricate system would severely perturb the efficient control of posture, muscle tone and movement (Penney and Young 1993).

*Figure 1.10. A simplified diagram of the interconnections between the motor areas of the cortex and the basal ganglia.*




*The above diagram indicates some of the interconnecting pathways of neurones between the pre-motor and basal ganglia (corpus striatum, globus pallidus, subthalamus, ST, and substantia nigra, SN). These connections are shown as solid lines, and the reciprocal neuronal connections as dotted lines. The individual basal ganglia are also interconnected as described above, and the globus pallidus is connected to the reticular formation, a motor centre in the brain stem (dashed line).*

*1.7.ii. Morphological, Pharmacological and Biochemical Changes in PD.*

There are many related yet pathologically different syndromes that fall under the umbrella of Parkinsonism. The most common is idiopathic PD. The general

syndrome appears as a gradual loss of the initiation of movement (akinesia), rigidity of voluntary muscles, and tremor at rest (Hoehn and Yahr 1967), which disappears upon use of the affected limb. Posture and balance are also affected (Martin 1967). The primary underlying cause of PD is the degeneration of the dopamine containing neurones in the substantia nigra (Hornykiewicz and Kish 1986, Hornykiewicz 1989). However, patients do not develop clinical symptoms until degeneration of 70 to 80 % of dopaminergic substantia nigral cells occurs. This may in part be due to the plasticity of dopamine receptors. There are two distinct dopamine receptors $D_1$ and $D_2$ coupled to adenylate cyclase, inducing activation or inhibition respectively. In PD as the substantia nigra degenerates presynaptic turnover is vastly increased in the remaining neurones (Schultz 1982, Bernheimer et al 1961, Donnan et al 1991). This process will relieve the feedback inhibition promoted by DA within the cells, thus promoting increased DA synthesis. Additionally, a supersensitivity of postsynaptic receptor sites occurs (Rinne 1982, Rinne et al 1980, Lee et al 1978). However, the increased presynaptic DA turnover appears well before postsynaptic supersensitivity (Creese and Snyder 1979). These related systems explain why clinical symptoms appear only after 70 to 80 % of DA neurone loss in the substantia nigra. Furthermore, the degeneration of the substantia nigra reduces DA concentrations in all related structures, such as the caudate nucleus and putamen (Hornykiewicz 1973 and Hornykiewicz 1982). The degeneration of these areas relieves the inhibition upon the excitatory cells of basal ganglia, resulting in the over stimulation of motor neurones and subsequent shaking of the extremities. Additionally, the presence of Lewy bodies is characteristic of PD (Forno 1986), however, they are often found in patients with AD (Jellinger and Riederer 1984). Furthermore, neurofibrillary tangles, cognitive and

psychiatric disturbances (Scatton et al 1983) may be present in both AD and PD patients. The substantia nigra is by no means the only area of the brain involved in the neurodegenerative disease. Indeed, there are reports that PD patients have degeneration of the noradrenergic locus ceruleus (Fornstedt et al 1990), and 5HT levels have been reportedly reduced in the caudate nucleus (Scatton et al 1983), hippocampus and the dorsal raphe (Scatton et al 1982). Moreover, in some PD patients the degeneration of the cholinergic neurones of the substantia innominata occurs (Tagliavin et al 1984, Jenner et al 1992, Arendt et al 1983, Whitehouse et al 1983). There are various potential causes of PD, however none have been proven conclusively and the exact aetiology remains unclear.

*1.7.iii. Genetic Predisposition.*

It has been postulated that there is a genetic predisposition to inherit and subsequently develop PD. However, examination of both mono- and dizygotic twins showed that there was a very low rate of concurrent PD in both twins (Ward et al 1983). These findings infer that non-genetic factors play a large role in PD (Ward et al 1983). There is evidence that the prevalence of PD may be lower in certain ethnic groups (Harada et al 1983, Kessler 1972a and b, Schoenberg et al 1981). However, data also indicate no sex related difference in the frequency of developing PD (Marttila and Rinne 1981). Additionally, there appears to be no distinction between social status, living environment or occupation (Kessler 1972, Marttila and Rinne 1976). Furthermore, both HLA and serological markers have been examined to attempt to find a predictor for PD. However, no correlation was found (Leheny et al 1983, Kondo 1984, Marttila

and Rinne 1981, Takagi et al 1982). Thus it appears that a genetic element of PD is unlikely.

Despite a general lack of conclusive evidence linking genetic deficiencies with PD, there is some evidence that PD patients may have mitochondrial insufficiencies. A recent study has examined variations in the genetic alleles for the important degradative enzyme MAO, in both PD patients and age matched controls (Hotamisligil et al 1994). MAO catabolises the degradation of catecholamines, and has been linked with the activation of toxins (Naoi 1993b), Furthermore, increased DA turnover by MAO can produce increased oxidative stress, which in turn may be important in PD (Spina and Cohen 1989). Hotamisligil et al (1994) found no association between individual allele types and PD. However, there were significant differences in frequency distribution of all alleles between the PD and age matched controls. Additionally, several studies have found genetic alterations in the mitochondrial DNA of PD patients (Ikebe et al 1990, Ozawa et al 1991, Golbe 1990, Mizuno et al 1990, DiDonato et al 1993, Ikebe et al 1995). These alterations may be related to the deficiencies in mitochondrial complex I also reported to occur in PD (Schoffner et al 1991, Mann et al 1992, Schapira et al 1990, Benecke et al 1993, Blin et al 1994, Tritschler 1994, Reichmann et al 1994). In animals, the inhibition of mitochondrial oxidative phosphorylation in the brain can induce motor defects similar to PD (Singer et al 1987, Nicklas et al 1985, Ginsberg 1980, Uitti et al 1985, and Rosenberg et al 1989). Since the inhibition of mitochondrial electron transport can lead to formation of reactive oxygen species (Rossetti et al 1988, Ksenzenko et al 1983), this may cause cell damage by oxidative damage to DNA, proteins and lipids. Therefore, abnormalities in mitochondrial function and MAO activity may play a role in PD.

*1.7.iv. A Toxic Aetiology for PD ?*

A possible environmental or endogenous toxin, which may induce PD is an attractive hypothesis. Indeed, intensive research has been centred on the identification of potential neurotoxins. The role of transition metals ($Fe^{2+}$ and $Mn^{2+}$), and the possibility of exogenous or endogenous toxins are discussed.

The Role of Transition Metals.

Transition metals such as iron and zinc are integrally linked to the correct functioning of many cellular enzymes. However, iron, copper, zinc, manganese, selenium, aluminium and lithium have all been linked to neurological disorders (Garby et al 1985). For example copper accumulation results in Wilson's disease (Patten 1993), and chronic manganese intoxication can induce PD syndrome (Mena et al 1967, Poltis et al 1980, Tanaka and Lieben 1969, Yamada et al 1986). However, iron is the transition metal most commonly linked with the aetiology of PD. Iron is not present in the brain at birth, but accumulates with age (Hallgren and Sourander 1958, Olanow et al 1989). Iron distribution in the brain is not uniform (Spatz 1922, Morris et al 1992, Griffiths and Crossman 1993). As the age of the brain increases iron accumulates in the substantia nigra, especially the melanin-containing cells of the substantia nigra pars compacta of PD patients (Hirsch et al 1991, Good et al 1992). This accumulation of iron is far above any biochemical requirement and the site specificity may indicate a relationship to PD (Dexter et al 1991).

The chemistry of iron enables a redox cycling between the ferrous and ferric forms ($Fe^{2+}$ and $Fe^{3+}$). Furthermore, free ferrous ($Fe^{2+}$) iron can interact with oxygen to induce free radical damage to cells. $Fe^{2+}$ can also promote the breakdown of the

intermediates produced from the enzymic degradation of reactive oxygen species. These interactions are called Fenton reactions and lead to the production of highly reactive oxygen radicals such as the hydroxyl radical, which can cause irreparable damage to cells.

Generation of Reactive Oxygen Species (ROS): Interactions of $Fe^{2+}$.

The reduction of $O_2$ to $H_2O$ involves the addition of four electrons during oxidative phosphorylation in the mitochondria. During this process oxygen radicals and reactive oxygen species (ROS) are formed.

1. $O_2 + e^- + H^+ \rightarrow HO_2^{\cdot}$ (hydroperoxyl radical)
2. $HO_2^{\cdot} \leftrightarrow H^+ + O^-_2{}^{\cdot}$ (superoxide radical)
3. $HO_2^{\cdot} + e^- + H^+ \rightarrow H_2O_2$ (hydrogen peroxide)
4. $H_2O_2 + e^- \rightarrow OH^- + OH^{\cdot}$ (hydroxyl radical)
5. $OH^{\cdot} + e^- + H^+ \rightarrow H_2O$

$Fe^{2+}$ can promote the production of ROS. In these reactions $Fe^{2+}$ acts as a catalyst.

1. $H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+}$ (Fenton Reaction)
2. Lipid peroxide 2(LOOH) + $Fe^{2+} \rightarrow LO^{\cdot} + LO^{\cdot}_2 + H_2O$

This process is normally under tight control to prevent interaction of radicals and cellular material that would lead to cell damage. Therefore, a number of enzymes exist to catalyse the safe disposal of radicals and ROS.

1. $2H_2O_2$ + Catalase $\rightarrow 2H_2O + O_2$
2. $2O^-_2{}^{\cdot} + 2H^+$ + superoxide dismutase$\rightarrow H_2O_2 + O_2$

3. $H_2O_2$ + 2GSH + glutathione peroxidase $\rightarrow$ GSSH + $2H_2O$

Antioxidants, such as vitamins C and E, and glutathione (GSH) can inhibit the chain reactions initiated by ROS by donating electrons to radicals (Halliwell and Gutteridge 1990).

The main enzymes that catabolise the safe removal of reactive oxygen species in the brain are superoxide dismutase and glutathione peroxidase (Weisinger and Fridovich 1973, Fridovich 1978, Marklund 1982, Flohe 1982, Donaldson and Barbeau 1985, Damier et al 1993). There is increasing evidence that the levels of glutathione and glutathione peroxidase are decreased in PD (Riederer et al 1989, Sofic et al 1992). In addition, evidence for an increased state of oxidative stress exists in PD is found with the increased lipid peroxidation products found in post-mortem brains (Dexter et al 1989, Sanchez-Ramos et al 1994). Furthermore, raised oxidative stress in cells has been linked to perturbation of $Ca^{2+}$ homeostasis (Jones et al 1983, Thor et al 1985, Kass et al 1989, Moore et al 1986, Crompton et al 1987, Beatrice et al 1980, De Erausquin 1994), and in some cases increased susceptibility to excitotoxicity (Siesjo and Bengtsson 1989, Blaustein 1988, Choi 1988, Beal 1992, Dugan and Choi 1994). Additionally, raised oxidative stress may be present due to increased $H_2O_2$ produced during the rapid turnover of DA by MAO by the remaining neurones of the substantia nigra (Spina and Cohn 1989, Schultz 1982, Bernheimer et al 1961). However, it is not clear whether these findings are initiated, or potentiated by elevated iron concentrations in the SN of PD brains. Thus either iron alone or in combination with another factor(s) may play a part in the aetiology of PD.

Exogenous and Endogenous Toxins.

The potential of many candidate toxins to induce PD has been examined. For instance epidemiological evidence inferred that a factor found in more rural environments may explain the relative increase in the rate of PD found in these populations (Barbeau et al 1986, 1987). The investigators suggested that the pesticide paraquat may be responsible, primarily since its structure was similar to that of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was originally determined as a parkinsonian toxin, when drug addicts in California presented with symptoms of PD after intoxication of a synthetic meperidine analogue, which contained trace elements of the impurity, MPTP (Langston et al 1983, Langston et al 1984). Subsequent investigation of the mode of MPTP action, has revealed that MPTP is able to cross the blood brain barrier, where it is metabolised by MAO (especially MAO B) (Chiba et al 1985, Salach et al 1984). However, the cells most affected by MPTP contain virtually no MAO B (Demarest et al 1980). This enzyme appears to be concentrated in glia and serotoninergic cells (Westlund et al 1985, Levitt et al 1982). Despite the high expression of MAO B neither cell type appears very susceptible to $MPP^+$. The action of MAO upon MPTP produces 1-methyl-4-phenyl pyridinium ($MPP^+$), which is actively taken up by dopaminergic neurones via the specific dopamine high affinity re-uptake transporter (Jarvitch et al 1985). Once inside the cell $MPP^+$ is known to actively accumulate in mitochondria down its electrochemical gradient (Ramsay and Singer 1986, Ramsay et al 1986, Davey et al 1992). Here, $MPP^+$ is known to inhibit complex I of the mitochondrial electron transport chain (Nicklas et al 1985). Consequently, cell death occurs due to perturbation of the energy balance. There is also evidence that $MPP^+$ may redox cycle with MPTP to cause oxidative damage to

cells (Chacon et al 1987, Korytowski et al 1987, 1988). However, the relevance of this process in the aetiology of PD is disputed (Walker et al 1991). In addition $MPP^+$ is reported to elicit a rapid and substantial efflux of DA from dopaminergic cells (Rollema et al 1988, Chiueh et al 1993). Subsequent extracellular non-enzymic oxidation of DA may play a role in cellular death through the production of oxygen radicals (Obata and Chiueh 1992, Chiueh et al 1993). $MPP^+$ stimulated dopamine release appears to be dependent upon $Ca^{2+}$ influx through L-type channel opening, as it can be blocked by nimodipine (Huang and Chuieh 1990, Lang et al 1990, Chiueh and Huang 1991). $MPP^+$ stimulated $Ca^{2+}$ influx may also activate the second messenger systems required to open L-type channels. Since excessive $Ca^{2+}$ entry can induce cell death, $MPP^+$ toxicity may also be related to increased intracellular $Ca^{2+}$ concentrations similar to that seen with excitatory amino acid neurotoxicity (Siesjo 1988, Garthwaite and Garthwaite 1988, Fransden and Schousboe 1991, 1992).

Accumulation of $MPP^+$ also occurs in noradrenergic neurones (Jarvitch et al 1985), but to a lesser degree, due to differences in the binding affinities of the individual re-uptake transporters for $MPP^+$. However, both dopaminergic and noradrenergic cells susceptible to the toxic effects of MPTP (Burns et al 1983, Davis et al 1979, Hallman et al 1984, Schneider et al 1987, Williams and Schnieder 1989). In addition, the pigmented cells of the substantia nigra may be more susceptible to $MPP^+$, since they contain neuromelanin, which binds and sequesters $MPP^+$ (D'Amato et al 1986).

As a consequence of the discovery of MPTP and its toxic effects in man, investigation of many related compounds has followed. The majority of these compounds are synthetic analogues of $MPP^+$ which do not naturally occur in the human brain. This

thesis will concentrate upon compounds that either may exist endogenously, or whose presence has been demonstrated in vivo.

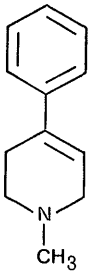
Tetrahyroisoquinolines.

1,2,3,4-Tetrahydroisoquinoline is a natural trace element in the human brain, and is reported to be increased in PD patients (Niwa et al 1987). When administered to monkeys it produced a parkinsonian syndrome (Niwa et al 1988), which disappeared upon cessation of treatment (Nagatsu and Yoshida 1988). Further examination has shown that N-methylation of TIQ can occur to produce N-methyl TIQ (NMTIQ) (Naoi 1989b). This compound is a substrate for MAO and is converted to the charged tertiary nitrogen compound $NMTIQ^+$ (Naoi 1989a). Moreover, $NMTIQ^+$ is structurally similar to $MPP^+$ and its toxicity is greater than either TIQ or NMTIQ (Naoi et al 1989b, Naoi et al 1991, Naoi et al 1993a,b). In addition these compounds can be synthesised naturally in the brain via Pictet-Spengler condensation (Rommelspacher and Susilo 1985). Pictet-Spengler reactions condense catecholamines (DA and NA) and indoleamines (5HT) with aldehydes, such as acetaldehyde or pyruvate (Robbins 1968, McIsaak 1961). By this process another TIQ alkaloid, 6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol) can be produced from dopamine and acetaldehyde (Robbins 1968). Salsolinol can also be synthesised by the condensation of dopamine and pyruvate, followed by decarboxylation (Dostert 1988). Since the substrate for salsolinol synthesis is dopamine one may expect that the condensation reactions would occur primarily in dopaminergic cells. Furthermore, salsolinol is a trace element in the mammalian central nervous system (Sjoquist and Magnuson 1980, Collins 1982, and Sjoquist et al 1981), and is a natural plant alkaloid

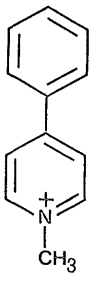
found in many foods and beverages (Riggin et al 1976, Duncan and Smythe 1982, and Duncan et al 1984). It has been shown that salsolinol can be N-methylated (Maruyama et al 1992), and is a substrate for MAO (Naoi et al 1993). Moreover, salsolinol is toxic to neurones in vitro, and is known to accumulate in mitochondria (Maruyama et al 1993b), where it may inhibit mitochondrial complex I respiration (Suzuki et al 1990, Melzig and Zipper 1993). There are reports that other TIQ derivatives may be cytotoxic (Maruyama et al 1993b), and TIQ is a mitochondrial respiratory inhibitor (Suzuki et al 1988, Suzuki et al 1989). Salsolinol has been implicated in the addictive properties of alcohol (Cohen and Collins 1970), and linked to the premature ageing of the brain from chronic alcoholics (Ryan and Butters 1980). Another TIQ related compound, the adrenaline derivative, 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ), depletes catecholamine, and indolamine concentrations from various areas of the rat brain after intracerebroventricular infusion (Liptrot et al 1993). Therefore, TIQ derivatives may have a role in the aetiology of PD and other neurodegenerative disorders.

Figure 1.11. Structures of MPTP, $MPP^+$ and the potential neurotoxins NMN, Salsolinol and TMIQ.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

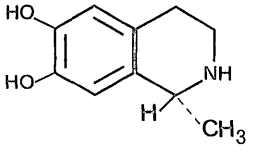



1-Methyl-4-phenylpyridinium ($MPP^+$)

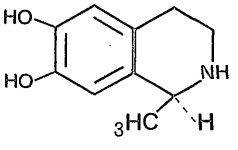



6,7-Dihydroxy-2-methyl-1,2,3,4-tetrahydroisoquinoline

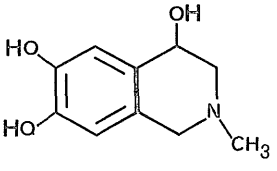
(R)-Salsolinol




(S)-Salsolinol




1,2,3,4-Tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ)



β-Carbolines.

This family of compounds are found in many plants and fungi (Allen et al 1980). Furthermore, β-carbolines can be found in foods, beverages, soy hydrolysates, and wood smoke (Rommelspacher and Susilo 1985). In addition, β-carbolines can be formed in vivo through the non-enzymic Pictet-Spengler condensation of trytophan or tryptamine with aldehydes or α-keto acids. Moreover, N-methylation of tryptamine, followed by P450 mediated oxidation and cyclation of the product, could produce tetrahydro-β-carboline (Rommelspacher and Susilo 1985). One β-carboline, 2-N-methyl β-carboline is neurotoxic, but at greater concentrations than $MPP^+$ (Neafsey et al 1989), whilst others are known to be cytotoxic to PC12 cells (Cobuzzi et al 1994). However, 2,9 [indole]-di-N-methylated β-carbolines are equipotent inhibitors of mitochondrial respiration, when compared to $MPP^+$.

Deficiencies in Xenobiotic Metabolism.

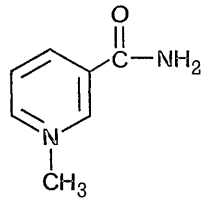
The study of xenobiotic metabolism has revealed defects in certain detoxification systems in PD patients. Steventon et al (1989) found that PD patients had altered sulphur metabolism. On treatment with S-carboxymethyl-cysteine the normal response is to produce S-oxide metabolites. Yet, 35 % of PD patients produced no S-oxides. In the latter case defects in cysteine metabolism are thought to be present. The enzyme responsible is cysteine dioxygenase, which has a role in xenobiotic metabolism. In the liver, cysteine dioxygenase catalyses the formation of organic sulphate, which is required for conjugation of xenobiotics in phase II detoxification reactions. To emphasise the inability of some PD patients to produce sulphate, treatment of the same patients with paracetamol resulted in reduced capacity to from paracetamol sulphate

before excretion. These data indicate that PD patients have a low sulphate pool, resulting in decreased ability to detoxify xenobiotics. In addition, it is possible that the brain cysteine dioxygenase sulphoxidation process is similar to that of the liver. If this is the case then poor sulphate-linked metabolism of xenobiotics may occur in the brain, resulting in neuronal cell toxicity. A more recent study examined the ability of Parkinsonian patients to metabolise D-penicillamine, a process that usually yields 50-70 % of inorganic sulphate in the urine, via the action of cysteine dioxygenase (Peters et al 1994). The results showed increased S-methylation of D-penicillamine, an alternative detoxification pathway, indicating a difficicency in cysteine dioxygenase activity.

Another study examined nicotinamide metabolism in PD patients (Green et al 1991). Nicotinamide was orally administered and the metabolites measured in the urine of patients. In PD patients elevated concentrations of one metabolite, N'-methyl nicotinamide (NMN), were found. Another metabolite, N'-methyl-2-pyridone-5-carboxyamide was also increased. Metabolic formation of NMN is thought to be carried out by N-methyl transferases and its accumulation may occur due to an enzymic insufficiency (Williams et al 1993). The enzyme dysfunction appears to involve the conversion of NMN to N'-methyl-2-pyridone metabolites possibly mediated by either an isoenzyme of cytochrome P450, or perhaps xanthine oxidase or aldehyde dehydrogenase. Initial examination of P450 activity proved inconclusive (Steventon et al 1989, Ferrari et al 1990), however examination of debrisoquine hydroxylase gene polymorphism found that those with the poor metaboliser phenotype were at greater risk to Parkinson's disease (Smith et al 1992). The raised levels of NMN may indicate an inability to detoxify N-methylated compounds such as NMTIQ

and NM-salsolinol. Additionally, NMN has structural similarity to MPTP, and thus may be neurotoxic.

Figure 1.12. Structure of N-methyl-nicotinamide.



## 1.8. Summary.

Notwithstanding the clinical, pathological and morphological differences, the three neurodegenerative disorders described herein have several features in common. For example dementia can be present in PD, AD and HD (Arendt et al 1983, Caine et al 1978, Kay et al 1956, Terry and Katzman 1983). Cholinergic (Arendt et al 1983, Whitehouse et al 1983, Hohmann et al 1988, Coyle et al 1983), dopaminergic and noradrenergic neurones can be affected in AD, HD and PD (Tabaton et al 1985, Mayeux and Stern 1983, Reisine et al 1977, Seeman 1987, Joyce et al 1988). Additionally, the symptoms of AD, PD and HD manifest due to decreased neurotransmitter concentrations within the brain. The process of neuronal degeneration in each disease is unclear. However, HD has a proven genetic link, but this is not the case for the majority of PD and AD cases. Where there is a link, the genetic factor may be of primary importance, or alternatively may predispose the patient to be more susceptible to environmental or endogenous compounds. There is evidence that inorganic metal ions may play a role in the pathogenesis of both AD and PD. However, most of this evidence relies upon the accumulation of these metals in the areas affected. Despite a positive correlation, to be active in the production of reactive oxygen species, these ions would have to be in a free state. In AD aluminium is found bound in PHF's and neurofibrillary tangles (Crapper et al 1973), and plaques (Edwarson et al 1986, Candy 1986), and in PD most iron is bound to neuromelanin and other intracellular proteins, and is therefore may be unavailable for free interaction with pro-oxidants (Good et al 1992).

However, there is one finding that encompasses AD, PD and HD. In all cases evidence suggests that glucose metabolism and mitochondrial insufficiency may play a role in aetiology prior to onset of clinical symptoms. This may occur due to genetic defects in mitochondria DNA (Schoffner et al 1991, Mann et al 1992, Schapira 1990, Singer et al 1987, Ginsberg 1980, Benecke et al 1993, DiDonato et al 1993, Reichmann et al 1994, Blin et al 1994), or through the action of metabolic poisons (Rosenberg et al 1989, Uitti et al 1985). Synthetic neurotoxins such as MPTP have been demonstrated to cause mitochondrial damage in PD (Nicklas et al 1985, Nicklas et al 1987, Ramsay and Singer 1986, Ramsay et al 1989), and alteration in catecholamine homeostasis (Rollema et al 1988, Chiueh et al 1993). Thus if synthetic toxins can produce syndromes similar to that found in neurodegenerative diseases, there is a strong case for the existence of environmental or endogenous potential neurotoxins. There exists a growing body of evidence that potential endogenous compounds related to indoleamines and catecholamines may be able to induce neuronal damage in vitro and in vivo. For example TIQ is known to induce a PD syndrome in monkeys (Nagatsu 1988), and its derivatives are cytotoxic to neurones in culture (Naoi et al 1989b, Naoi et al 1991, Naoi et al 1993, Maruyama et al 1993). TIQ derivatives are also linked to the neurodegeneration seen in chronic alcoholism (Ryan and Butters 1980). The adrenaline derivative TMIQ depletes catecholamine, indoleamine and other neurotransmitters from the rat brain after intracerebral infusion (Liptrot et al 1993). In addition certain cyclation products of 5HT are cytotoxic to neurones in vitro (Neafsey et al 1989). Thus the mechanism of the potential toxicity produced by these related compounds and their possible involvement in the process of neurodegeneration warrants further investigation.

## 1.9. Aims of the Investigations.

The principal aim of this investigation will be to examine the neurotoxicity of the naturally occurring potential endogenous compounds, NMN, TMIQ and salsolinol, and thus to build toxicological profiles for each compound.

Plan of Investigation.

1). Three cell neuroblastoma cell lines will be examined to determine their suitability for further use. The ability to take up, store and release the catecholamines, DA and NA will be assessed. Additionally, the potential to block uptake with $uptake_1$ inhibitors will be examined. Furthermore, the profile of released catecholamines will be determined.

2). The potential toxicity of three naturally occurring compounds will be investigated using data from point one above. N-methyl nicotinamide is elevated in the urine of PD patients after oral ingestion of nicotinamide (Green et al 1991). This finding may highlight potential problems arising from the normal detoxification of endogenous compounds, or it may indicate a build up of a potential toxin, NMN. TMIQ, an adrenaline derived compound can be formed by Pictet-Spengler condensation (Bates 1981), depleted catecholamine and indoleamine concentrations in the rat brain after intracerebroventricular infusion (Liptrot et al 1994a). Thus as neurotransmitter loss can be related to neuronal cell death, TMIQ may also be toxic to neurones. The third compound investigated, salsolinol, is cytotoxic to PC12 cells, and is associated with chronic alcoholism (Ryan and Butters 1980, Myers and Melchior 1977a, Myers and Melchior 1977b). However, PC12 cells rely mainly on glycolysis instead of the mitochondrial metabolism for their energy requirements (Warberg 1967, Racher and

Spector 1981, Morelli et al 1986) and as such may not a good cell model with which to study potential mitochondrial toxins.

3). Assessment of Toxicity.

To determine the effects of these potential endotoxins on cell viability, three separate methods were chosen to give an assessment of the integrity of different cellular functions. For example [$^3$H]thymidine incorporation gives an assessment of cellular growth and division. Whereas, lactate dehydrogenase release will give an assessment of membrane integrity, and MTT reduction, an assessment of mitochondrial function in relation to cell numbers.

4). Since the PD neurotoxin $MPP^+$ is known to interfere with catecholamine uptake and release, and as both TMIQ and salsolinol are reported to interact with catecholaminergic systems in vivo, the effect of the potential neurotoxins upon catecholamine homeostasis will be examined. This will be conducted by the use of tritiated labelled DA and NA as described later (material and methods, section 2.3).

# CHAPTER 2

# GENERAL MATERIAL AND METHODS

(Specific methodology can be found in the appropriate chapters)

## 2.1. Cell Culture.

All tissue culture media and supplements were from Gibco BRL (Paisley, U.K.). Culturing flasks and multitrays (Nunc) were obtained from Life Technologies (Uxbridge, U.K.). The rat B65 neuroblastoma cell line, (Schubert et al 1974), passage >80, was obtained from P.H.L. Centre for Applied Microbiology and Research (Porton Down, Salisbury, U.K.). Human SH-SY5Y neuroblastoma cells (Ross and Biedler 1985), passage 69, were a kind gift from J.L. Biedler, Sloan-Kettering Institue for Cancer Research (Rye, New York, U.S.A.). Human IMR32 neuroblastoma cells (Tumilowicz et al 1970), passage 69, were obtained from E.A.C.C. (Porton Down, Salisbury, U.K.).

### *2.1.i. Methods.*

Stock cultures of rat B65 (passage >80), and human SH-SY5Y cells (passages 69 to 100), were maintained in 80cm$^2$ culture flasks using RPMI 1640, and minimal essential media (MEM) respectively, supplemented with foetal calf serum (10 % v/v), L-glutamine (2mM), penicillin (100 IU / mL), streptomycin (100μg / mL), and incubated at 37°C, in 5 % $CO_2$ / humidified air. Undifferentiated cells were used except where stated. When confluent, stock cultures were harvested by trypsinization (0.05 % EDTA) and split at 1:20 for B65 and 1:10 for SH-SY5Y cells respectively. Human IMR32 cells (passages 69 to 80), were maintained in similar conditions to that described above. However, they were grown in α-MEM (containing ribonucleosides), supplemented as above, with the addition of 1 % non-essential amino acids. Confluent IMR32 cells were harvested by trypsinization (0.05 % EDTA) and split at 1:10. In all

cultures the media was changed every two days, except during 72 hour incubation periods when the effects of toxins were examined.

For experimental purposes confluent stock cultures were harvested by trypsinization (0.05 % EDTA), seeded at 1 x $10^5$ cells / well, in either 12 or 24 well multitrays, and cultured in either supplemented RPMI 1640, MEM or α-MEM. However, for uptake of catecholamines in whole cell suspensions and for binding assays confluent cells were harvested by the addition of HEPES (10mM, Sigma, Poole, Dorset, U.K.) / EDTA (0.02 %, Sigma, Poole, Dorset, U.K.), NaCl (0.9 % w/v) buffer, pH 7.4, prior to use. Following harvesting cells were resuspended in the appropriate experimental buffer after centrifugation.

**2.2. Toxicity Experiments.**

Toxicity of test agents can be measured by numerous methods. These methods usually adapt a universal property of the cell. The most common is the assessment of cell membrane integrity. Loss of membrane integrity can be determined by several techniques, usually involving the leakage of intracellular substances into the assay medium, or the loss of active transport (exclusion, e.g. trypan blue) of other compounds by dead cells. Leakage of cellular contents can be determined by several methods. In practise analysis of the activity of a cytosolic enzyme (e.g. lactate dehydrogenase) is the commonest method. Lactate dehydrogenase (LDH) is an ubiquitous intracellular protein contained in every cell, which catalyses the reversible conversion of L-pyruvate to L-lactate, and requires the cofactor nicotinamide adenine

dinucleotide ($NAD^+$). The presence of this enzyme in culture medium can be linked to leakage from damaged cell membranes. Therefore, measurement of the activity of this enzyme can allow an assessment of cell membrane and hence cell integrity (Bergmeyer and Bernt 1974). However, large holes must be present in the cell membrane before detectable levels of enzyme activity (above basal) are found. This assay therefore indicates a late stage of cellular toxicity when cells are usually dead. However, this assay is inexpensive and easy to perform.

Additionally, loss of membrane integrity can result in an inability to exclude from (e.g. trypan blue, Patterson 1979) or take up various dyes into (e.g. neutral red) damaged cells. Assessment of cell death by trypan blue exclusion is made by counting both viable and non-viable (blue) cells and is expressed as a percentage. However, cell counting is laborious and error prone, due either to the accuracy of the counter, and additionally with a haemocytometer, one counted cell represents 20,000 cells per mL. Furthermore, viable cells start to take up dye after a short time as trypan blue is toxic. The assessment of cell membrane integrity requires a large amount of damage to be present before significant detection of damage is observed. Therefore, more sensitive assessments of cell integrity have been developed. These include the assessment of mitochondrial function. Specific compounds such as 3[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue (MTT) or 2,3 bis [2-methoxy-4-nitro-5-solfophenyl]-2H-tetrazolium-5-caroxanilelide inner salt (XTT) are absorbed by cells and metabolised by the enzyme succinate dehydrogenase in the mitochondria of living cells. MTT is converted to an insoluble, coloured formazan dye. The ability of cells to reduce MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell viability or number. Determination of

their ability to reduce MTT to the formazan dye after exposure to test compounds, compared to the untreated control, enables the relative toxicity of test compounds to be assessed (Denizot and Lang 1986). Therefore, mitochondrial activity (i.e. viable cell number) is proportional to the intensity of coloured dye measured spectrophotometrically. Thus data may be expressed as the percentage of cell survival, when compared to the appropriate control. This assay is suited to cells that clump as well as those that fail to form colonies. However, it is not suitable to static cultures or cells with low mitochondrial activity. Despite the sensitivity of this assay, there are problems if a potential toxin interferes with mitochondrial respiration. For example, if a potential toxin either inhibited the activity or was a substrate of succinate dehydrogenase the relative amount of MTT converted to formazan dye would be reduced thus over estimating toxicity.

Another property that can be exploited in the assessment of toxicity is the rate of growth of a population of dividing cells when exposed to potential toxins. This is achieved by the addition of potential toxins to exponentially growing cells for a relevant time period followed by the addition of [$^3$H]thymidine. After pulsing with [$^3$H]thymidine the rate of growth (label incorporation) can be expressed when compared to untreated cells. Reduction of cell growth indicates toxicity of a compound. However, this assay is not suited to slow growing or static populations of cells.

There are other assays which can be utilised to determine the potential toxicity of a compound. For example measurement of the activity of the membrane bound enzyme acid phosphatase can give an indication of the total biomass (Purchase 1990). The enzyme converts p-nitrophenyl phosphate to a coloured product which is measured

spectrophotometrically. The assay can be run for up to six hours and can be adjusted to suit a broad range of cells. However, acid phosphatase activity is also found in dead cells and membrane fragments. Therefore, it would be impossible to determine viable from non-viable cells with this method. Another method for measuring cell death utilises the ability of ethidium bromide to bind to non-membrane bound DNA. After exposure to potential toxins ethidium bromide is added to cells and the fluorescence measured at 610nm. Finally, the cell membranes are solubilized with the addition of detergent and the fluorescence measured again, to give an assessment of cell death. However, despite the apparent ease of this assay ethidium bromide is a highly toxic compound, and the assay requires a fluorescence plate reader. Therefore, in this thesis a combination of the MTT, lactate dehydrogenase and [$^3$H]thymidine incorporation protocols have been used to assess the toxicity of several potential neurotoxins. These methods were chosen because they gave a range of both sensitivity and a number of different sites within the cells to assess the action of potential toxins. They are also easy to undertake, inexpensive and are highly reproducible.

**Methodology.**

*2.2.i. Lactate Dehydrogenase Release.*

Neuroblastoma cells were seeded at 1 x $10^5$ cells / well and grown to confluence as described previously (for 7 days). The cells were exposed to varying concentrations of the appropriate potential toxic agent. After 24, 48 or 72 hours LDH activity was determined in the media and from the cell monolayers via the following method. The media from each well was removed and the cell monolayers lysed by the addition of

phosphate buffered saline (10mM PBS, 2.7mM KCl and 13.7mM NaCl, pH 7.4) containing 10 % (v/v) triton x 100 . 100μL aliquots of each sample (media or cell lysate) were added to 843μL of PBS (pH 7.4) and 33μL of NADH (3.5mM). The reaction was initiated with the addition of 33μL of L-pyruvate (20mM). The activity of LDH is measured spectrophotometrically by the rate of decrease in absorbance via the reduction of NADH to NAD at 340nm, using a Corning 259 spectrophotometer. A specimen trace is shown in figure 2.1. The resulting LDH activity was determined using the Beer-Lambert law:

$A = \Sigma cl$

Where: A= absorbance; $\Sigma$ = coefficient of absorption (6.3 for NADH); c = concentration; l= path length (= 1cm).

One unit of lactate dehydrogenase is defined as that amount which will catalyse the conversion of 1μmole of NADH per minute, and 1μmole of NADH / mL gives an absorbance of 6.3 at 340nm.

Therefore, the total NADH converted to NAD per minute is equal to:

($\Delta$ / 6.3) moles of NADH / mL,

and the activity of LDH per mL is equal to:

$$\frac{\Delta \text{ absorbance / min}}{6.3} \times \text{dilution factor of sample}$$

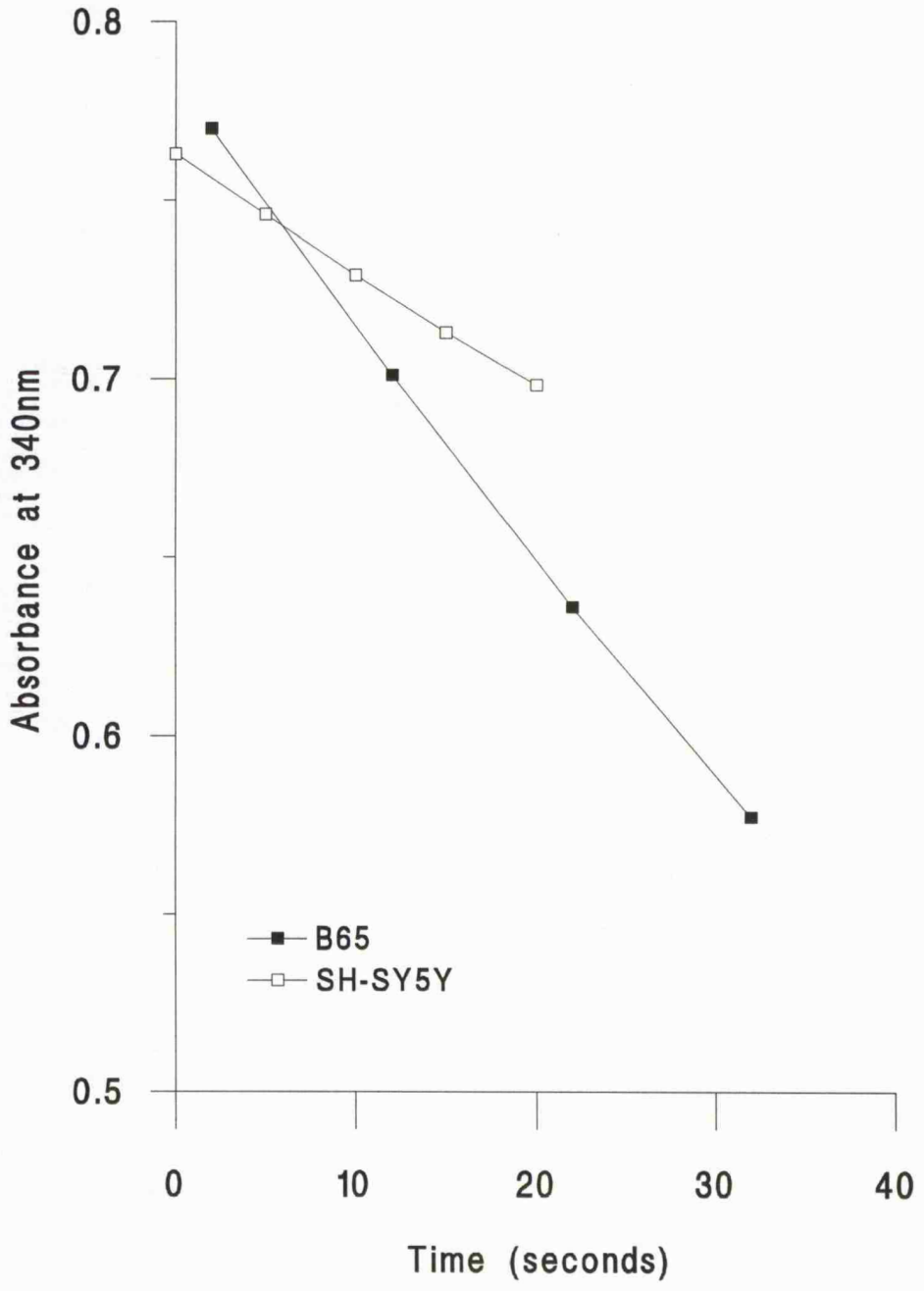
LDH activity was then expressed as a percentage of the total activity present for each sample, i.e.:

$$\text{LDH Activity (\%)} = \frac{\text{LDH(m)}}{\text{LDH(l)} + \text{LDH(m)}} \times 100$$

Where LDH(m) = the activity of LDH from the medium of each sample; and LDH(l) = the activity of LDH from the cell lysate of each corresponding sample.

All the reagents used in this assay were from Sigma (Poole, Dorset, U.K.).

Figure 2.1. Specimen trace showing the rate of NADH reduction in B65 and SH-SY5Y cell lysates.




LDH activity was determined by the decrease in absorbance at 340nm over time.

*2.2.ii. MTT Reduction: The assessment of mitochondrial activity.*

Undifferentiated neuroblastoma cells were seeded at 1 x $10^5$ cells / well in 24 well multitrays and incubated in supplemented media as described previously for 3 days. After 3 days, the cells were exposed to varying concentrations of individual potential neurotoxins. MTT (120μM, in Hanks Balanced Salts), was added to cells 24, 48 and 72 hours after addition of a potential toxin, and incubated for one hour at 37°C. Both MTT and Hanks Balanced salts, of the following composition (mM): KCl (5.4), $H_2PO_4$ (0.44), NaCl (13.7), $Na_2HPO_4$ (0.34), D-glucose (5.6), $CaCl_2$ (1.7), $MgSO_4$ (0.2), $NaHCO_3$ (4.2), were obtained from Sigma Poole, Dorset, U.K. The resulting dye was extracted with acidified isopropanol (Fisons, Loughborough, Leicestershire, U.K.) and the absorbance measured at 550nm with a 620nm reference, on an Anthos 2001 plate reader. The wavelength 550nm was chosen as it gave the highest absorbance for the formazan dye product of reduced MTT. A reference measurement was taken at 620nm, where the formazan dye does not absorb to a high degree, to eliminate any interference from the plate, cellular matter or any compound that may overlap to increase the absorbance at 550nm. In each individual experiment potential interaction between MTT and the appropriate toxins, which may produce interfering coloured dyes, was determined by the addition of the highest concentration of toxin alone, or the appropriate combination of compounds utilised in each assay, in 1mL of media without cells, as a blank. The blank was then subtracted from all appropriate samples, and expressed as a percentage of the control (untreated) absorbance value.

*2.2.iii. [$^3$H]Thymidine Incorporation.*

Undifferentiated neuroblastoma cells were seeded at 1 x $10^5$ / well in 12 well multitrays and grown for 3 days, for SH-SY5Y and IMR32 cells, and 1 day for B65 cells, in the appropriate supplemented media as described previously, to reach exponential growth. They were then exposed to varying concentrations of the appropriate potential toxin for 24, 48 and 72 hours. After exposure to the potential toxins the monolayers were washed with 0.5mL of supplemented media, without serum for 5 minutes. Next 0.5mL of [methyl, 1',2'-$^3$H]thymidine (1μCi / mL, 5nM, Amersham International, Buckinghamshire, U.K., in supplemented media minus serum) was added to each well, and incubated for 4 hours at 37°C. After further washing with 0.5mL of supplemented media, minus serum (5 minutes), the incorporated [$^3$H]thymidine was extracted by the addition of perchloric acid (PCA, 0.4M, Fisons, Loughborough, Leicestershire, U.K.) for 30 minutes. The resultant activity was quantified by liquid scintillation spectroscopy, and the amount of thymidine incorporation expressed as a percentage of basal (untreated control).

## 2.3. Catecholamine Uptake and Release.

### *2.3.i. Catecholamine Uptake.*

Catecholamine uptake was undertaken, in all cell lines, using the method of Atcheson et al (1993), as described below. Confluent monolayers of undifferentiated SH-SY5Y, IMR32 or B65 cells, in 12 well multitrays were washed with Krebs / HEPES buffer, pH 7.4, of the following composition (mM): $Na^+$ (143.3), $K^+$ (5.9), $Ca^{2+}$ (2.5), $Mg^{2+}$ (1.2), $Cl^-$ (153.2), $H_2PO_4^-$ (1.2), $SO_4^{2-}$ (1.2), glucose (11.7) and HEPES (10), supplemented with pargyline (0.2mM, Sigma, Poole, Dorset, U.K.) and ascorbic acid (0.2mM, Sigma, Poole, Dorset, U.K.), for 5 minutes. Pargyline was included to prevent catecholamine metabolism by monoamine oxidase, and ascorbic acid to prevent the non-enzymic oxidation of labelled catecholamines. The monolayers were then loaded with 0.5mL of either [7,8-$^3$H]dopamine (DA, 44nM) or [7,8-$^3$H]noradrenaline (NA, 48nM), obtained from Amersham International (Buckinghamshire, U.K.), for up to one hour, at 37°C. Compounds under analysis were included in the loading buffer, with the exception of basals. After one hour the monolayers were washed again and the catecholamines taken up by the cells were extracted with PCA, (0.4M) for 30 minutes. Samples of the extracts were quantified for labelled catecholamines by liquid scintillation spectroscopy (Packard 1900 TR). Catecholamine uptake was expressed as a percentage of the total available activity.

Catecholamine uptake for some experiments using IMR32 cells was also undertaken with whole cell suspensions. The method was similar to that used in monolayers, however confluent stock cultures were harvested by addition of HEPES (10mM) /

ethylenediamine-tetraacetic acid (EDTA, 0.02 %), NaCl (0.9 %) buffer, pH 7.4. Following centrifugation, cells were re-suspended in 4mL of Krebs / HEPES buffer, pH 7.4 (as for uptake in monolayers). Whole cell suspensions (260μL) were incubated in the presence of 20μL of either [$^3$H]NA (600nM) or [$^3$H]DA (710nM), and 20μL of Krebs / HEPES buffer, for varying times. Impiramine (10μM) or benztropine (10μM) were included in place of 20μL of Krebs / HEPES buffer, to block uptake of [$^3$H]NA or [$^3$H]DA, respectively (Rang and Dale 1991). After incubation, uptake was terminated by the addition of ice-cold Krebs / HEPES buffer and the cells harvested onto GF/B filters (Whatman, Maidstone, U.K.) under vacuum filtration. The filters were added to 4mL of Optiphase Safe (LKB, Scintillation Products, Loughborough, U.K.) for extraction overnight. The extracted catecholamines were quantified by liquid scintillation spectroscopy. Uptake was expressed as a percentage of the total available activity (20μL of either [$^3$H]DA, 48nM, or [$^3$H]NA, 44nM were counted as the total available activity).

*2.3.ii. [$^3$H]Nisoxetine Binding.*

[N-methyl-$^3$H]Nisoxetine (Amersham International, Buckinghamshire, U.K.) is a ligand which binds specifically to the catecholamine re-uptake transporter (uptake$_1$) and has a higher affinity than many other ligands such as imipramine (Sigma, Poole, Dorset, U.K.), which binds to uptake$_1$ sites and blocks catecholamine uptake (Tejani-Butt 1991). Concurrent incubation of increasing concentrations of test compound and a constant concentration of [$^3$H] nisoxetine allows competition between both compounds for the catecholamine uptake transporter. The concentration of [$^3$H]nisoxetine is set around the $K_d$ (i.e. 50 % saturation of the available binding sites,

0.8nM). Displacement of nisoxetine binding indicates interaction of the test compound with the uptake transporter.

Displacement Studies.

Whole cell suspensions (100μL) were incubated with a fixed concentration of [$^3$H]nisoxetine (200μL) set at the $K_d$ of ~0.8nM (Tejani-Butt 1991). In an attempt to displace [$^3$H]nisoxetine binding the concentration of the potential toxins TMIQ and salsolinol (200μL) were included in increasing concentration ($10^{-10}$ to $10^{-3}$M, table 2.1). Non-specific [$^3$H]nisoxetine binding was measured by the addition of excess imipramine (100μM). After incubation for one hour at 37°C, the bound and free radioligand were separated by rapid vacuum filtration with a Brandel harvester. The filters were removed and added to 4mL of Optiphase Safe scintillant overnight. The concentration of [$^3$H]nisoxetine added to the whole cell suspensions were determined by the addition of 200μL of each to 4mL of scintillant. The activity of both standards and samples were quantified by liquid scintillation spectroscopy. Displacement of [$^3$H]nisoxetine binding was expressed as a percentage of the total [$^3$H]nisoxetine binding (i.e. no TMIQ or salsolinol), after subtraction of non-specific binding.

Table 2.1. Nisoxetine binding displacement studies with SH-SY5Y cells; table of additions.

| Tube Sample | Buffer (μL) | Imipiramine (μL) | Nisoxetine (μL) | Toxin (μL) | Cells (μL) |
|---|---|---|---|---|---|
| Total | 700 | | 200 | | 100 |
| NSB | 500 | 200 | 200 | | 100 |
| SAL/TMIQ | 500 | | 200 | 200 | 100 |
| SAL/$TMIQ_x$ | 500 | | 200 | 200 | 100 |

The above table illustrates the additions of each compound used during [$^3$H]nisoxetine displacement binding studies with SH-SY5Y cells. The total sample indicates the total binding of [$^3$H]nisoxetine to SH-SY5Y cells, whilst in the NSB sample imipramine (100μM), displaces the [$^3$H]nisoxetine from the catecholamine uptake transporter recognition sites to provide an assessment of any non-specific [$^3$H]nisoxetine binding (NSB). Salsolinol ($SAL_X$) and TMIQ ($TMIQ_X$) were added to the whole cell suspensions in varying concentrations ($10^{-10}$ to $10^{-3}$M).

*2.3.iii. Catecholamine release.*

Catecholamine release was undertaken, in all cell lines, by the method of Atcheson et al (1994a), as described below. Confluent monolayers of SH-SY5Y cells, in 12 well multitrays, were loaded with [$^3$H]DA or [$^3$H]NA, as described above (0.5mL of NA, 48nM or DA, 44nM). Following loading of labelled DA or NA, the monolayers were washed with Krebs / HEPES buffer (0.5mL), for three 15 minute periods. Release of stored catecholamines was achieved by addition of the cholinergic agonist carbachol (CCH 1mM) or isotonic potassium (100mM, $Na^+$ adjusted buffer) and compared to basal release in the absence of stimuli for 3 minutes. 100mM potassium and 1mM CCH were applied as they, via depolarization, or receptor occupation, elicit maximal release of catecholamines (Atcheson et al 1994a). Released catecholamines were removed and the remaining, non-released catecholamines were extracted with PCA (0.4M) for 30 minutes. All incubations were carried out at 37°C. Samples of released and non-released catecholamines were quantified by liquid scintillation spectroscopy. The release of catecholamines was expressed as shown in the following equation:

$$\text{Release (\%)} = \frac{[^3H](R)}{[^3H](R) + [^3H](M)} \times 100$$

Where [$^3$H](R) = the amount of catecholamines released from the cells during the 3 minute release period, (in disintergrations per minute, DPM), and [$^3$H](M) = the amount of catecholamines retained in the cells after the 3 minute release period (DPM).

When analysing the effect of test compounds on the release of catecholamines, compounds were included at various doses in the washing buffer after loading of [$^3$H]NA (48nM) was completed ( i.e. all three washes, 15 minutes each, for the N'-methyl nicotinamide, and the final 15 minute wash for TMIQ and salsolinol). Isotonic potassium (100mM) and CCH (1mM) were used to study the effects of the compounds under investigation on evoked release of [$^3$H]NA over a 3 minute period. Direct stimulation of loaded catecholamines was also attempted by the addition of compounds under study to the monolayers after three 15 minutes washes with Krebs / HEPES buffer for 3 minutes.

*2.3.iv. HPLC analysis of released catecholamines.*

SH-SY5Y cells were seeded at 3 x $10^5$ cells / well in 6 well multitrays to allow greater release for HPLC analysis and cultured as described previously. Confluent cultures were loaded with [$^3$H]DA (44nM) or [$^3$H]NA (48nM) and release stimulated with CCH (1mM) or isotonic potassium (100mM) as described above. Released catecholamines were removed from the monolayers after 3 minutes and acidified with PCA (0.4M). The remaining catecholamines were extracted from the cells with PCA (0.4M) for 30 minutes. Samples (0.5mL) were separated by the HPLC method of Herdon et al 1985, using a Partisil reverse-phase, C18, 5μM column. The mobile phase consisted of citric acid (27mM), sodium acetate (50mM), sodium hydroxide (60mM), sodium octyl sulphate (0.5mM) and 10 % methanol. The flow rate was 0.5mL min$^{-1}$, and fractions were collected every 30 seconds (0.25mL per fraction) for 30 minutes. Resulting peaks were compared to 0.5mL of authentic [$^3$H]DA and [$^3$H]NA standards (1μCi in 4mL of 0.4M PCA).

## 2.4. Synaptosomal Catecholamine Uptake.

### *2.4.i. Preparation of Synaptosomes.*

Cortical synaptosomes were isolated by the method of Dunkley et al (1988) from female Wistar rats (between 150-200g) and used to study the uptake of catecholamines in the presence and absence of test compounds. Rats were killed by stunning and then decapitated before removal of the cortex. Once removed, the cortex was homogenised in ice-cold sucrose (0.32M) / HEPES (20mM) buffer (pH 7.4), using 10 strokes of a tight-fitting Potter S, teflon / glass homogeniser, at 700 r.p.m. The homogenate was spun at 1000g for 10 minutes. The supernatant was removed and layered onto percoll gradient (3, 10, 15, 23 %, pH 7.4) prior to centrifugation at 20,000g for 10 minutes. The synaptosomal pellet was resuspended in Krebs / HEPES buffer, pH 7.4 (as described previously) and centrifuged at 20,000g. This procedure was repeated before the synaptosomes were ready for experimental use.

### *2.4.ii. Catecholamine Uptake.*

Rat cortical synaptosomes in 260μL volumes of Krebs / HEPES buffer, pH 7.4 (as described previously), were incubated at 37°C, in the presence or absence of test compound. Uptake of catecholamines was initiated by the addition of 20μL of either [$^3$H]NA (48nM, final concentration) or [$^3$H]DA (44nM, final concentration), in Krebs / HEPES buffer, pH 7.4. Incubation continued for up to one hour. After incubation was completed, uptake was terminated by the addition of ice-cold Krebs / HEPES buffer, pH 7.4, and the synaptosomes harvested onto GF/B filters (Whatman) under vacuum filtration. The filters were added to 4mL of Optiphase Safe scintillant overnight, and

finally the catecholamines taken up were quantified by liquid scintillation spectroscopy. Uptake was expressed as a percentage of the total available activity (20μL of either [$^3$H]DA (44nM)or [$^3$H]NA (48nM) were counted as the total available activity).

**2.5. Muscarinic Binding.**

To investigate the effects of both TMIQ and salsolinol upon muscarinic receptor mediated binding and reinforce the catecholamine release studies, displacement of the specific muscarinic ligand 1-[N-methyl-$^3$H]scopolamine methyl chloride (NMS, Amersham International, Buckinghamshire, U.K.) was attempted using the method of Lambert et al (1989). Whole cell suspensions (100μL) were incubated with a fixed concentration of [$^3$H]NMS set at the $K_d$ of ~0.202nM. In an attempt to displace [$^3$H]NMS binding, increasing concentrations of either TMIQ or salsolinol were included ($10^{-8}$ to $10^{-3}$M Table 2.2) in the incubation buffer. Non-specific [$^3$H]NMS binding was measured by the addition of excess atropine (1μM). After incubation for one hour at 37°C, bound and free NMS were separated by rapid vacuum filtration using a Brandel harvester. The filters were removed and added to 4mL of Optiphase Safe overnight. The concentration of [$^3$H]NMS added to the whole cell suspensions were determined by the addition of 200μL of each to 4mL of scintillant. The activity of both standards and samples were quantified by liquid scintillation spectroscopy. Displacement of [$^3$H]NMS binding was expressed as a percentage of the total [$^3$H]NMS binding (i.e. no TMIQ or salsolinol), after subtraction of non-specific binding.

Table 2.2. N-methyl scopolamine (NMS) binding displacement studies with SH-SY5Y cells; table of additions.

| Tube Sample | Buffer (μL) | Atropine (1μM) (μL) | NMS (μL) | Toxin (μL) | Cells (μL) |
|---|---|---|---|---|---|
| Total | 700 | | 200 | | 100 |
| NSB | 500 | 200 | 200 | | 100 |
| SAL/TMIQ | 500 | | 200 | 200 | 100 |
| SAL/TMIQ$_X$ | 500 | | 200 | 200 | 100 |

The above table illustrates the additions of each compound used during [$^3$H]NMS displacement binding studies with SH-SY5Y cells. The 'total' sample indicates the total binding of [$^3$H]NMS to SH-SY5Y cells, whilst in the NSB sample atropine (1μ M), displaces the [$^3$H]NMS from muscarinic binding sites to provide an assessment of any non-specific [$^3$H]NMS binding (NSB). Salsolinol (SAL$_X$) and TMIQ (TMIQ$_X$) were added to the whole cell suspensions at varying concentrations ($10^{-3}$ to $10^{-8}$M).

## 2.6. Determination of Protein Concentration.

Protein concentrations were determined by the method of Lowry (1951). Samples were prepared by addition of either 25μL (1:20) or 50μL (1:10) to sodium hydroxide (0.1M) to make a final volume of 0.5mL. To each sample 2.5mL of the following mixture was added, 2 % $Na_2CO_3$, (w/v) (in 0.1M NaOH), 1 % $CuSO_4$, (w/v), 2 % sodium potassium tartrate (w/v), in the ratio (v/v) 100:1:1, respectively. The samples were then incubated for 10 minutes before the addition of 0.25mL of Folin / Ciocalteu's phenol reagent (1:4 v/v in distilled water, Fisons, Loughborough, Leicestershire, U.K.), for 30 minutes at room temperature. After 30 minutes incubation the absorbance of both samples and standards (bovine serum albumin, 0, 50, 100, 150, 200, 250, 300 μg/ mL, Sigma, Poole, Dorset, U.K.), were determined spectrophotometrically, at 750nm. The protein concentration was extrapolated from the standard curve (a sample curve is shown in figure 2.2), run simultaneously to the samples, and corrected for dilution as appropriate.

## 2.7. Data Analysis and Statistical Analysis.

In all cases the data is represented as the mean ± SEM for 3 or more separate experiments. In LDH release and [$^3$H]thymidine incorporation experiments the data is expressed as a percentage of the basal level of LDH release from, or [$^3$H]thymidine incorporation in untreated cells. The data from MTT studies is expressed as a percentage of the basal MTT reduction (i.e. dye accumulation in an equivalent untreated control). With the exception of the catecholamine uptake experiments conducted in synaptosomes, all catecholamine uptake is expressed as the percentage of

the available activity during uptake. The synaptosomal data is expressed as the amount of uptake, in DPM mg protein$^{-1}$.

For displacement binding studies the $IC_{50}$ (the concentration giving 50 % displacement of the radio-labelled ligand) was calculated by non-linear regression analysis of the displacement curves using Graphpad (Version 2.0). The $K_{50}$ values ($IC_{50}$ values corrected for the competing mass of radioligand) were calculated according to the Cheng and Prusoff equation (1973), which is quoted below:

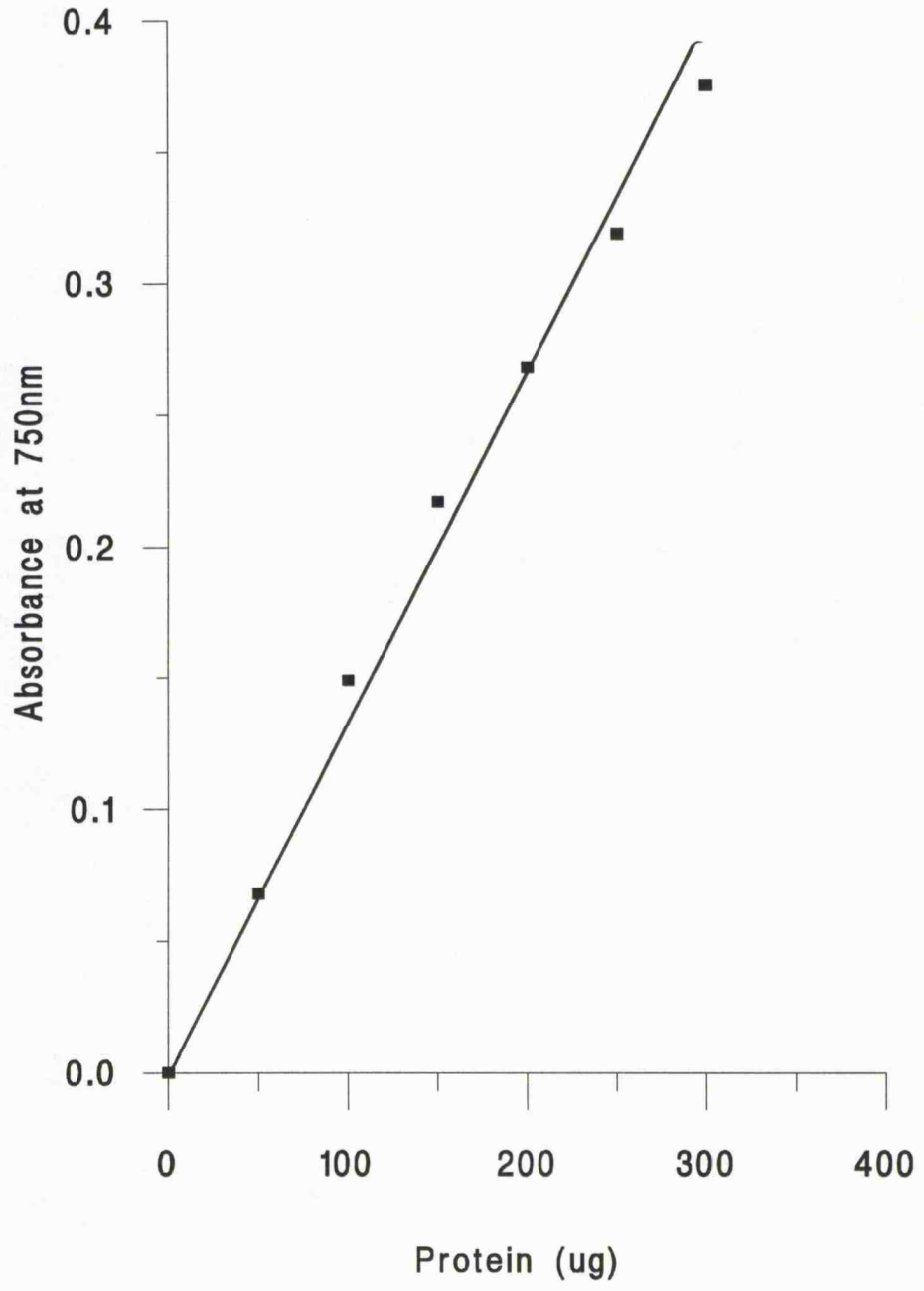
$$K_{50} = \frac{IC_{50}}{1 + \frac{(L)}{K_d}}$$

Where $K_{50}$ ($K_i$) equals the ; $IC_{50}$ equals the concentration giving 50 % displacement of the radio-labelled ligand; L equals the concentration of radioactive ligand; and $K_d$ is the concentration of the radioligand that occupies 50 % of the total available binding sites.

Both $EC_{50}$ (the concentration of a compound giving 50 % of the maximum response) and $IC_{50}$ (the concentration of a compound causing 50 % inhibition of a response) were calculated by non-linear regression using the Graphpad (Version 2.0) curve analysis program.

All data were analysed by one way analysis of variance, followed, where appropriate by unpaired Student's t-test or Duncan's multiple range post hoc test. Significant differences between groups was determined with $P<0.05$.

Figure 2.2. A sample protein standard curve determined by the method of Lowry (1951).




The graph represents a single protein determination using bovine serum albumin as standard at the concentrations stated. The method is described previously, page 82. The correlation coefficient for this curve had an r = 0.9966.

# CHAPTER 3

# Suitability of B65, SH-SY5Y and IMR32 neuroblastoma cells as models for 'in vitro' neurotoxicity testing

### 3.1. Introduction.

There is much debate about whether whole animal or isolated cultured cell models are the best with which to study the effects of potential neurotoxins on cellular biochemistry and physiology, and the possible interactions between neurotoxins. There are advantages and disadvantages with both models. Firstly, with an animal model one can study the interaction of a potential neurotoxin with many cell types, as well as assessing effects on behaviour and mortality. Cell lines offer a defined and constant system with which to study neurotoxins, as long as the passage number is known and thus variation in cellular responses minimised. Many neuronal cell lines can be differentiated to allow the study of neurotoxins on cell types similar to mature neurones occurring in vivo (Pahlman et al 1984, Yu et al 1988). Cell lines are also relatively inexpensive when compared to animal experimentation, and their quick growth allows many results to be obtained rapidly. However, there are some disadvantages when using cellular models as the investigation of long term toxins is not easily undertaken, due to the rapid achievement of confluence.

The potential endogenous neurotoxins under investigation in this thesis are reported to interact with catecholaminergic systems in vitro and in vivo (Birkmayer and Birkmayer 1989, NMN; Liptrot et al 1993, TMIQ; Heikkila et al 1971, salsolinol). Therefore, to examine the effects of these potential endogenous neurotoxins, in vitro, an appropriate cell line was required, with properties as close to those which occur in vivo. For example, it would be preferable if the cell line were catecholaminergic in origin, and it should possess the ability to take up, store and release neurotransmitters. There are many cell lines which are classified as catecholaminergic in origin. However, this classification is usually on the basis of the presence of tyrosine

hydroxylase, the first, and rate limiting enzyme in catecholamine production (figure 1.6). Unfortunately this only provides a starting point for the examination of the catecholaminergic properties of any cell line. Previous literature reports are limited to the analysis of few cell lines, and these studies are often incomplete. For example, the presence of an uptake transporter, which is blocked with catecholamine uptake inhibitors, such as imipramine, is also often considered sufficient to classify a cell line as catecholaminergic. However, this definition is not always adequate. Therefore, a full analysis of catecholamine uptake, release (both stimulated and unstimulated), and the determination of the release components is required to ascertain the nature of the cell type.

From the many available cell lines, rat B65 (Schubert et al 1974), human SH-SY5Y (Ross and Biedler 1985), and human IMR32 (Tumilowicz et al 1970) were chosen for further analysis. All were derived from neuroblastomas, reported to be of catecholaminergic origin, and to possess significant tyrosine hydroxylase activity. SH-SY5Y (Murphy et al 1991, and Atcheson et al 1993a) and IMR32 (Clementi et al 1986) human neuroblastoma cell lines are reported to take up noradrenaline and dopamine, respectively. Uptake of catecholamines is blocked by the addition of either imipramine (SH-SY5Y cells) or desmethylimipramine (IMR32), indicating the presence of a catecholamine uptake$_1$ transporter in both cell lines. Stimulated release of NA has been demonstrated in SH-SY5Y cells (Murphy et al 1991, and Atcheson et al 1994b) by both receptor mediated (carbachol) and potassium depolarization-induced mechanisms. Catecholamine release has also been reported in IMR32 cells (Clementi et al 1986). B65 cells have significant tyrosine hydroxylase activity (Schubert 1974), however no examination of catecholamine uptake and release has been reported.

Thus, uptake and release of both [$^3$H]DA or [$^3$H]NA was examined. In addition, the profile of catecholamine release was measured using HPLC.

### 3.2. Methods and Materials.

*Cell Culture.*

Undifferentiated neuroblastoma cells were maintained as described previously, materials and methods, chapter 2, page 67.

*Catecholamine Uptake and Release.*

Catecholamine uptake and release were undertaken with B65, SH-SY5Y and IMR32 cells, by the methods of Atcheson et al (1993, 1994a) as described previously, materials and methods, chapter 2, pages 77 and 81. Released catecholamines were also collected and analysed by the HPLC method of Herdon et al (1985) outlined in materials and methods, chapter 2, page 82.

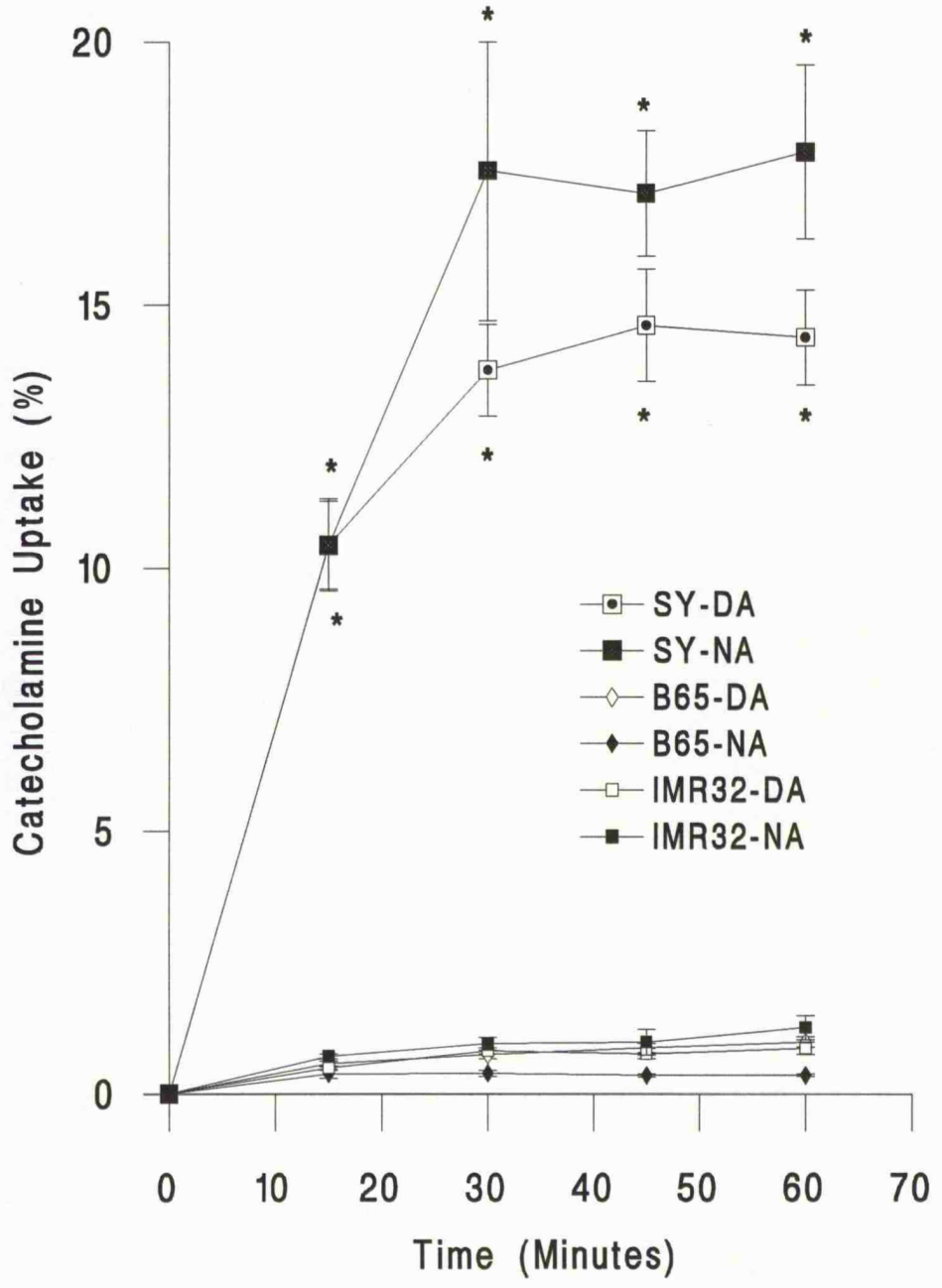
### 3.3. Results.

In confluent monolayers SH-SY5Y cells take up both [$^3$H]DA and [$^3$H]NA with $t_{1/2}$ of 3.7 and 5.4 minutes and maximum uptake at 45 and 30 minutes respectively. Maximum uptake for DA and NA amounted to 14.1 ± 2.4 and 17.1 ± 2.7 % respectively (data is expressed as mean ± SEM of the percentage of the total available activity during uptake) (figure 3.1). In contrast, B65 cells, in monolayer culture, did not take up significant amounts of either catecholamine (<1 % at 60 minutes). The uptake of DA and NA into SH-SY5Y cells was blocked by the catecholamine uptake inhibitor, imipramine (10μM, table 3.1). IMR32 cells were able to take up catecholamines (figures 3.1 and 3.2). However, the amount depended upon which experimental protocol was undertaken. When confluent IMR32 cell monolayers are exposed to either [$^3$H]DA (44nM) or [$^3$H]NA (48nM) they take up around 1 % of the total available activity (figure 3.1). The use of whole cell suspensions increased the amount of catecholamine uptake to a maximum of 5.6 ± 0.3 % and 3.6 ± 0.5 % for [$^3$H]DA and [$^3$H]NA, respectively (figure 3.2). If the IMR32 cells in suspension were agitated, then the uptake of catecholamines was significantly ($P<0.05$) increased to levels of 12.6 ± 2.0 and 8.7 ± 1.7 % for DA and NA respectively (figure 3.2). These data are comparable with the findings of Clementi et al (1986). However, it was not possible to significantly block the uptake of catecholamines in IMR32 cells with imipramine or benztropine (10μM) in the presence or absence of agitation during uptake (table 3.1), indicating non-specific uptake.

The small amount of DA and NA taken up by IMR32 cells, in monolayer culture, was not stored in a releasable pool, since neither CCH (1mM) or isotonic potassium (100mM) significantly increased release above basal (background release, table 3.2).

HPLC analysis of the catecholamines released from IMR32 cells after preloading of catecholamines gave similar results (data not shown). In contrast SH-SY5Y appeared to store catecholamines in a releasable pool, with carbachol (1mM) the cholinergic agonist, stimulating release over a range of 2 to 2.5 fold, and isotonic potassium (100mM) greater than 3 fold above basal, respectively (figure 3.3). However, analysis of the released catecholamines indicated that when preloaded with DA, virtually all of the DA is converted to NA (92.4 ± 0.5 %, n = 12) upon uptake prior to storage and release, probably due to the action of dopamine-β-hydroxylase. Moreover, only NA is stored in a releasable pool since addition of CCH (1mM) or isotonic potassium (100mM) elicited an increase the NA, but not DA release (table 3.3 and figure 3.4).

Figure 3.1. Catecholamine uptake in confluent monolayer cultures of neuroblastoma cells, over a one hour incubation.



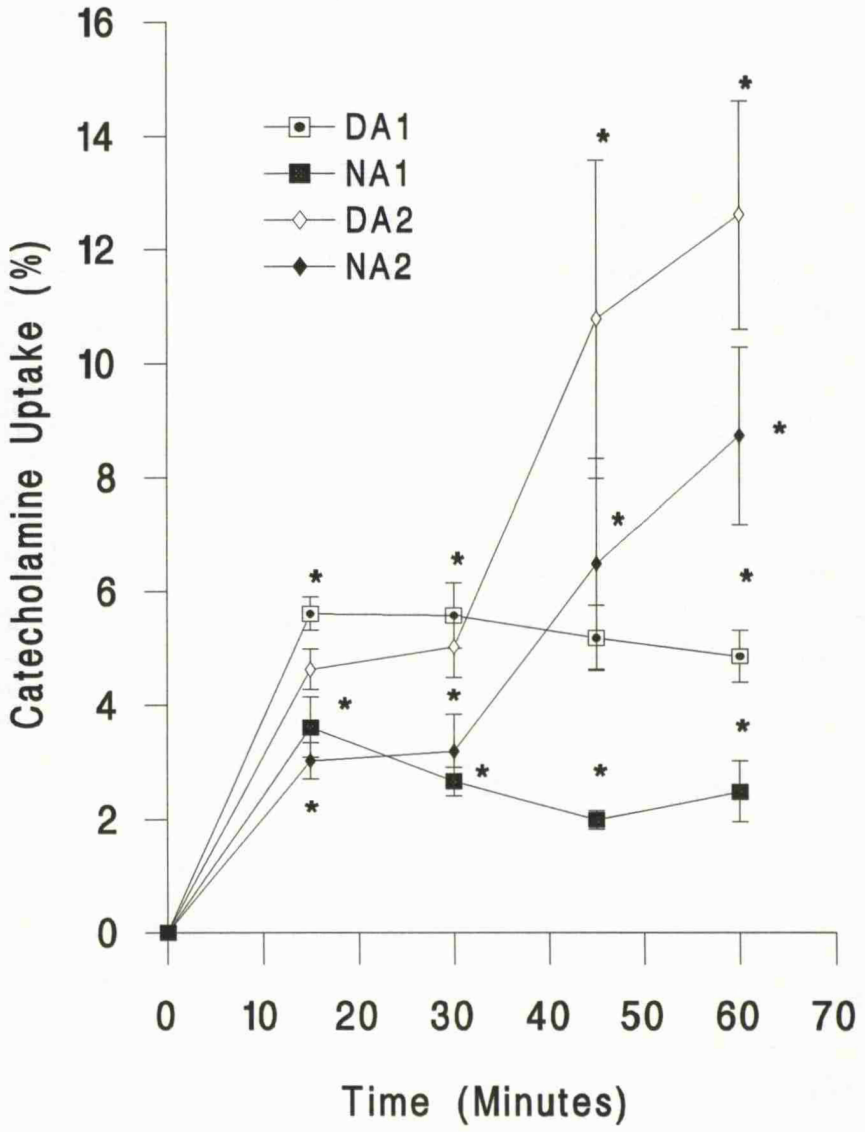

The data is expressed as a percentage of the total available activity, and is represented as the mean ± SEM for n = 4 to 5 experiments. SY represents SH-SY5Y cells, DA represents dopamine and NA represents noradrenaline. * Signifies significant ($P<0.05$) uptake when compared to time zero.

Table 3.1. Inhibition of catecholamine uptake by benztropine (10μM) and imipramine (10μM) in neuroblastoma cells.

| | Inhibition of Catecholamine Uptake (%) | |
|---|---|---|
| Cells | DA + Benztropine (10μM) | NA + Imipramine (10μM) |
| SH-SY5Y | 90.1 ± 0.3* | 85.7 ± 0.9* |
| IMR32 | 19.4 ± 21.4 | 23.6 ± 15.6 |

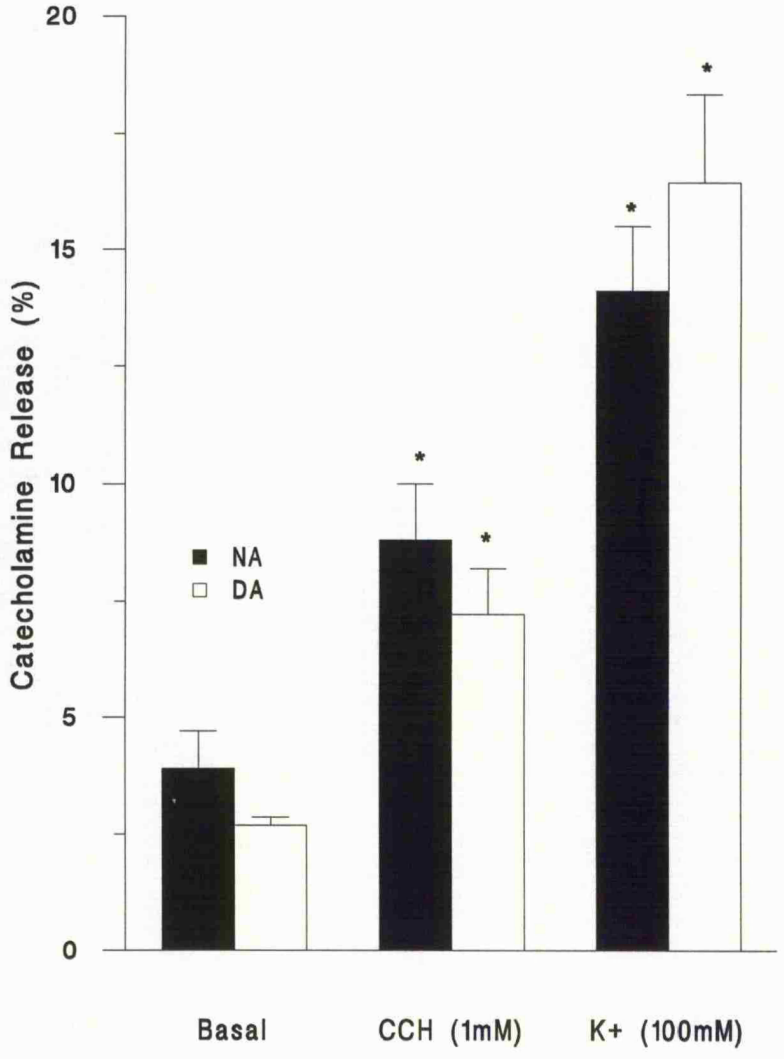
The data is expressed as the mean ± SEM of the percentage inhibition of uptake compared to a concurrent sample run without either benztropine or imipramine, for n = 3 for SH-SY5Y cells, and n = 4 experiments for IMR32 cells, respectively. * Represents $P < 0.05$ when compared to basal uptake.

Figure 3.2. Catecholamine uptake in IMR32 cells in suspension culture.



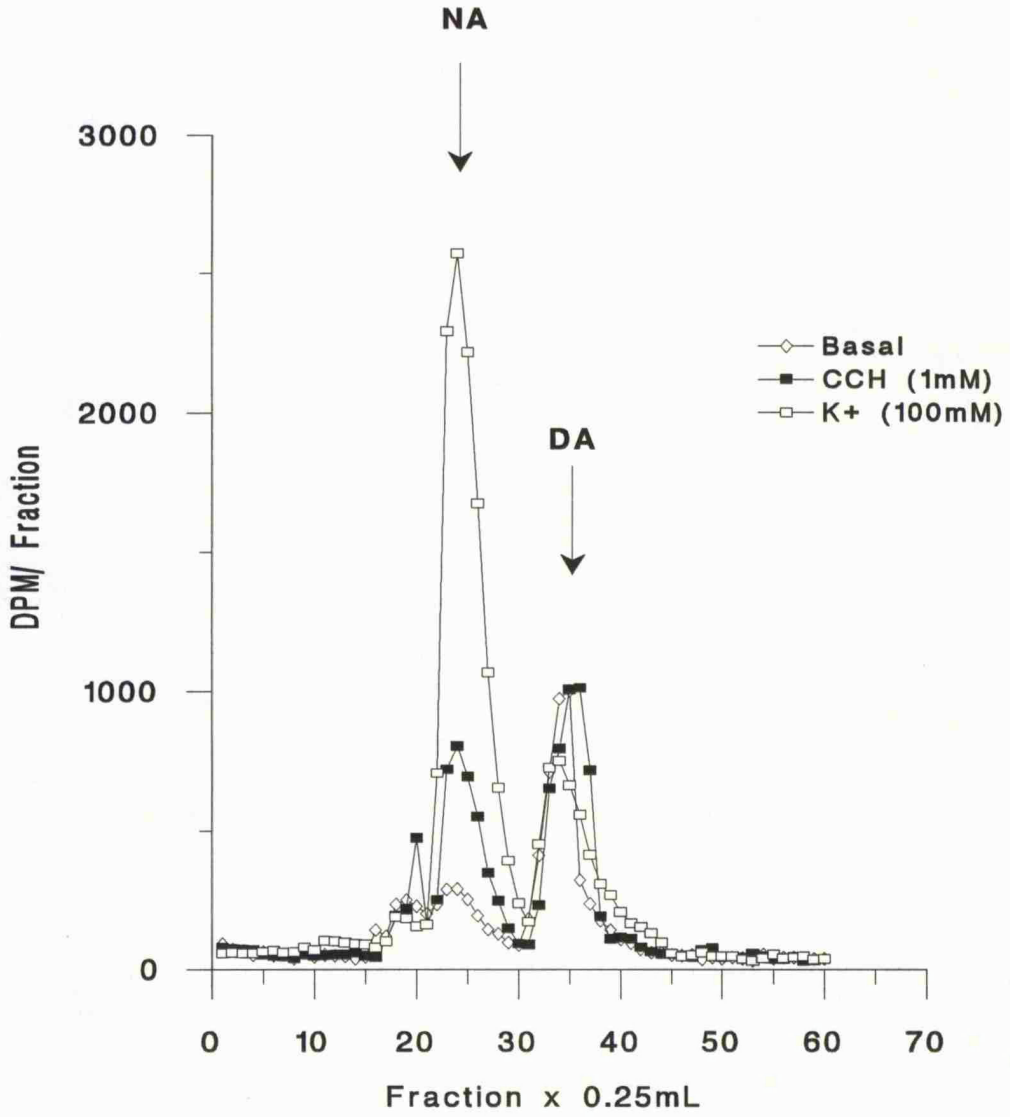

DA1 and NA1 represent suspension culture, whilst DA2 and NA2 represent agitated suspension culture, during catecholamine uptake over one hour. The data is expressed as a percentage of the total available activity, and represented as the mean ± SEM, for n = 4 experiments. * Signifies significant uptake ($P<0.05$) when compared to time zero.

Figure 3.3. Catecholamine release from SH-SY5Y cells loaded with either noradrenaline or dopamine.




Catecholamine release was performed in 12 well multitray monolayer culture. Carbachol (CCH 1mM) and potassium ($K^+$ 100mM, $Na^+$ adjusted buffer), were added for 3 minutes to stimulate release, when compared to basal (i.e. no additions). The data are expressed as a the percentage release (as described previously, materials and methods, page ). Data are given as the mean ± SEM, for n = 5 to 10 experiments. * Signifies $P<0.05$ when compared to basal.

Figure 3.4. HPLC determination of catecholamine release profile from SH-SY5Y cells loaded with dopamine.




The data presented is from a single experiment, where confluent SH-SY5Y cells, in 6 well multitrays, were loaded with dopamine prior to either, basal (no additions), carbachol (CCH 1mM) or potassium ($K^+$ 100mM) stimulated release. The released catecholamines were collected, separated by the HPLC method of Herdon et al (1985) and determined by liquid scintillation spectroscopy. The highlighted peaks indicate the elution of either noradrenaline (NA) or dopamine (DA) as determined by authentic standards.

Table 3.2. Release of catecholamines from IMR32 and SH-SY5Y neuroblastoma cells after loading with either DA or NA as described previously.

| | Catecholamine Release (%) | |
|---|---|---|
| | DA Loaded | NA Loaded |
| **IMR32** | | |
| Basal | 7.35 ± 0.83 | 9.82 ± 1.34 |
| CCH (1mM) | 7.69 ± 1.23 | 9.80 ± 0.68 |
| $K^+$ (100mM) | 8.66 ± 1.02 | 11.0 ± 0.75 |
| **SH-SY5Y** | | |
| Basal | 2.40 ± 0.10 | 5.26 ± 1.4 |
| CCH (1mM) | 6.00 ± 1.30 | 8.82 ± 0.30* |
| $K^+$ (100mM) | 13.4 ± 1.30 | 12.3 ± 3.30* |

The data is expressed as mean ± SEM of the percentage release of loaded catecholamines (i.e. NA and DA). * Signifies $P<0.05$ when compared to basal release. (n = 7 or 8 experiments, except for DA loaded SH-SY5Y cells where n = 2) These data suggest that IMR32 cells release substantial amounts of catecholamines. However, this is due to the small amount of catecholamines taken up by IMR32 cells. This can be seen by analysing the set of sample data below:

Table 3.3. Comparison of the absolute values, in DPM, of [$^3$H]NA released in SH-SY5Y and IMR32 cells after 3 minute incubation with either Krebs / HEPES buffer or potassium (100mM, $Na^+$ adjusted) buffer.

| Sample | Released (DPM) | Retained (DPM) | NA Release (%) |
|---|---|---|---|
| **SH-SY5Y** | | | |
| Basal | 7532 | 149985 | 4.78 |
| $K^+$ (100mM) | 17119 | 153002 | 10.1 |
| **IMR32** | | | |
| Basal | 554 | 5857 | 8.65 |
| $K^+$ (100mM) | 535 | 4890 | 9.86 |

Table 3.4. HPLC analysis of catecholamines released from SH-SY5Y cells preloaded with [$^3$H]DA.

| Treatment | Catecholamine Release (%) | | |
|---|---|---|---|
| DA Loaded Cells | Total | NA | DA |
| Basal | 3.69 ± 0.33 | 1.59 ± 0.33 | 30.90 ± 3.05 |
| CCH (1mM) | 6.18 ± 0.36* | 3.30 ± 0.29 * | 41.78 ± 0.69 |
| $K^+$ (100mM) | 7.04 ± 0.53* | 4.60 ± 0.69* | 35.99 ± 6.83 |

All data are expressed as mean ± SEM for n = 3 or 4 experiments. In all NA loaded cells DA was not detected. Substantial quantities of DA appear to be released from DA loaded cells. However, this reflects the low DPM related to dopamine in the cells since over 92 % is converted to NA prior to storage in vesicles (see table 3.2 legend for further explanation).

### 3.4. Discussion.

In this study SH-SY5Y and IMR32 cells were shown to take up both [$^3$H]DA and [$^3$H]NA. This data is in agreement with that of Atcheson et al (1993, 1994a) and Clementi et al (1986). In contrast, in monolayer culture, B65 cells do not take up significant amounts of either [$^3$H]DA or [$^3$H]NA, indicating the absence of a catecholamine uptake transporter in this cell line. The uptake of catecholamines in SH-SY5Y is significantly blocked by the inclusion of imipramine (10μM, table 3.1). Moreover, in contrast with the data of Clementi et al (1986), neither imipramine (10μ M) nor benztropine (10μM) significantly inhibited catecholamine uptake in IMR32 cells. However, the amount of catecholamine uptake in IMR32 cells depended upon which experimental protocol was undertaken. In monolayers, catecholamine uptake was less than 2 % of the total available activity, unless IMR32 cells were agitated during incubation. Thus, it appears that catecholamine uptake in IMR32 cells relies more upon the surface area than the number of catecholamine uptake sites, and may suggest a non-specific uptake of catecholamines. However, direct comparison between monolayer and suspension cultures is not attainable without a measure of cell numbers. This is difficult to undertake in monolayer cultures as correction for protein density would require measurement of radioactively contaminated cells after experimentation. Without a correction for cell density the observed differences in catecholamine uptake in monolayers and suspension may be artifactual and not a real biological difference. Therefore, within these confines the data suggest that SH-SY5Y cells possess active uptake$_1$ in monolayer culture, whilst IMR32 and B65 cells probably do not.

We have confirmed that SH-SY5Y cells released [$^3$H] labelled catecholamines approximately 2 fold and 3 fold above basal (non-stimulated) release with carbachol

(CCH, 1mM) and potassium (100mM, $Na^+$ adjusted buffer), respectively. However, analysis of the released catecholamines, when loaded with DA, revealed that virtually all of the DA is converted to NA (92.4 ± 0.5 %, n = 12) upon uptake prior to storage and release. The conversion of DA to NA in SH-SY5Y indicates the activity of dopamine-β-hydroxylase in these cells. These data also indicate that SH-SY5Y cells store NA in a releasable pool.

Although IMR32 cells take up catecholamines, they store neither [$^3$H]DA or [$^3$H]NA in a releasable pool, because neither CCH (1mM) or potassium (100mM, $Na^+$ adjusted buffer) significantly stimulated catecholamine release.

This study demonstrates that SH-SY5Y cells, of the three cell lines examined, appear to have the greatest resemblance to an in vivo catecholaminergic cell. They possess catecholamine $uptake_1$, and store NA in a releasable pool. Catecholamine release may be achieved by the addition of CCH or potassium stimulation, indicating that SH-SY5Y cells respond to both ligand / receptor based and depolarization stimuli (Scott et al 1986, Murphy et al 1991, and Atcheson et al 1994a). Both CCH and potassium evoked catecholamine release are calcium dependent. CCH induced catecholamine release is possibly mediated by increased $IP_3$ (Atcheson et al 1994b, Purkiss et al 1995), and subsequent calcium release from intracellular stores (Lambert and Nahorski 1990), whereas, potassium depolarization opens voltage sensitive calcium channels to evoke NA release (Atcheson et al 1994b). I have therefore chosen SH-SY5Y cells with which to study the effects of potential neurotoxins that are reported to interact with catecholaminergic cells in vivo. However, SH-SY5Y cells convert virtually all DA to NA upon uptake prior to storage and release only NA in a releasable pool. Therefore, they are not ideal to study the effects of toxins that evoke the release of DA

as a component of their toxicity. However, SH-SY5Y cells possess an active catecholamine uptake transporter, which shows little selectivity for DA or NA. This uptake transporter also facilitates $MPP^+$ toxicity in SH-SY5Y cells (Spina et al 1992). Additionally, none of the potential toxins examined in this study are known to induce DA or catecholamine release from cells in vitro. PC12 cells are reported to be dopaminergic, but they also produce many other catecholamines including noradrenaline and adrenaline (Hatanaka 1981). Study of the uptake of the 1-methyl-4-phenylpyridium ion ($MPP^+$, the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) indicated uptake occurred through a carrier system common to both DA and NA (Takahashi et al 1987). Choi et al (1991) have reported that they possess a cell line which appears to take up, store, and synthesize dopamine alone. However, it was not possible to obtain any of these cells to assess their suitability as dopaminergic neurones. Therefore, the SH-SY5Y cell line has been used as the 'in vitro' model with which to study the effects of the potential neurotoxins NMN, TMIQ and salsolinol.

# Chapter 4

## Studies on the potential neurotoxicity of the nicotinamide derivative N-methyl nicotinamide

## 4.1. Introduction.

The study of neurodegenerative diseases has produced several candidate neurotoxins from either environmental or endogenous sources. For example 1,2,3,4-tetrahydroisoquinoline, from dietary source, or produced endogenously via spontaneous Pictet-Spengler cyclization of tryptamines and catecholamines in the brain, has been reported to be a likely candidate (Niwa et al 1987, Nagatsu and Yashida 1988, Niwa et al 1989). In addition transition metals such as aluminium and iron have also been implicated in the aetiology of neurodegenerative disorders, including Alzheimer's (Perl 1988) and Parkinson's diseases (Youdim et al 1993, Michel et al 1992). Poor detoxification of xenobiotics could represent a mechanism for the accumulation of potential neurotoxins (Steventon et al 1989a). For example the study of nicotinamide metabolism, after oral administration, in PD and motor neurone disease patients, and age matched controls showed that levels of N'-methyl nicotinamide (NMN) were approximately 90 fold greater than the controls, in the urine of PD patients (Green et al 1991). The accumulation of NMN may be due to inactivity of an isoform of cytochrome P450, however initial investigation of the enzyme function was inconclusive (Steventon et al 1989b, Ferrari et al 1990).

This chapter investigates whether NMN is a potential neurotoxin. Cellular damage was assessed by means of thymidine incorporation and lactate dehydrogenase release to give an indicator of cellular growth and membrane integrity. This study also investigated whether NMN may alter catecholamine uptake and, or release as a potential mechanism for its toxicity. Human SH-SY5Y (Ross and Biedler 1985) neuroblastoma cells were used based on the data from chapter 3 and their expression of tyrosine hydroxylase. In addition the effects of NMN were assessed with B65 cells

(Schubert et al 1974), which appear to lack any catecholamine uptake transporter (Chapter 3), and as such should be unaffected by any potential toxins that require catecholamine uptake to facilitate their toxicity. Furthermore, as a common factor in many neurodegenerative diseases is the disturbance of mitochondrial metabolic function (see introduction), and as metabolic inhibitors are known to induce PD-like symptoms (Uitti et al 1985, Rosenberg et al 1989), the metabolic poison rotenone was included as a positive control during most experiments. Similarly, NMN may express any potential toxicity through such a pathway.

## 4.2. Materials and Methods.

*Cell culture and incubation protocols.*

Human SH-SY5Y and rat B65 neuroblastoma cells were maintained as outlined in chapter 2, page 67. For experimental purposes, confluent stock cultures were harvested by trypsinization (0.05 %) and seeded at 1 x $10^5$ cells / well in twelve well multitrays, and grown until confluent under the conditions described previously. Fresh media was supplied every two days. When confluent, the cell monolayers were exposed to either N'-methyl nicotinamide or, nicotinamide ($10^{-4}$ to $10^{-2}$M), (Aldrich, Gillingham, Dorset, UK) rotenone (10μM) or dimethyl sulphoxide (DMSO, vehicle for rotenone, both from Sigma, Poole, Dorset, UK) (0.1% v/v) in the appropriate supplemented media. α-Tocopherol (Sigma) was added at varying doses (between 0.5 μM to 500μM), in ethanol (0.1 % v/v), concurrently with NMN (10mM), to both confluent SH-SY5Y and B65 cells, for up to 72 hours. Additionally, the effects of NMN and nicotinamide were assessed using differentiated SH-SY5Y cells. Differentiation was induced by the addition of retinoic acid (10μM, Pahlman et al 1984, Yu et al 1988) to the supplemented media 24 hours after seeding in 12 well multitrays. Cells were cultured for a further 7 days to allow differentiation. Cell differentiation was determined by the presence of long neuritic processes and an observable reduction in growth rate. In contrast undifferentiated SH-SY5Y cells have an irregular shaped cell body with short processes.

*Lactate Dehydrogenase Release.*

Following incubation for 24, 48 or 72 hours both the media and cell monolayers were analysed for lactate dehydrogenase activity as described in chapter 2, page 71. LDH

release was calculated as the percentage of activity in the media compared to the total activity per well. Confluent SH-SY5Y cells were also incubated with α-tocopherol (50, 100 and 500μM) for 24 hours, prior to incubation with NMN for 24 hours.

*Thymidine Incorporation.*

SH-SY5Y and B65 cells were seeded at 1 x $10^5$ cells / well in twelve well multitrays and grown in supplemented media as described previously, materials and methods, chapter 2, page 76. After three days (SH-SY5Y), or one day (B65), the cells were exposed to NMN, nicotinamide, ($10^{-2}$ to $10^{-5}$M), rotenone (10μM) or DMSO (0.1 % v/v), for 24, 48 and 72 hours. NMN, nicotinamide ($10^{-2}$ to $10^{-5}$M), rotenone (10μM) or DMSO (0.1 % v/v) were included in the appropriate wells during [$^3$H]thymidine incorporation. α-Tocopherol (50 to 500μM) was included concurrently with NMN (10mM), in some experiments to try and reverse the effects of NMN upon [$^3$H]thymidine incorporation, in SH-SY5Y cells. The amount of thymidine incorporation was expressed as a percentage of the total available activity.

*Catecholamine Uptake.*

Catecholamine uptake was undertaken by the method of Atcheson et al (1993) summarized in chapter 2, page 77. NMN or nicotinamide were included in the loading buffer (1 and 10mM), with the exception of basal samples. Catecholamine uptake is expressed as a percentage of basal.

Catecholamine uptake was also studied using rat cortical synaptosomes in Krebs / HEPES buffer, pH 7.4, outlined in materials and methods, chapter 2, page 83. NMN or nicotinamide were included in the synaptosomal incubations during catecholamine

uptake over various incubation periods. Uptake was expressed as a percentage of the total available activity.

*Catecholamine release.*

Catecholamine release was undertaken by the method of Atcheson et al (1994a) as described in chapter 2, page 81. Samples of released and non-released catecholamines were quantified by liquid scintillation spectroscopy. The release of catecholamines was expressed as a percentage of the total (both released and retained). When analysing the effect of nicotinamide derivatives on release of catecholamines, NMN and nicotinamide were included at the stated doses (1 and 10mM) in the washing buffer after loading of [$^3$H]NA was completed (all three washes). Isotonic potassium (100mM) was used to study the effects of NMN and nicotinamide on evoked release of [$^3$H]NA. Direct stimulation (3 minutes) of loaded catecholamines was also attempted by the addition of nicotinamide derivatives (1 and 10mM) to the monolayers after three 15 minutes washes with Krebs / HEPES buffer.

**4.3. Results.**

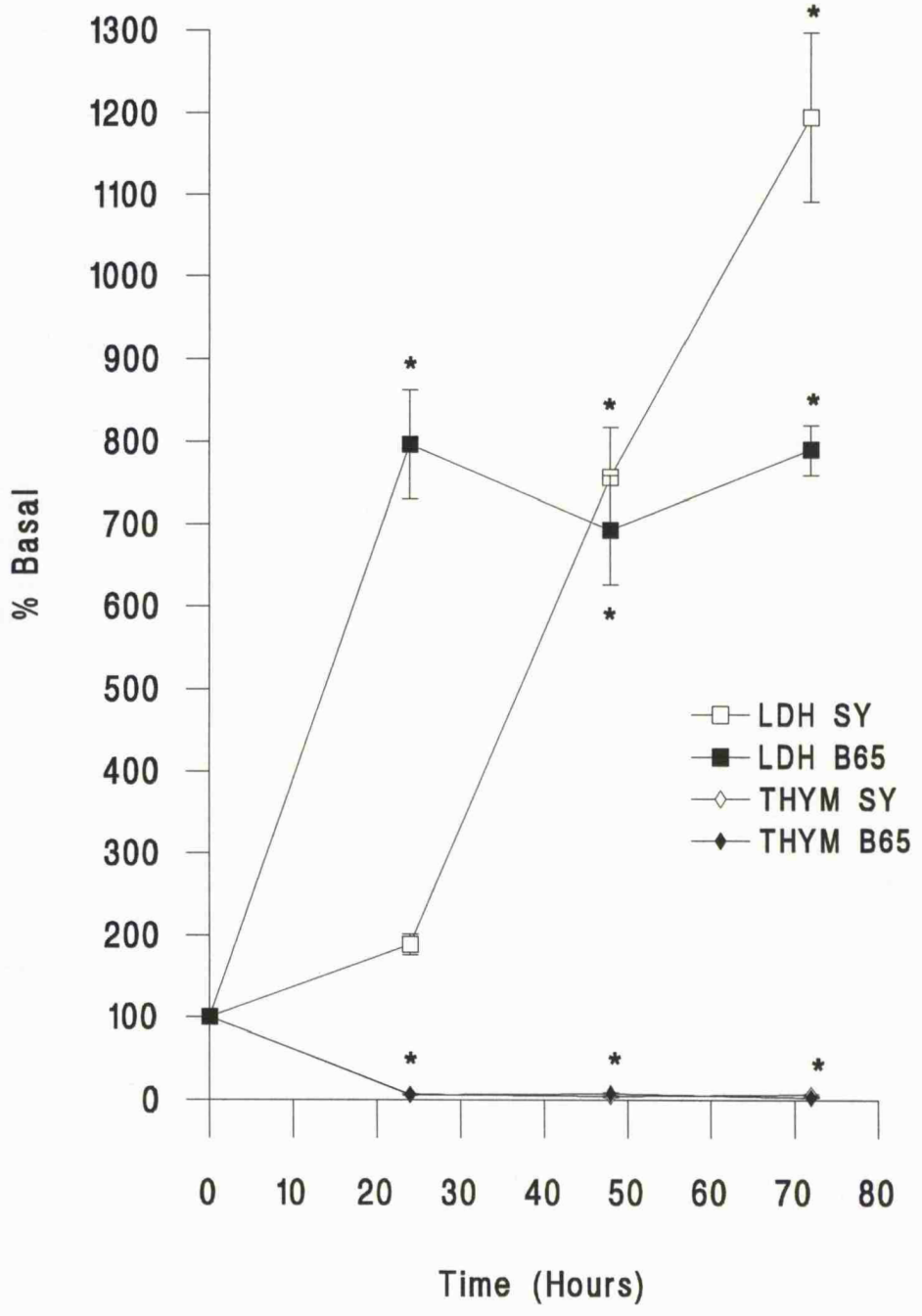
Maximal release 796.4 ± 114.2 %, of LDH was elicited by rotenone (10μM), after 24 hours incubation in B65 cells. SH-SY5Y cells were less susceptible to rotenone (10μ M) over 24 hours, followed by a large increase in LDH release over the next 48 hours (figure 4.1). However, in SH-SY5Y cells [$^3$H]thymidine incorporation was reduced to 6.8 ± 1.3 % of basal after 24 hours and remained at this level at 48 and 72 hours (figure 4.1). NMN (10mM) significantly increased ($P<0.05$) the release of LDH from both B65 and SH-SY5Y cells from 24 hours to 72 hours (figure 4.2 A and B). NMN (10mM) also significantly reduced ($P<0.05$) [$^3$H]thymidine incorporation in SH-SY5Y cells at 48 and 72 hours (figure 4.2A). Nicotinamide (10mM) increased [$^3$H]thymidine incorporation in SH-SY5Y cells to a maximum of 45.1 ± 15.8 % greater than basal at 72 hours (figure 4.2A). In B65 cells, NMN and nicotinamide (10mM) had no effect upon [$^3$H]thymidine incorporation at 24 hours, compared to basal (figure 4.2B). However, at 48 hours NMN and nicotinamide (10mM) produced an increase in [$^3$H]thymidine incorporation of 64.5 ± 6.6 % and 43.8 ± 10.6 %, respectively. The increase in [$^3$H]thymidine incorporation was reversed after 72 hours incubation of NMN and nicotinamide (10mM) to 32.7 ± 3.3 and 36.2 ± 3.1 % below basal, respectively. Lower doses ($10^{-3}$ to $10^{-5}$M) of NMN and nicotinamide had no significant effect upon LDH release or [$^3$H]thymidine incorporation in either cell line (data not shown). Nicotinamide (10mM) had no effect upon the release of LDH from SH-SY5Y cells. In B65 cells nicotinamide (10mM) increased LDH release up to approximately 2.5 times basal at 48 and 72 hours (figure 4.2B). However, NMN (10mM) induced a 5 fold increase in LDH release over the same time period (figure 4.2B).

In differentiated SH-SY5Y cells NMN (10mM) significantly increased LDH release from 48 to 72 hours of 130.4 ± 10.9 % to 211 ± 17.8 %, respectively (figure 4.3). In contrast nicotinamide did not significantly increase LDH release over 72 hours (116.3 ± 11.9 %). Significant increase in LDH release was not observed at doses of NMN or nicotinamide below 10mM (data not shown). Rotenone (10μM), the positive control induced similar levels of LDH release as NMN (10mM), 252.3 ± 5.4 % to 279.7 ± 21.2 % at 24 to 72 hours, respectively (figure 4.3).

The release of LDH by NMN (10mM) in SH-SY5Y cells, was inhibited by the addition of α-tocopherol (100μM) (figure 4.4). This inhibition was dose related with an $IC_{50}$ of 31.6μM (figure 4.5), and occurred either with concurrent incubation of α-tocopherol and NMN (figure 4.4 and 4.5), or with a 24 hour pre-incubation of α-tocopherol (50, 100 or 500μM) prior to NMN incubation (figure 4.6). The inhibition of LDH release by α-tocopherol in SH-SY5Y cells was not observed in B65 cells (table 4.1). However, addition of α-tocopherol (25, 50 or 100μM) did not inhibit the effects of NMN upon [$^3$H]thymidine incorporation in SH-SY5Y cells.

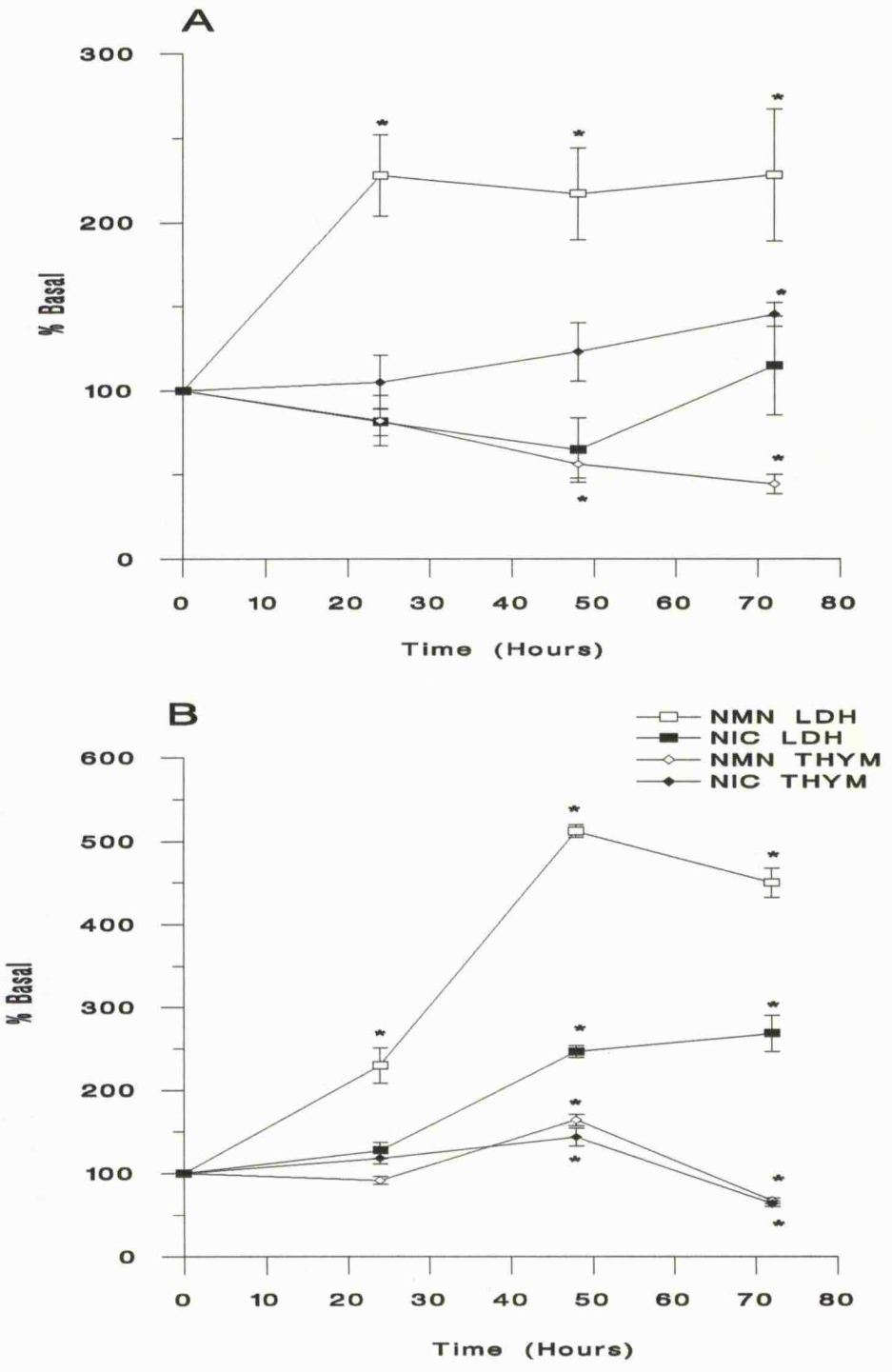
At 10mM, NMN, but not nicotinamide, inhibited NA and DA ($P<0.05$) uptake (table 4.2). No effect was observed at lower doses. Rotenone at 50μM did not affect the uptake of [$^3$H]DA (basal 100 %, 50μM rotenone 106 %). Both basal and $K^+$ stimulated release of [$^3$H]NA were unaffected by NMN, or nicotinamide (table 4.2). In addition, neither NMN or nicotinamide, at 1 or 10mM, affected [$^3$H]DA uptake in rat cortical synaptosomes (figure 4.7).

Figure 4.1. The effect of rotenone (10μM) upon LDH release and [$^3$H]thymidine incorporation in SH-SY5Y and B65 neuroblastoma cells.



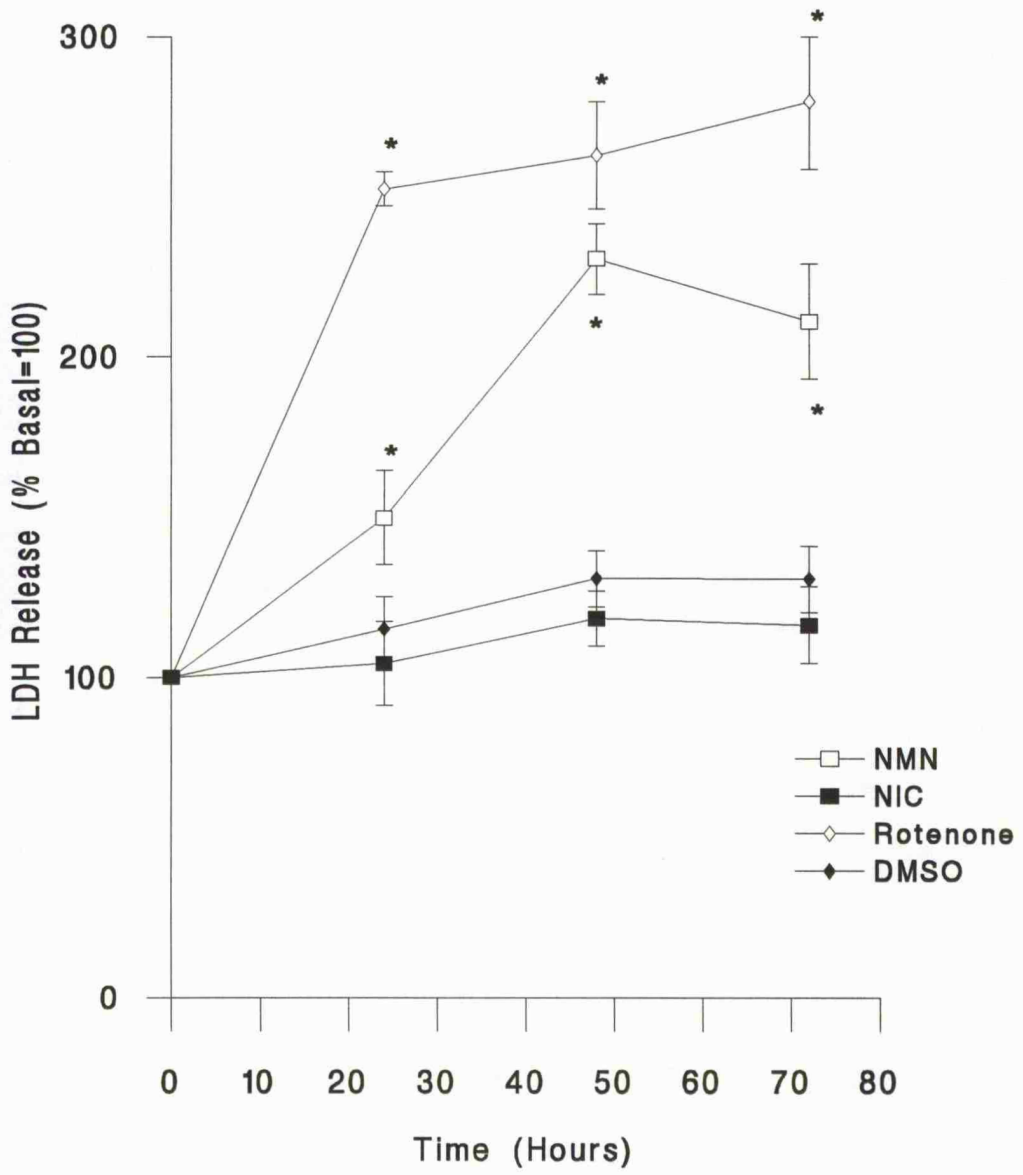

All data are expressed as mean ± SEM of the percentage of basal (no additions = 100), for n = 3 to 6 experiments. * Signifies $P<0.05$ when compared to basal.

Figure 4.2. The effects of NMN and nicotinamide (NIC) upon LDH release and [$^3$H]thymidine incorporation in SH-SY5Y (A), and B65 (B) neuroblastoma cells.



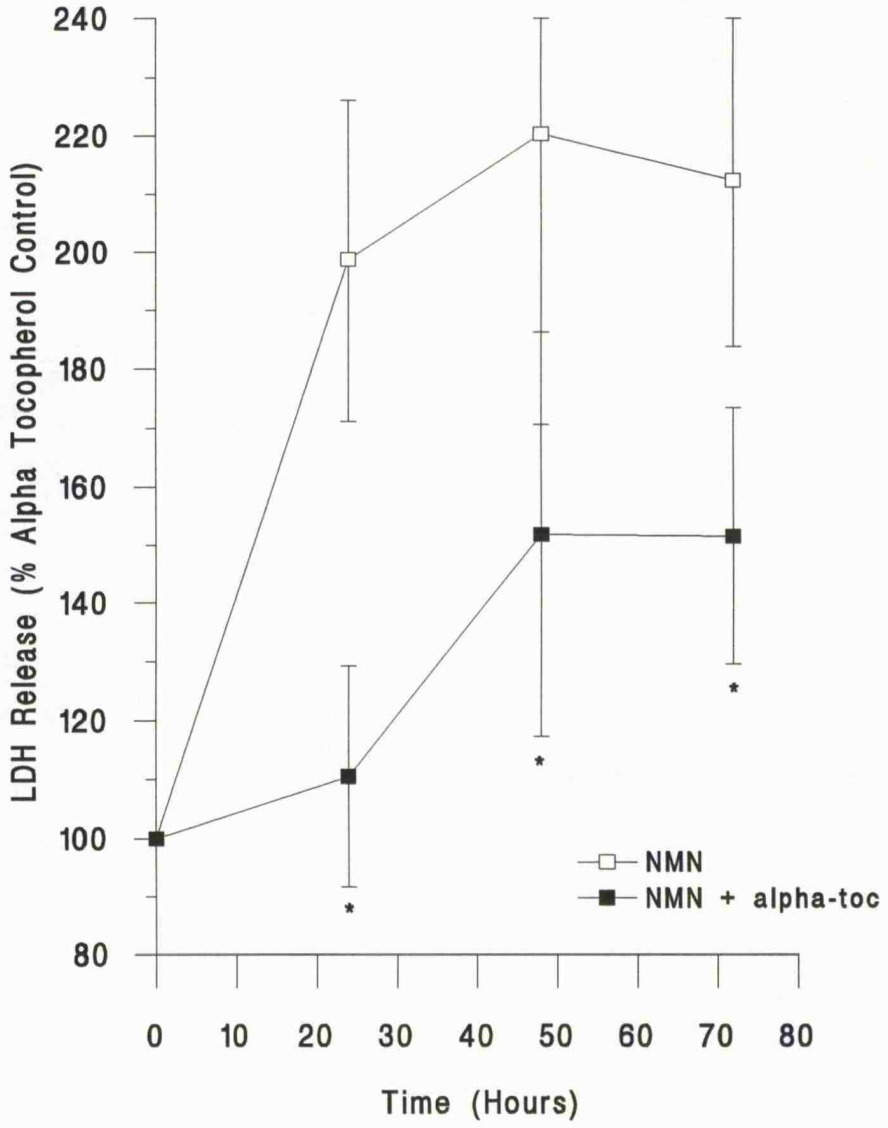

All data are expressed as the mean ± SEM of the percentage of basal (no additions = 100), for n = 3 to 6 experiments. * Signifies $P<0.05$ when compared to basal.

Figure 4.3. The effect of NMN, nicotinamide (NIC), rotenone and dimethyl sulphoxide (DMSO) upon LDH release in differentiated SH-SY5Y neuroblastoma cells.



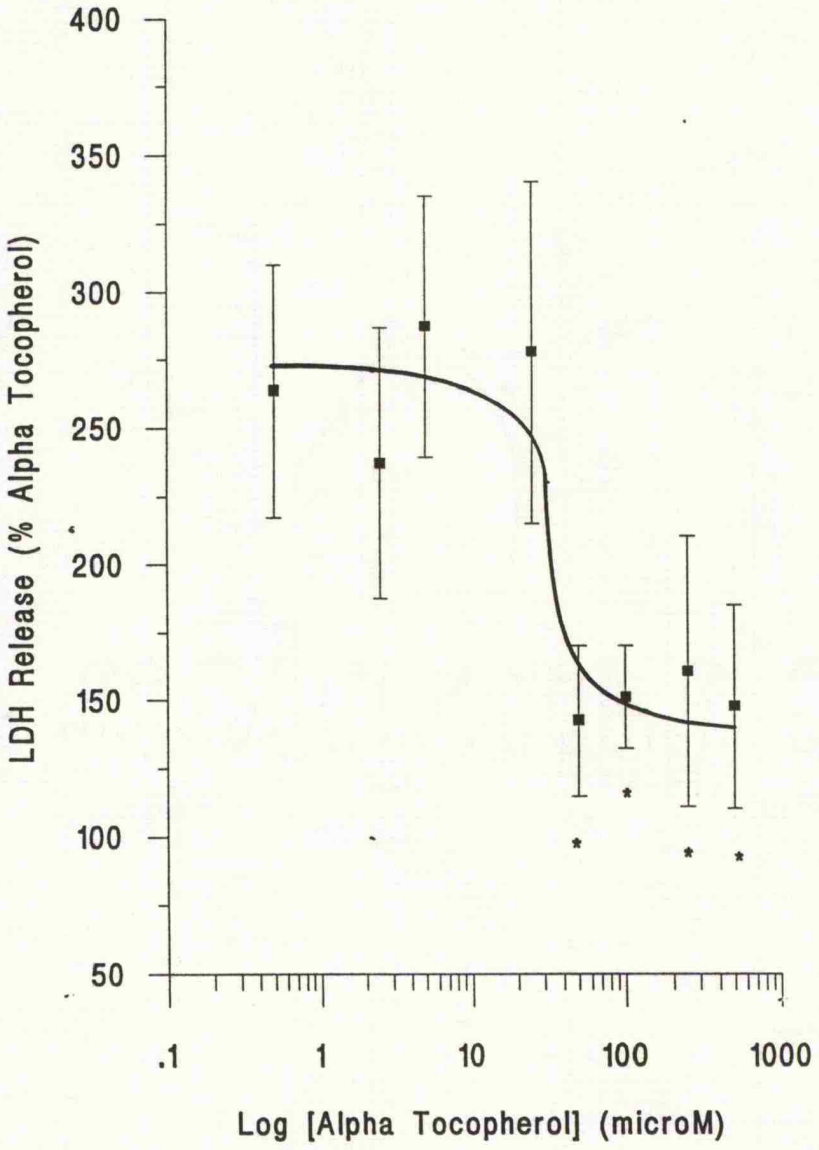

All data are expressed as mean ± SEM of the percentage of basal (no additions = 100), for n = 3 to 4 experiments. NMN and NIC were used at 10mM, rotenone at 10μM and DMSO, the vehicle for rotenone at 0.1 % v/v. SH-SY5Y cells were differentiated for seven days by the addition of retinoic acid ( 10μM). * Signifies $P<0.05$ when compared to basal.

Figure 4.4. The effect of concurrent incubation of α-tocopherol (100μM, α-toc) and NMN (10mM) over 72 hours.



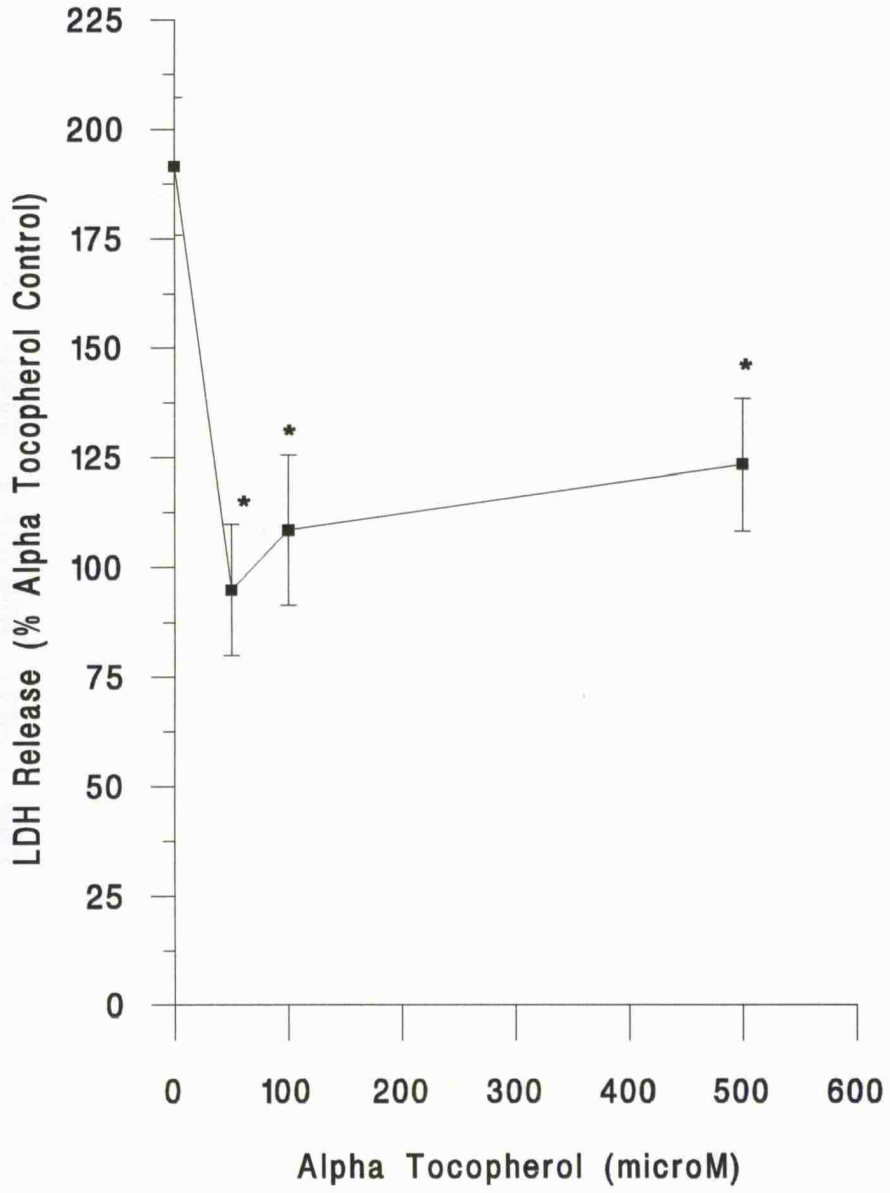

All data are expressed as mean ± SEM of the percentage of the control (i.e. cells incubated with 100μM α-tocopherol), for n = 5 to 8 experiments. * Signifies $P<0.05$ when compared to NMN (10mM) in the absence of α-tocopherol.

Figure 4.5. Dose related inhibition of LDH release from SH-SY5Y cells by α-tocopherol concurrently incubated with NMN (10mM).




All data are expressed as mean ± SEM of the percentage of the control, i.e. cells incubated with the highest dose of α-tocopherol (500μM), for n = 4 experiments. * Signifies $P<0.05$ when compared to NMN (10mM) in the absence of α-tocopherol.

Figure 4.6. Inhibition of LDH release from SH-SY5Y cells, following the preincubation of α-tocopherol for 24 hours, prior to NMN insult for a further 24 hours.




All data are expressed as the mean ± SEM of the percentage of the control, which contained the highest concentration of α-tocopherol (500μM), for n = 9 experiments.

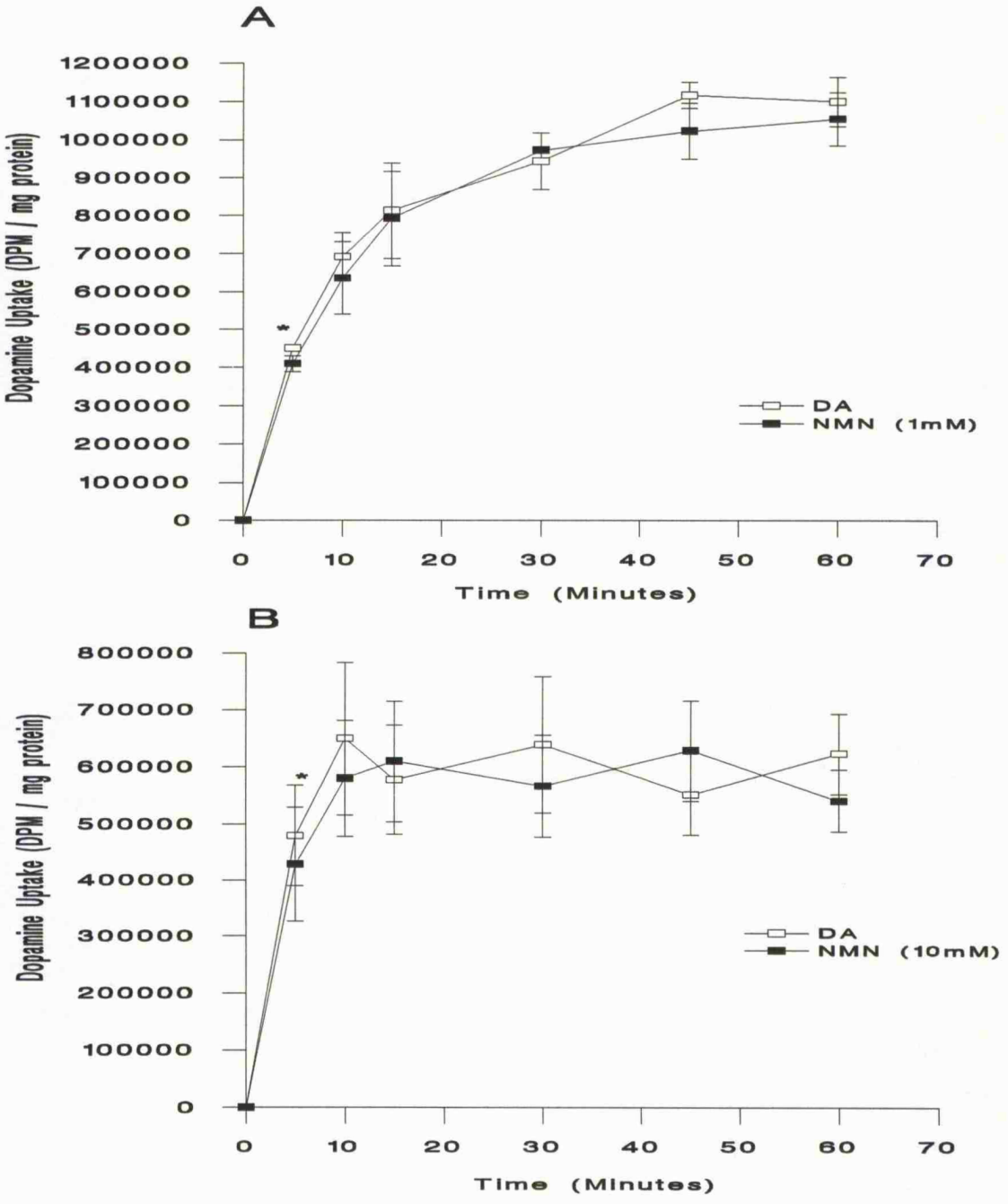
* Signifies $P<0.05$ when compared to NMN (10mM) in the absence of α-tocopherol.

Table 4.2. The effects of nicotinamide and NMN on dopamine and noradrenaline uptake and noradrenaline release from SH-SY5Y human neuroblastoma cells.

| | Nicotinamide | NMN |
|---|---|---|
| A. [$^3$H]Dopamine uptake (n = 5) | | |
| 0 | 100 | 100 |
| 1mM | 116.3 ± 6.1 | 124.7 ± 8.1 |
| 10mM | 114.4 ± 5.6 | 86.60 ± 3.5* |
| B. [$^3$H]Noradrenaline uptake (n = 5) | | |
| 0 | 100 | 100 |
| 1mM | 117.6 ± 6.5 | 109.8 ± 9.8 |
| 10mM | 100.5 ± 7.1 | 80.50 ± 6.9* |
| C. [$^3$H]Noradrenaline release (n = 3-4) | | |
| Basal | 100 | 100 |
| $K^+$, 100mM | 234.4 ± 13.2 | 234.4 ± 13.2 |
| + 1mM | 257.0 ± 15.5 | 230.2 ± 7.95 |
| + 10mM | 272.0 ± 8.5 | 216.7 ± 13.8 |

All data (% basal = 100) are expressed as mean ± SEM. * Signifies $P<0.05$ compared with nicotinamide.

Figure 4.7. The effect of NMN 1mM (A) and 10mM (B) on [$^3$H]dopamine (DA) uptake in rat cortical synaptosomes over one hour.




All data are expressed as the mean ± SEM measured in disintergrations per minute (DPM) mg protein$^{-1}$, for n = 3 experiments. * Signifies $P<0.05$ DA uptake after 5 minutes.

**4.4. Discussion.**

These data suggest that NMN and nicotinamide have different effects upon both B65 and SH-SY5Y cells, and NMN may be neurotoxic. However, the high concentrations of NMN (10mM) required to produce significant levels of LDH release and reduced [$^3$H]thymidine incorporation may indicate a non-specific membrane effect. Indeed the inhibition of NMN induced LDH release from SH-SY5Y cells by α-tocopherol, would support a membrane associated or extra-cellular site of action. In contrast, NMN induced LDH release from B65 cells was unaffected by α-tocopherol, implying that NMN may be acting at other site(s) in this cell line. α-Tocopherol is known to stabilise membranes by preventing autocatalytic free radical chain reactions which could disrupt them (Gutteridge 1978). The NMN induced LDH release was reversed by either concurrent incubation or pre-incubation of α-tocopherol, indicating that NMN may increase membrane fluidity. However, both NMN and nicotinamide (1 and 10mM) caused no significant increase membrane fluidity in erythrocyte ghosts (less than 1% increase in anisotropy, B.L. Appadu, 1994, personal communication). In SH-SY5Y cells, nicotinamide (10mM) had no effect upon LDH release or [$^3$H]thymidine incorporation, whilst NMN increased LDH release and reduced [$^3$H]thymidine incorporation. These actions cannot therefore be attributed to simple alterations in the osmotic balance of the buffer. In addition, NMN caused similar LDH release from differentiated SH-SY5Y cells at 10mM but not 1mM, indicating that both differentiated and undifferentiated cells are equally affected by NMN. Undifferentiated cells appear to be more susceptible to NMN than differentiated cells. This is because the absolute amount of LDH is related to the number of cells in each

experiment. Therefore, as differentiation reduces the total number of cells, equivalent levels of LDH release are not possible.

Whilst nicotinamide derivatives are reported to interact with catecholaminergic systems in vivo (Birkmayer and Birkmayer 1989), NMN has little effect upon catecholamine uptake in SH-SY5Y cells over an hour incubation. We have shown that B65 cells have negligible catecholamine uptake (Chapter 3), due to the apparent absence of $uptake_1$. Therefore, if uptake is required to facilitate toxicity, NMN should have little effect upon B65 cells. However, this was not observed. Furthermore, the failure of α-tocopherol to reverse the effects of NMN indicate that NMN may be acting at sites other than the cell membrane.

NMM appears to be cytotoxic to both SH-SY5Y and B65 neuroblastoma cells, albeit at relatively high concentrations (10mM). However, the exact mechanism of NMN induced LDH release and decreased [$^3$H]thymidine incorporation in both cell lines is unclear.

Increased turnover of catecholamines initiated by compounds that elicit catecholamine release, such as $MPP^+$, may contribute to their cytotoxicity. This is thought to occur via free radical formation as a consequence of increased catecholamine metabolism (Chiueh et al 1993). Therefore, in an attempt to examine the potential mechanism of toxicity of NMN the effects of NMN and nicotinamide on catecholamine uptake and release in SH-SY5Y cells were investigated.

SH-SY5Y cells take up both NA and DA, however, the latter catecholamine is converted to NA prior to release indicating the presence and activity of $uptake_1$ and dopamine-β-hydroxylase in these cells. In this study nicotinamide derivatives appear to have little effect upon catecholamine uptake or release in these cells. However,

after incubation with NMN (10mM), a small reduction in DA and NA uptake was observed. Furthermore, the small reduction in uptake seen with NMN at high doses (10mM) is not observed with nicotinamide, implying that the inhibition is not merely due to changes in the ionic composition of the buffer. If NMN was taken up via $uptake_1$, a dose related reduction of catecholamine uptake would be anticipated. This is not supported by these data. Therefore, if NMN is gaining access to the cell it does not appear to be through $uptake_1$. Since NMN is a charged compound it would clearly require a carrier system to allow intracellular accumulation. Thus one suspects that NMN has mainly extracellular effects upon cells perhaps by inference with essential substrate availability. Therefore, as the concentration of NMN required to induce cytotoxicity is so large, its role as an endogenous neurotoxin is in doubt.

# Chapter 5

# Studies on the potential neurotoxicity of 6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol), and its effects upon catecholamine homeostasis in SH-SY5Y cells

## 5.1. Introduction.

The tetrahydroisoquinoline alkaloid salsolinol (6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline, is found naturally in trace amounts in the mammalian central nervous system (Sjoquist and Magnuson 1980, Collins 1982 and Sjoquist et al 1981). Salsolinol can be formed in vivo by two separate mechanisms. Firstly, through non-enzymic Pictet-Spengler condensation of dopamine and aldehydes, such as acetaldehyde. This reaction forms racemic isomers of salsolinol (R or S). Secondly, salsolinol may be formed via the condensation of dopamine and pyruvate to form 1-carboxyl tetrahydroisoquinoline. Further reduction of this compound followed by decarboxylation and reduction produces (R)-salsolinol (Dorset et al 1988). (R)-salsolinol is thought to be the predominant enantiomer in humans, and was found exclusively in the urine of healthy volunteers (Dostert et al 1987, 1990, Strolin Benedetti et al 1989). Additionally elevated levels of salsolinol have been detected in the urine of L-dopa-treated patients (Sandler et al 1973). Moreover, (R)-salsolinol has been detected in both human (Origitano et al 1981 and Sjoquist et al 1982) and animal (Sjoquist and Magnusson 1980) brains. (S)-Salsolinol is the predominant enantiomer in many beverages and food stuffs, such as beer, port, soy sauce and bananas (Riggin et al 1976, Duncan and Smythe 1982 and Duncan et al 1984).

Since salsolinol can be synthesised from the condensation of dopamine, the primary site for any biochemical effects of salsolinol could be catecholaminergic nerve terminals. In addition, N-methylation of salsolinol has recently been demonstrated by in vivo microdialysis of rat brain (Maruyama et al 1992), and N-methylated derivatives of salsolinol have been detected in rat brain (Niwa et al 1991). Therefore, salsolinol may undergo a similar bioactivation pathway as MPTP, to produce a compound with

similar chemical properties as $MPP^+$. Salsolinol has been linked, in chronic alcoholics, to the behavioural, physical dependence and addictive properties of alcohol (Cohen and Collins 1970). Indeed, chronic intracerebroventricular infusion of salsolinol in the rat brain, evoke an increase in the preference of alcohol ingestion in the test animal (Myers and Melchior 1977a, Myers and Melchior 1977b). Chronic alcoholism has even been linked to premature ageing of the brain (Ryan and Butters 1980).

Since salsolinol has been linked to both Parkinson's disease and damage to the brain from chronic alcoholism, much research has been undertaken to evaluate the potential effects of salsolinol upon neuronal cells. (R)-Salsolinol is reported to inhibit the growth of PC12h cells, and inhibit the activities of both tyrosine hydroxylase (TH) (Maruyama et al 1993b and Minami et al 1992), and monoamine oxidase (Bembenek et al 1983). However, PC12 cells are known to rely far more upon glycolysis that the tricarboxylic acid cycle for their energy requirements (Warburg 1967, Racker and Spector 1981 and Morelli et al 1986), and since TIQ derivatives are purported to be putative mitochondrial toxins (Suzuki et al 1989), PC12 cells would appear to be a poor cell model with which to examine such potential toxins. In addition, salsolinol is reported to inhibit the uptake of catecholamines in rat brain synaptosomes (Heikkila et al 1971), and to elicit the release of catecholamines from rat brain (1mM salsolinol) during microdialysis studies (Maruyama et al 1993a).

Therefore, in this chapter the potential cytotoxicity of salsolinol has been examined in the human SH-SY5Y neuroblastoma cell line (Ross and Biedler 1985). The effects of salsolinol upon catecholamine homeostasis was also examined, as a possible mechanism for its cytotoxicity in an isolated cellular system.

## 5.2. Materials and Methods.

*Matherials.*

6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol), was obtained from the Sigma Chemical Co., Poole Dorset, U.K., as a racemate, consisting of a 50:50 mixture of R and S enantiomers. The Roche compounds Ro 20-9747 (Trolox C) and Ro 31-8220 were gifts from Roche Products Limited, Welwyn Garden City, Hertfordshire, U.K. All other compounds were supplied as stated in the general materials and methods section, unless otherwise stated.

*Cell Culture.*

Undifferentiated human SH-SY5Y neuroblastoma cells were cultured by the method stated in materials and methods, chapter 2, page 67. For experimental purposes cells were harvested, and seeded in 12 (for LDH and catecholamine studies), and 24 (for MTT studies) well multitrays.

*Toxicological Incubation Procotols.*

In the majority of the following protocols toxicity was assessed by MTT reduction. The exceptions are the initial assessment of the effect of salsolinol on LDH release and the inclusion of $\alpha$-tocopherol in an attempt to prevent salsolinol induced LDH release (see LDH release section below for full method). In all experiments salsolinol was dissolved in Krebs / HEPES, pH 7.4, containing ascorbate (0.2mM) in order to prevent oxidation prior to a 1:10 dilution into the incubation media (supplemented MEM). The resulting ascorbate (20$\mu$M) had no effect upon the degree of salsolinol toxicity. In an attempt to prevent or determine the mechanism of toxicity of salsolinol antioxidants

(α-tocopherol and Trolox C), monoamine oxidase inhibitors (clorgyline and deprenyl) or imipramine, were incubated with further cell cultures. In these experiments examining the mechanism of salsolinol toxicity, relatively high concentrations (30 and 100μM) and longer incubation periods (48 hours) were chosen to provide a large window in which to observe any perturbation or increase in toxicity. Imipramine (1 and 10μM) was added for a 30 minute pre-incubation, followed by concurrent addition with salsolinol (30 or 100μM) for 48 hours. The monoamine oxidase inhibitors deprenyl (RBI, Natick, U.S.A., type B inhibitor) and clorgyline (Sigma, Poole, Dorset, U.K., type A inhibitor) either alone or in combination, were pre-incubated for 30 minutes prior to concurrent addition of salsolinol (30 or 100μM). The antioxidant, α-tocopherol (2.5 to 500μM) was added to SH-SY5Y cells for 24 hours, prior to addition of salsolinol (30 or 100μM) for 48 hours. In addition a synthetic, water soluble α-tocopherol analogue, Trolox-C (10 to 500μM) was concurrently incubated with salsolinol (30 or 100μM) for 48 hours.

*LDH Release.*

Confluent SH-SY5Y cells were exposed to various concentrations of salsolinol (1 to 1mM) for up to 72 hours. LDH levels were assessed in both the cell media and the cell lysates as described previously in chapter 2, page 71. Where α-tocopherol was used various concentrations (2.5 to 500μM) were pre incubated with SH-SY5Y cells for 24 hours prior to salsolinol incubation for a further 24 hours.

*MTT Reduction.*

Salsolinol, at various doses (1μM to 1mM), was incubated with SH-SY5Y cells three days after seeding, and incubated for up to 72 hours. After 24, 48 and 72 hours MTT (0.12mM) was added to all cells and left for one hour at 37°C. The resulting dye was extracted and measured spectrophotometrically as outlined in materials and methods, chapter 2, page 75. Additional incubation protocols were used, as above, and toxicity was assessed after 48 hours.

*Catecholamine Uptake.*

Catecholamine uptake was undertaken by the method of Atcheson et al (1993). With the exception of controls (to determine basal uptake), salsolinol was included during uptake at various concentrations (1μM to 1mM). To investigate the mechanism of action of salsolinol upon catecholamine uptake in SH-SY5Y cells, the effects of sodium nitroprusside (100μM) a guanylate cyclase (GC) activator (Murad et al 1978, Bohme et al 1978), Roche compound 31-8220 (1μM) a protein kinase C (PKC) inhibitor (Davies et al 1989), and forskolin (10μM) an adenylate cyclase (AC) activator (Seamon et al 1981, Metzger and Lindner 1981) were incubated for 15 minutes, at 37°C, prior to further incubation in the presence or absence of salsolinol (20 or 400μM) during uptake.

*[$^3$H]Nisoxetine Displacement Binding Studies.*

Displacement of [$^3$H]nisoxetine binding was undertaken by the method described previously, chapter 2, page 78. Salsolinol was included at increasing concentrations between $10^{-10}$ to $10^{-3}$M.

*Catecholamine Release.*

Catecholamine release was undertaken by the method of Atcheson et al (1994a). When analysing the effects of salsolinol upon catecholamine release, salsolinol was included at various concentrations (1μM to 1mM) in Krebs / HEPES buffer during the last of the three 15 minutes washes applied after catecholamine loading, prior to either potassium (100mM) or CCH (1mM) evoked [$^3$H]NA release. Direct stimulation of loaded [$^3$H]NA release was attempted by the addition of salsolinol (1μM to 1mM) for three minutes after the three, 15 minute washing steps.

*Muscarinic Displacement Binding Studies.*

[$^3$H]NMS displacement binding studies were undertaken with the method described in chapter 2, page 84. Salsolinol was included in increasing concentrations between $10^{-8}$ to $10^{-3}$M.

**5.3 Results.**

Salsolinol caused a time and dose related inhibition of MTT reduction in SH-SY5Y cells (figure 5.1, and table 5.1), with significant ($P<0.05$) inhibition of dye production (compared to untreated control) at 3μM salsolinol at 72 hours (89.3 ± 2.97 % of untreated control, mean ± SEM) (figure 5.1). $IC_{50}$ values (MTT reduction) at 24, 48, and 72 hours were 308 ± 25μM, 92 ± 11μM, and 54 ± 12μM, respectively. After 72 hours salsolinol induced toxicity to SH-SY5Y cells, with a similar $IC_{50}$ (54 ± 12μM) to that reported by Melzig and Zipper in aortic endothelial cells ($IC_{50}$ = 38μM). Cytotoxicity was unaffected by the small concentration of ascorbate (20μM) included to prevent autoxidation of salsolinol prior to incubation with cells (figure 5.1). There was no significant difference between the degree of salsolinol toxicity in the presence or absence of ascorbate (20μM, table 5.1). Salsolinol also induced a dose and time related increase in LDH release from SH-SY5Y cells, with significantly ($P<0.05$) increased LDH release after 24 hours with 500μM (186 ± 15.7 %), and with 100μM after 48 hours (168.1 ± 3.7 %) (figure 5.2).

The inclusion of the uptake$_1$ inhibitor, imipramine (0.1, 1 and 10μM) for 30 minutes prior to incubation in the presence or absence of salsolinol (30 or 100μM) did not prevent the related decrease in MTT reduction after 48 hours (table 5.2). Pre-incubation with the MAO-A inhibitor clorgyline (0.1, 1 and 10μM), for 30 minutes prior to concurrent incubation in the presence of salsolinol (30 or 100μM) did not affect salsolinol inhibition of MTT reduction (table 5.3). This pattern was repeated with deprenyl (0.1, 1 and 10μM, MAO-B inhibitor) or a combination of clorgyline and deprenyl (10μM, table 5.4 and 5.5). Furthermore, pre-incubation of SH-SY5Y cells with the antioxidant, α-tocopherol (2.5 to 500μM) for 24 hours prior to incubation of

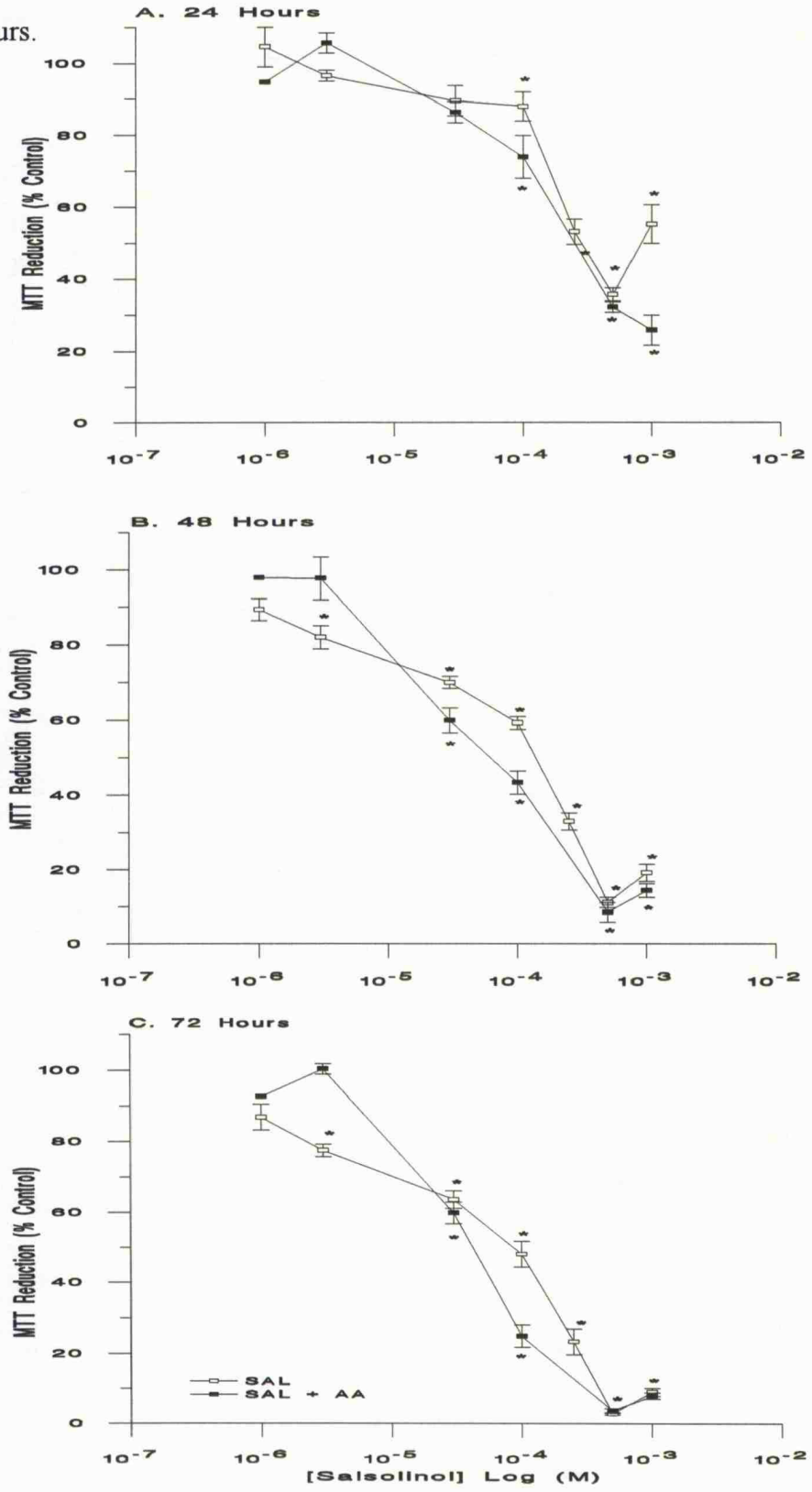
salsolinol (30 or 100μM), did not prevent either salsolinol induced inhibition of MTT reduction (after 48 hours), or the increased LDH release (after 24 hours) (tables 5.6 and 5.7). The water soluble α-tocopherol analogue, Trolox C (10 to 500μM) when concurrently incubated with salsolinol (30 or 100μM) failed to prevent salsolinol induced inhibition of MTT reduction (table 5.8).

Salsolinol stimulated catecholamine uptake, at concentrations below 100μM (figure 5.3), with $EC_{50}$ values of 17 ± 3.3μM and 11 ± 3.8μM, for [$^3$H]NA and [$^3$H]DA, respectively. This was followed by a decrease in catecholamine uptake towards basal levels above 250μM (figure 5.3), with $IC_{50}$ values of 411 ± 25.7 and 379 ± 13.4μM, for [$^3$H]NA and [$^3$H]DA, respectively. Salsolinol (1mM) inhibited [$^3$H]nisoxetine binding by 71.9 ± 2.3 % to SH-SY5Y cells, with $IC_{50}$ of 818 ± 67.7μM, and $K_i$ of 353 ± 29.8μM (figure 5.4), but had no effect at lower concentrations. Pre-incubation with the PKC inhibitor Ro 31-8220, for 15 minutes prior to incubation in the presence or absence of salsolinol (20μM, ~$EC_{50}$ or 400μM, ~$IC_{50}$) did not affect the level of [$^3$H]NA uptake in SH-SY5Y cells (table 5.9). Additionally, pre-incubation of either forskolin (10μM, table 5.10) or sodium nitroprusside (100μM, table 5.11), for 15 minutes at 37°C, prior to incubation of either forskolin (10μM) or sodium nitroprusside (100μM) in the presence or absence of salsolinol (20 or 400μM) did not effect salsolinol stimulated [$^3$H]NA uptake in SH-SY5Y cells.

Salsolinol (250μM) also significantly ($P<0.05$) inhibited both potassium (100mM), and CCH (1mM) evoked [$^3$H]NA release from SH-SY5Y cells (in monolayer culture), at concentrations of 250μM to 1mM, and 100μM to 1mM, respectively (figures 5.5 and 5.6). Lower doses of salsolinol did not inhibit either CCH or potassium evoked [$^3$H]NA release. When salsolinol was directly incubated with SH-SY5Y, preloaded

with [$^3$H]NA, for 3 minutes, there was no significant difference of [$^3$H]NA release compared to basal (table 5.12). Salsolinol displaced [$^3$H]NMS binding to whole SH-SY5Y cells, with 69.9 ± 3.2 % displacement at 1mM, $IC_{50}$ of 398 ± 17.8 and $K_i$ of 215 ± 9.8μM, respectively (figure 5.7).

Figure 5.1. The effect of salsolinol upon MTT reduction in SH-SY5Y cells over 72 hours.




For legend see next page.

Legend to figure 5.1.

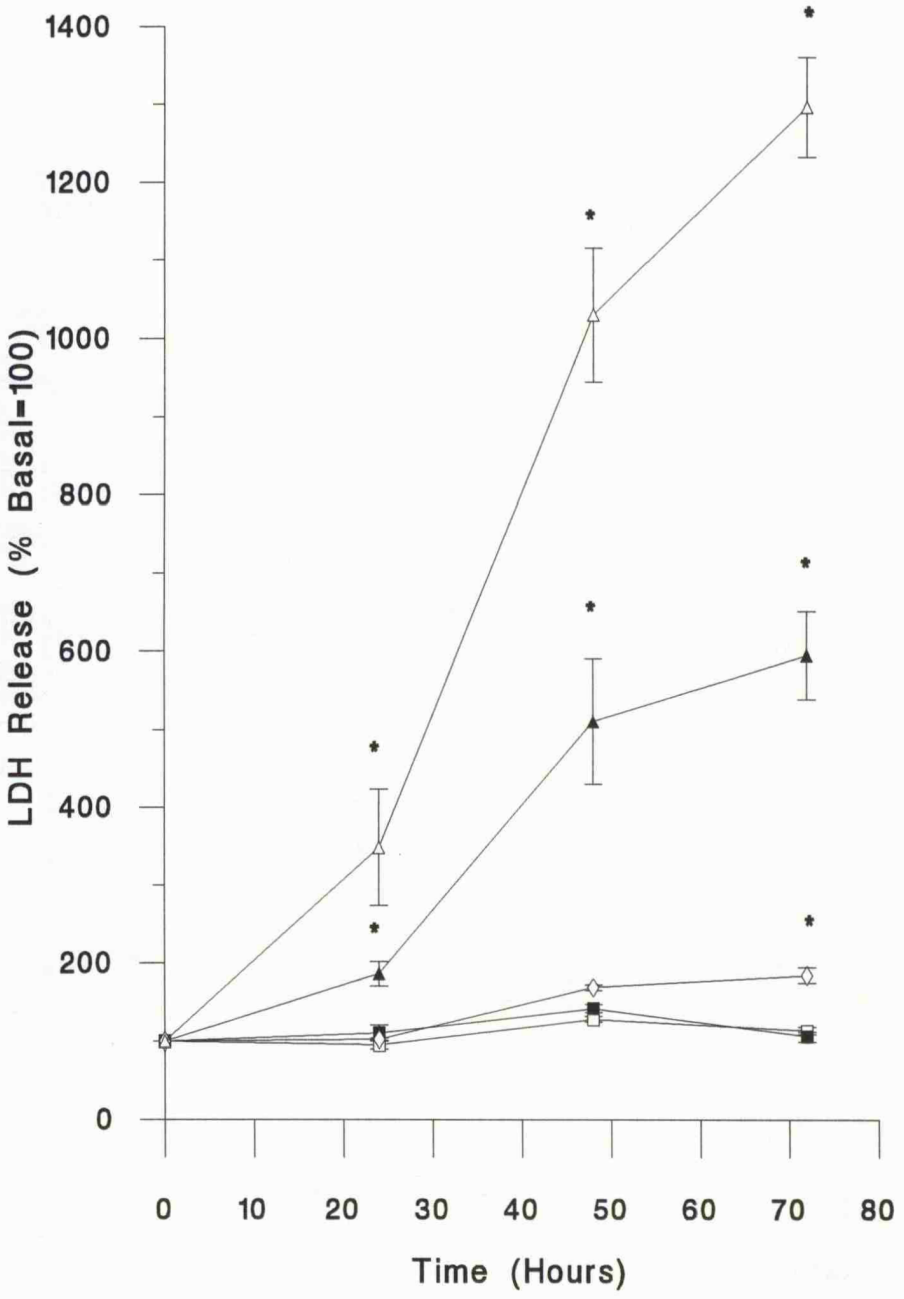
MTT reduction is expressed mean ± SEM of the percentage of the relevant untreated control (= 100), for n = 3 and 5 experiments, for salsolinol or salsolinol + ascorbic acid (AA) (20μM). Toxicity was measured in the presence or absence of AA (20μM), which was used to prevent salsolinol autoxidation prior to incubation with SH-SY5Y cells. * Signifies $P<0.05$ when compared to control.

Table 5.1. Comparison of the toxicity of salsolinol in the presence or absence of ascorbic acid (AA) prior to incubation with SH-SY5Y cells.

| | Krebs / HEPES buffer | |
|---|---|---|
| Time (Hours) | No AA (n = 5) | AA (20μM) (n = 3) |
| 24 | 308 ± 25.0 | 251 ± 25.9 |
| 48 | 94.2 ± 12.3 | 68.7 ± 6.27 |
| 72 | 54.4 ± 12.0 | 43.8 ± 4.96 |

The degree of toxicity was assessed by MTT reduction. The data is expressed as the mean $IC_{50}$ value ± SEM, in the presence or absence of ascorbic acid. The final concentration of ascorbic acid was 20μM. Salsolinol was included at various concentrations between 1μM to 1mM.

Figure 5.2. The effect of salsolinol upon LDH release from SH-SY5Y cells over 72 hours.




All data are expressed as the mean ± SEM, of the percentage basal (no additions), for n = 4 experiments. * Signifies $P<0.05$ when compared to basal LDH release.

Table 5.2. The effect of imipramine pre-incubated for 30 minutes, followed by concurrent incubation with salsolinol for 48 hours in SH-SY5Y cells. At 48 hours toxicity was assessed by MTT reduction.

| | Salsolinol (μM) | | |
|---|---|---|---|
| Imipramine (μM) | 0 | 30 | 100 |
| 0 | 100 | 73.3 ± 5.25* | 57.5 ± 6.25* |
| 1 | 105.6 ± 4.99 | 76.9 ± 5.38* | 63.2 ± 6.39* |
| 10 | 109.6 ± 10.22 | 72.9 ± 3.25* | 55.0 ± 5.71* |

The data are expressed as a percentage of the relevant untreated control, and are represented as the mean ± SEM for n = 4 experiments. * Signifies $P<0.05$ when compared to control. Impiramine did not affect salsolinol inhibition of MTT reduction.

Table 5.3. The effects of clorgyline pre-incubation, for 30 minutes, followed by concurrent incubation of salsolinol and clorgyline for 48 hours in SH-SY5Y cells. After 48 hours toxicity was assessed by MTT reduction.

| | Salsolinol (μM) | | |
|---|---|---|---|
| Clorgyline (μM) | 0 | 30 | 100 |
| 0 | 100 | 70.8 ± 3.64* | 52.6 ± 4.56* |
| 0.1 | 95.3 ± 1.16 | 66.3 ± 1.26* | 47.0 ± 1.87* |
| 1.0 | 92.1 ± 2.11 | 63.6 ± 1.74* | 49.2 ± 1.81* |
| 10 | 88.2 ± 2.80 | 60.9 ± 2.98* | 46.5 ± 1.76* |

The data are expressed as a percentage of the untreated control, and are represented as the mean ± SEM for n = 4 experiments. * Signifies $P<0.05$ when compared to control. Clorgyline did not prevent salsolinol inhibition of MTT reduction.

Table 5.4. The effects of deprenyl pre-incubation, for 30 minutes, followed by concurrent incubation of salsolinol and deprenyl for 48 hours in SH-SY5Y cells. After 48 hours toxicity was assessed by MTT reduction.

| | Salsolinol (μM) | | |
|---|---|---|---|
| Deprenyl (μM) | 0 | 30 | 100 |
| 0 | 100 | 70.8 ± 3.64* | 50.6 ± 4.56* |
| 0.1 | 90.8 ± 3.03 | 65.3 ± 3.84* | 46.5 ± 2.18* |
| 1.0 | 92.6 ± 1.80 | 61.1 ± 1.63* | 46.5 ± 1.73* |
| 10 | 93.5 ± 4.11 | 60.9 ± 2.14* | 45.3 ± 3.52* |

The data are expressed as a percentage of the relevant untreated control, and are represented as the mean ± SEM for n = 4 experiments. * Signifies $P<0.05$ when compared to control. Deprenyl did not prevent salsolinol inhibition of MTT reduction.

Table 5.5. The effects of concurrent pre-incubation of clorgyline and deprenyl (10μM) for 30 minutes followed by concurrent incubation with salsolinol in SH-SY5Y cells.

| Clorgyline + Deprenyl | Salsolinol 30μM | 100μM |
|---|---|---|
| 0 | 72.3 ± 1.91* | 51.1 ± 1.93* |
| C 10μM + D 10μM | 70.3 ± 3.57* | 55.5 ± 3.38* |

The data are expressed as a percentage of the relevant untreated control, and are represented as the mean ± SEM for n = 4 experiments. The degree of toxicity was measured after 48 hours by MTT reduction. * Signifies $P<0.05$ when compared to control. The combination of clorgyline and deprenyl did not prevent salsolinol inhibition of MTT reduction. In the absence of salsolinol clorgyline and deprenyl (10μM) did not significantly effect MTT reduction (99.5 ± 4.36 % c.f. control).

Table 5.6. The effects of pre-incubation of α-tocopherol for 24 hours with SH-SY5Y cells prior to salsolinol incubation for a further 48 hours. After 48 hours toxicity was assessed by MTT reduction.

| | Salsolinol (μM) | |
|---|---|---|
| α-Tocopherol (μM) | 30 | 100 |
| 0 | 77.4 ± 2.46* | 65.3 ± 4.97* |
| 2.5 | 96.7 ± 4.20 | 77.5 ± 3.58* |
| 5 | 88.2 ± 5.21* | 70.4 ± 1.52* |
| 25 | 84.9 ± 6.06* | 65.6 ± 7.11* |
| 50 | 83.6 ± 4.68* | 69.9 ± 3.31* |
| 100 | 78.1 ± 1.50* | 62.5 ± 2.40* |
| 250 | 79.8 ± 2.04* | 75.8 ± 2.50* |
| 500 | 74.7 ± 1.42* | 60.7 ± 2.76* |

The data are expressed as a percentage of a control treated with 500μM α-tocopherol for 24 hours and then incubated in supplemented media without further additions for 48 hours. The data represent the mean ± SEM for n = 4 separate experiments. * Signifies $P<0.05$ when compared to control.

Table 5.7. The effects of α-tocopherol pre-incubation (24 hours) prior to salsolinol insult to SH-SY5Y cells. After 48 hours incubation with salsolinol toxicity was measured by LDH release.

| α-Tocopherol (μM) | Salsolinol (100μM) |
|---|---|
| 0 | 316.2 ± 19.4* |
| 2.5 | 324.3 ± 13.0* |
| 5 | 325.3 ± 19.1* |
| 25 | 318.5 ± 20.8* |
| 50 | 317.4 ± 21.1* |
| 100 | 328.3 ± 15.7* |
| 250 | 318.3 ± 13.1* |
| 500 | 307.0 ± 4.5* |

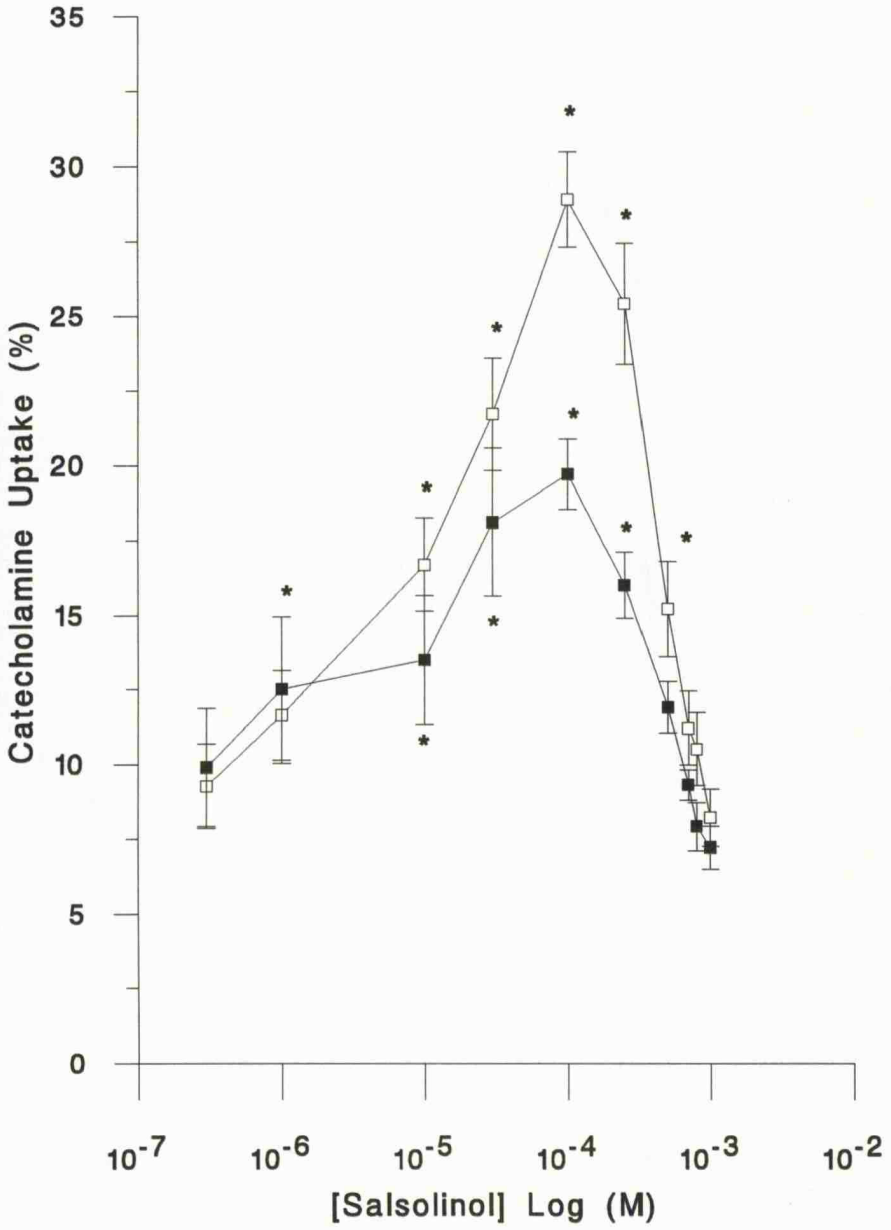
The data is expressed as the percentage of the control (cells treated with the highest dose of α-tocopherol, 500μM, for 24 hours followed by no further treatment), and is represented by the mean ± SEM for n = 3 separate experiments. * Signifies $P<0.05$ when compared to control (500μM α-tocopherol). α-Tocopherol had no effect upon salsolinol induced LDH release.

Table 5.8. The effects of concurrent incubation of Trolox C and salsolinol (100μM) with SH-SY5Y cells. After 48 hours incubation with salsolinol toxicity was measured by MTT reduction.

| Trolox C (μM) + Salsolinol (100μM) | MTT Reduction (% Basal) |
|---|---|
| 0 | 72.40 ± 4.18* |
| 10 | 69.40 ± 3.38* |
| 30 | 73.60 ± 2.69* |
| 100 | 75.50 ± 4.05* |
| 250 | 72.00 ± 10.10* |
| 300 | 71.10 ± 5.55* |
| 500 | 74.50 ± 9.08* |
| 500 (no SAL) | 103.7 ± 7.62 |

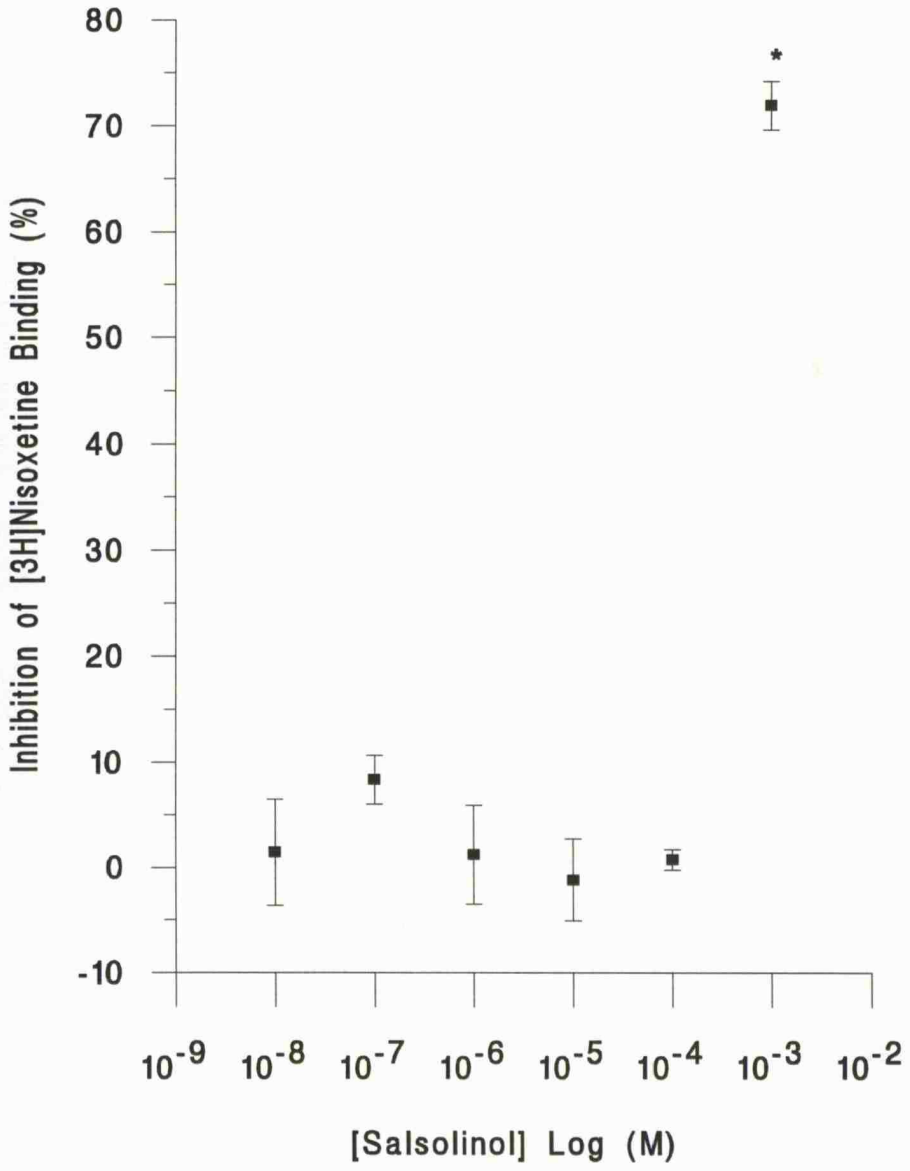
The data is expressed as the percentage of the control (untreated cells), and is represented by the mean ± SEM for n = 4 separate experiments. * Signifies $P<0.05$ when compared to control. Trolox C did not prevent salsolinol inhibition of MTT reduction.

Figure 5.3. The effect of salsolinol upon catecholamine uptake in SH-SY5Y cells, in monolayer culture, over one hour.




Catecholamine uptake is expressed as a percentage of the total available activity, and represented as the mean ± SEM, for n = 4 and 6 experiments for DA and NA, respectively. * Signifies $P<0.05$ when compared to basal uptake.

Figure 5.4. The effect of salsolinol upon displacement of [$^3$H]nisoxetine binding to SH-SY5Y cells.




Displacement of [$^3$H]nisoxetine binding is expressed as a percentage of the total [$^3$H]nisoxetine binding (i.e. no salsolinol), after subtraction of non-specific binding. All data is expressed as mean inhibition of [$^3$H]nisoxetine binding ± SEM, for n = 4 experiments. * Signifies $P<0.05$ when compared to maximum specific binding.

Table 5.9. The effect of Roche 31-8220 upon salsolinol (SAL) stimulated [$^3$H]noradrenaline uptake in SH-SY5Y cells.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 5.03 ± 1.25 |
| Ro 31-8220 (1μM) | 5.55 ± 1.24 |
| 20μM SAL + Ro 31-8220 (1μM) | 11.9 ± 0.73* |
| 20μM SAL | 14.9 ± 2.85* |
| 400μM SAL + Ro 31-8220 (1μM) | 9.89 ± 0.33* |
| 400μM SAL | 10.9 ± 0.33* |

The data is expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 6 experiments. Ro 31-8220 is a PKC inhibitor (Davis et al 1989). * Signifies $P<0.05$ when compared to basal uptake. Addition of Ro 31-8220 had no significant effect upon salsolinol stimulated NA uptake.

Table 5.10. The effect of forskolin (adenylate cyclase activator) upon salsolinol stimulated [$^3$H]noradrenaline uptake in SH-SY5Y cells.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 5.21 ± 1.43 |
| Forskolin (10μM) | 6.58 ± 1.39 |
| 20μM SAL + Forskolin (10μM) | 14.3 ± 3.11* |
| 20μM SAL | 13.7 ± 3.18* |
| 400μM SAL + Forskolin (10μM) | 11.3 ± 0.60* |
| 400μM SAL | 11.6 ± 0.62* |

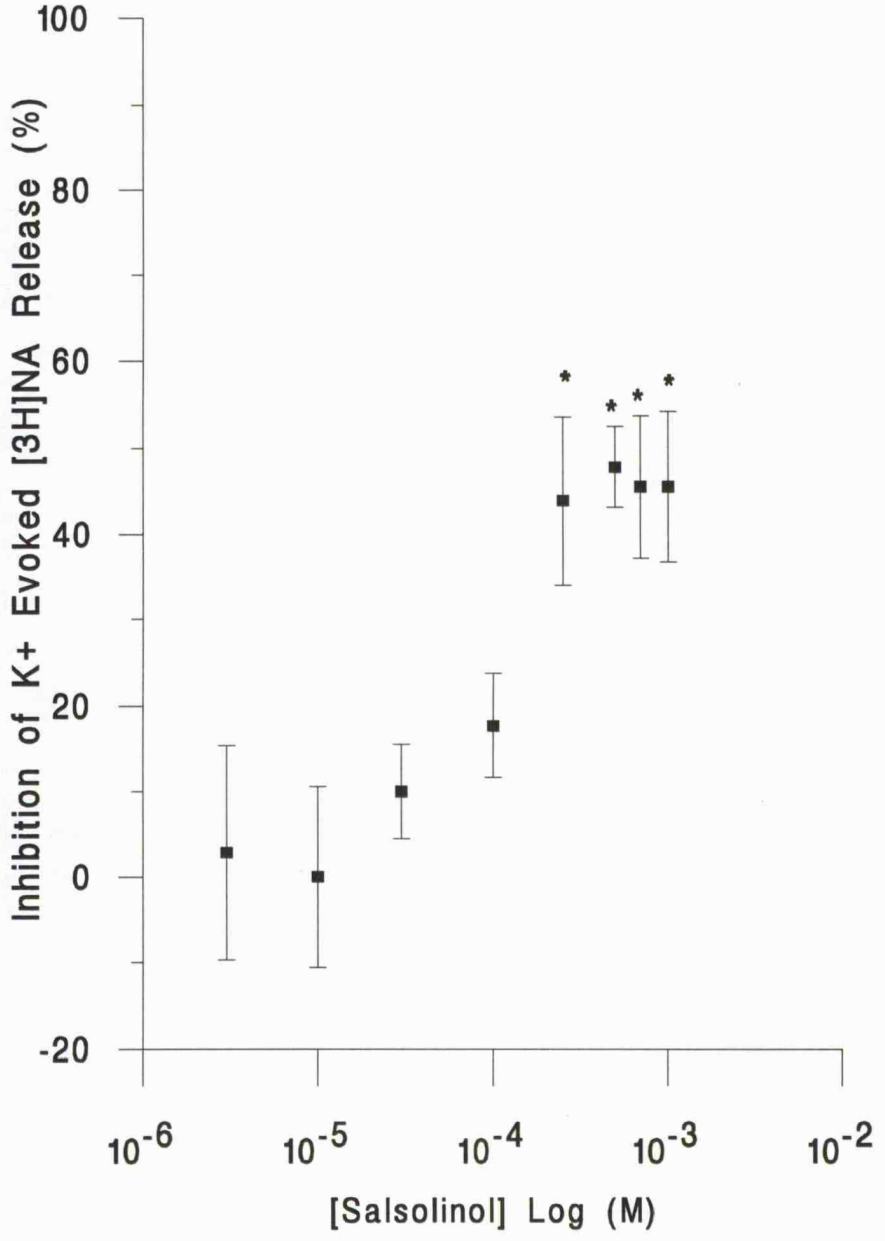
The data is expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 4 experiments. * Signifies $P<0.05$ when compared to basal uptake. The addition of forskolin had no significant effect upon basal or salsolinol stimulated NA uptake.

Table 5.11. The effect of sodium nitroprusside upon salsolinol (SAL) stimulated [$^3$H]noradrenaline uptake.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 12.5 ± 2.04 |
| Nitroprusside (100μM) | 15.4 ± 2.70 |
| 20μM SAL + Nitroprusside (100μM) | 22.9 ± 1.88* |
| 20μM SAL | 23.9 ± 0.99* |
| 400μM SAL + Nitroprusside (100μM) | 15.5 ± 1.00 |
| 400μM SAL | 15.3 ± 1.35 |

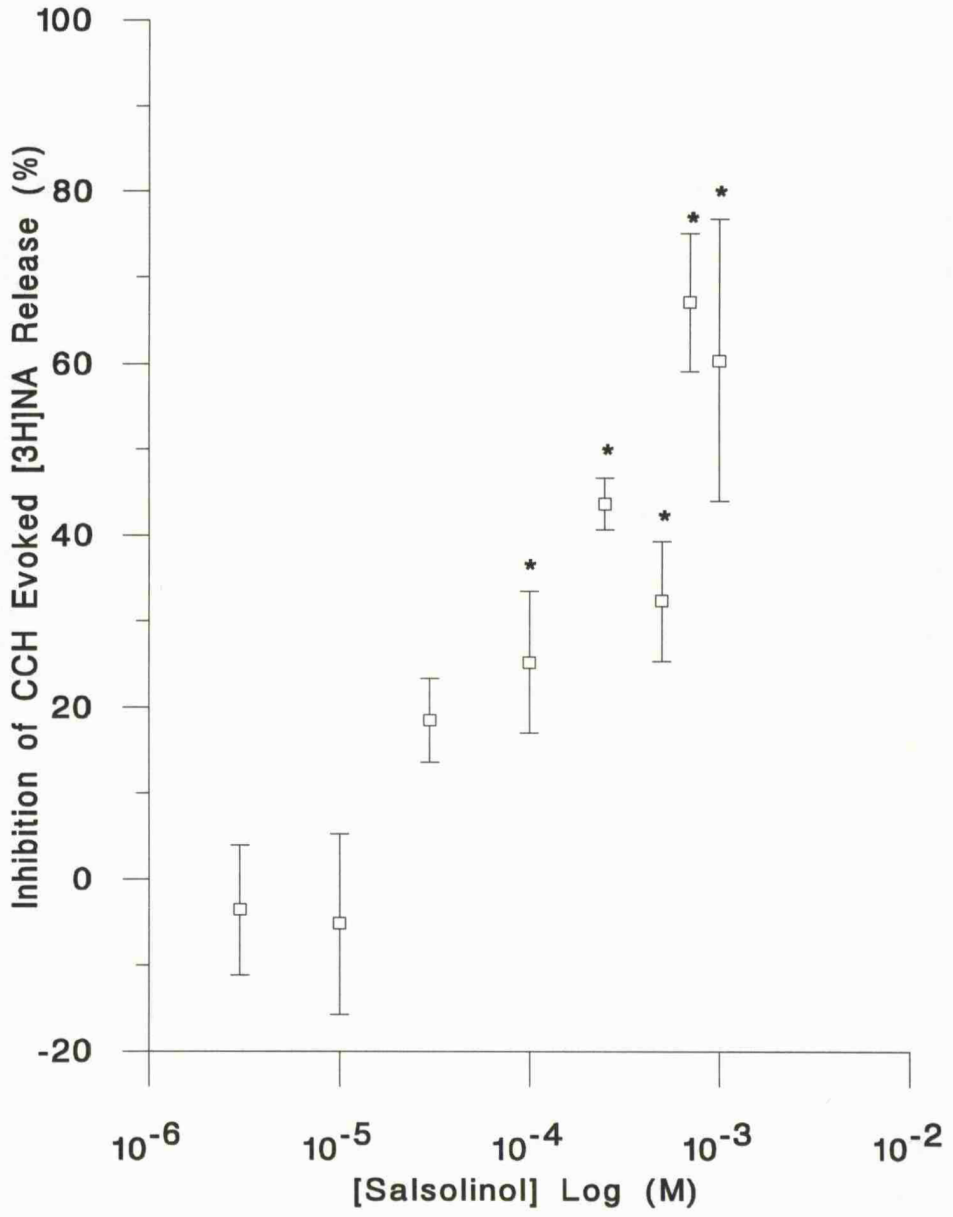
The data is expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 4. Sodium nitroprusside is a guanylate cyclase activator (Murad et al 1978, Bohme et al 1978). * Signifies $P<0.05$ when compared to basal uptake.

Figure 5.5. The effect of salsolinol upon potassium ($K^+$ 100mM, $Na^+$ adjusted) evoked [$^3$H]noradrenaline (NA) release from SH-SY5Y cells.



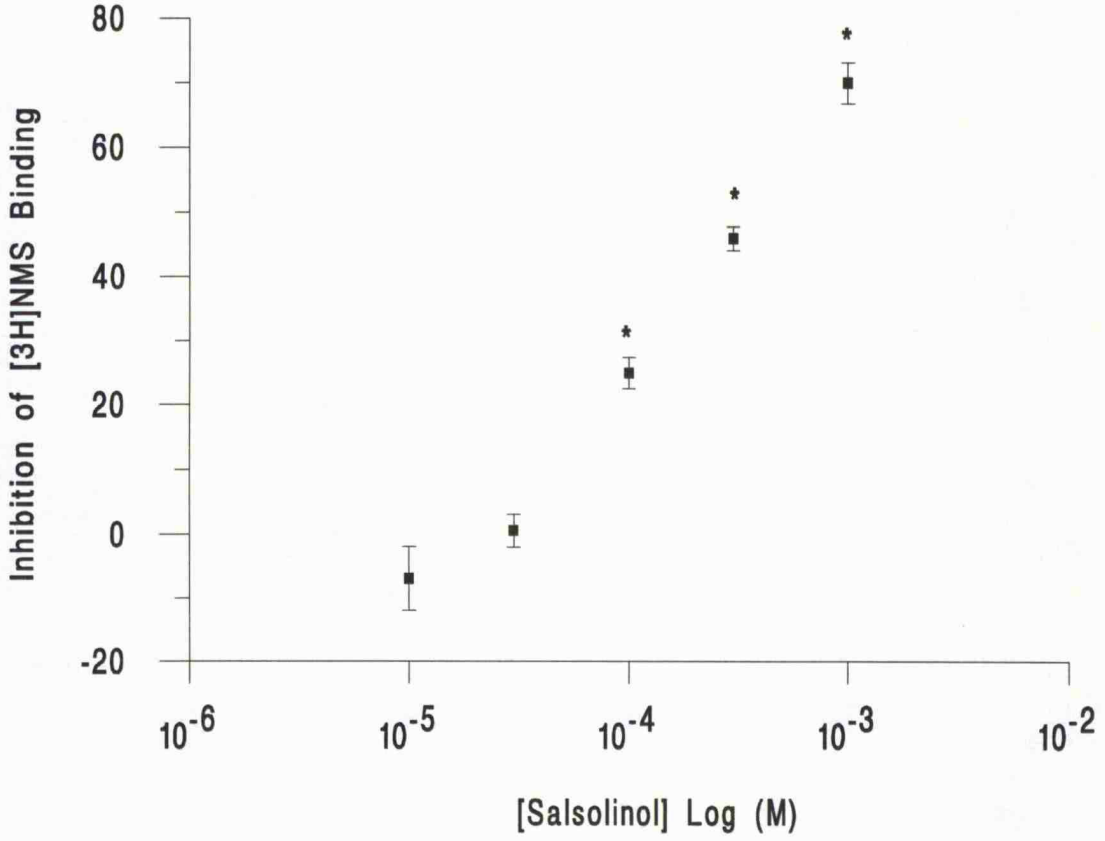

Inhibition NA release is expressed as a percentage of the maximum release evoked by $K^+$ (100mM), after subtraction of basal (background) release. All data represents the mean ± SEM, for n = 5 experiments. * Signifies $P<0.05$ when compared to maximum stimulated release.

Figure 5.6. The effect of salsolinol upon carbachol (CCH 1mM) evoked [$^3$H]noradrenaline release from SH-SY5Y cells.




Inhibition NA release is expressed as a percentage of the maximum release evoked by CCH (1mM), after subtraction of basal (background) release. All data represents the mean ± SEM, for n = 6 experiments. . * Signifies $P<0.05$ when compared to maximum stimulated release.

Figure 5.7. The effect of salsolinol upon [$^3$H]N-methyl scopolamine binding to SH-SY5Y cells.




Displacement of [$^3$H]NMS binding is expressed as a percentage of the total [$^3$H]NMS binding (i.e. no salsolinol), after subtraction of non-specific binding. All data is expressed as mean inhibition of [$^3$H]NMS binding ± SEM, for n = 6 experiments. * Signifies $P<0.05$ when compared to maximum specific binding.

Table 5.12. The effect of salsolinol added directly to SH-SY5Y cell monolayers after loading with [$^3$H]NA.

| [Salsolinol] (μM) | [$^3$H]NA Release (%) |
|---|---|
| 0 | 5.10 ± 0.39 |
| 0.3 | 4.70 ± 0.23 |
| 1 | 6.20 ± 0.94 |
| 10 | 5.10 ± 0.67 |
| 30 | 5.40 ± 0.95 |
| 100 | 4.10 ± 0.13 |
| 250 | 4.90 ± 0.53 |
| 500 | 3.90 ± 1.11 |
| 700 | 4.20 ± 0.34 |
| 800 | 4.10 ± 0.26 |
| 1000 | 3.90 ± 0.41 |

The data are expressed as the amount [$^3$H]NA release as a percentage of the total [$^3$H]NA present, and are represented as the mean ± SEM for n = 3 experiments.

## 5.4. Discussion.

This chapter has investigated the potential neurotoxicity of salsolinol, and its effect upon catecholamine homeostasis in the human SH-SY5Y neuroblastoma cell line. These data show that salsolinol is cytotoxic to SH-SY5Y, producing a time and dose related inhibition of MTT reduction, and increased LDH release. In contrast to PC12h cells, SH-SY5Y cells appear to be more susceptible to salsolinol, as the data from this study indicated a significant inhibition of MTT reduction over equivalent time periods (72 hours), at lower concentrations of salsolinol (3μM) in SH-SY5Y cells. This may be due to two factors; firstly, there may be sensitivity differences between the MTT assay utilised here, and the measurement of total protein used by Maruyama et al (1993b). Secondly, PC12 cells are known to gain little of their energy from mitochondrial metabolism, utilising glycolysis for the majority of carbohydrate based metabolism (Warburg 1967, Racker and Spector 1981, Morelli et al 1986). Since TIQ derivatives are thought to be putative mitochondrial toxins (Suzuki et al 1990), PC12 cells may well be less susceptible to salsolinol. The data in this chapter with $IC_{50}$ of 54 μM (at 72 hours), are of a similar order of magnitude to the work of Melzig and Zipper (1993), who report salsolinol toxicity to aortic endothelial cells with $IC_{50}$ of 38μM after 72 hours.

It has been postulated that TIQ derivatives are N-methylated in vivo (Naoi et al 1989b), prior to MAO activation to a cytotoxic equivalent of $MPP^+$ (Naoi et al 1989a). Indeed, Maruyama et al (1992) recently reported N-methylation of salsolinol during in vivo microdialysis, and N-methylated derivatives of salsolinol were detected in human brain (Niwa et al 1991). However, inhibition of MAO types A and B by clorgyline (10 μM) or deprenyl (10μM) did not prevent the decrease in MTT reduction induced by

salsolinol (30 or 100μM), after 48 hours. Therefore, despite being reported as a MAO inhibitor (Naoi et al 1993), it appears unlikely that salsolinol toxicity requires metabolic activation by MAO in SH-SY5Y cells. Pre-incubation with imipramine (10 μM), followed by concurrent incubation with salsolinol (30 or 100μM) did not prevent the inhibition of MTT reduction observed with salsolinol after 48 hours. Therefore, it also is unlikely that salsolinol toxicity, in SH-SY5Y cells, involves the catecholamine uptake transporter.

Salsolinol is derived from dopamine, an extremely labile compound, and as such could autoxidise outside cells to generate free radical species that may initiate oxidative damage to membranes, and subsequently lead to cell death. However, pre-incubation of α-tocopherol (2.5 to 500μM), for 24 hours, prior to addition of salsolinol, did not prevent either the inhibition of MTT reduction, or increased LDH release (after 48 hours). In addition, concurrent incubation of the water soluble α-tocopherol analogue, Trolox C (10 to 500μM), and salsolinol (30 or 100μM) had no effect upon the inhibition of MTT reduction observed with salsolinol after 48 hours. Thus, it seems unlikely that the mechanism of salsolinol toxicity is through membrane-directed autoxidation. Salsolinol is reported to accumulate in the mitochondria of PC12h cells (Maruyama et al 1993b), it is therefore possible that salsolinol toxicity may involve mitochondrial function.

If several assumptions are accepted one can roughly calculate the concentration of salsolinol in firstly the cerebral spinal fluid (CSF) and interstitial fluid (ISF), and secondly the whole brain, after intraventricular infusion (Myers and Melchior 1977a). The first assumption is that the average CSF volume in an adult rat is approximately 400μL, and that the ISF is approximately 15-20 % of the total rat brain volume of

2000μL (Cserr and Patlak 1992). Secondly, the CSF has a half life of 100 minutes and the ISF 400 minutes. However, in this study salsolinol was infused at a steady rate of 1μM per half hour, thus the half lives of the CSF and ISF are not important. Thirdly, assuming a complete mixing into CSF and ISF which would take approximately 20 minutes, one can calculate that if salsolinol was evenly distributed in the extracellular fluid it would have a concentration of 1.25nM. Furthermore, if one assumed that salsolinol were evenly distributed across the whole brain, then the concentration of salsolinol would be approximately 0.5nM. Unfortunately as Myers and Melchior did not assess the toxicological or pharmacological effects of salsolinol infusion, it is not posible to make a true comparison between the concentration of salsolinol required to increase the consumption of alcohol and the effects of salsolinol described in this thesis. However, if one does compare the two sets of data one can see that the closest comparisons are between salolinol toxixcity after 72 hours (3μM), and the stimulation of catecholamine uptake (11μM and 17μM, for DA and NA respectively).

The effects of salsolinol upon catecholamine uptake and release were also examined in SH-SY5Y cells. Salsolinol (100μM) is reported to inhibit the uptake of catecholamines in rat brain synaptosomes (Heikkila et al 1971), and stimulate the release of catecholamines from rat brain during in vivo microdialysis, at high concentrations (1mM, Maruyama et al 1993a). However, this study showed that in monolayer culture, salsolinol stimulated catecholamine uptake with a dose related increase with maximum at 100μM ($EC_{50}$ 17μM for NA, 11μM for DA), followed by decreased catecholamine uptake back to basal from 250μM ($IC_{50}$ 411μM for NA, 379μM for DA) to 1mM.

The stimulation (below 100μM), and subsequent inhibition (>100μM), of catecholamine uptake by salsolinol could be due to many potential interactions. These may include modification of the uptake recognition site, or alteration of one or more intracellular second messenger systems (figure 5.8). To investigate the potential mechanism behind salsolinol stimulated [$^3$H]NA uptake in SH-SY5Y cells, the interaction of salsolinol and [$^3$H]nisoxetine (a high affinity ligand for uptake$_1$), was examined. Salsolinol displaced [$^3$H]nisoxetine binding by 71.2 ± 2.32 % at 1mM, with an $IC_{50}$ of 818 ± 67.7μM, and $K_i$ of 353 ± 29.8μM. Therefore, inhibition of catecholamine uptake at higher concentrations of salsolinol (>250μM) is probably due to direct competition of salsolinol with either NA or DA for the uptake recognition site, and may explain why salsolinol has been reported to inhibit catecholamine uptake previously (Heikkila et al 1971).

A possible mechanism for the observed stimulation could involve second messenger mediated phosphorylation of the uptake transporter. Indeed, Casado et al (1993) have recently reported that the $Na^+$ dependent L-glutamate transporter in pig brain can be phosphorylated by PKC. Further evidence showed that inhibition of PKC inhibited glutamate uptake in rat dorsal horn cells of the spinal cord (Chen and Huang 1991). However, inhibition of PKC with Ro 31-8220 did not affect salsolinol stimulated [$^3$H]NA uptake in SH-SY5Y monolayer culture. Forskolin, a diterpene, directly activates AC in SH-SY5Y cells (Morgan et al 1993). Thus, if salsolinol stimulated catecholamine uptake through elevation of cAMP concentrations one would expect forskolin to stimulate catecholamine uptake. However, forskolin (10μM) alone did not increase [$^3$H]NA uptake, and had no effect upon salsolinol stimulated [$^3$H]NA uptake,

implying that salsolinol did not stimulate uptake through inhibition of AC. Another second messenger cGMP may be involved in the regulation of catecholamine uptake. Sodium nitroprusside is known to activate GC and increase the cellular concentration of cGMP (Murad et al 1978, Bohme et al 1978). However, nitroprusside had no effect upon basal or salsolinol stimulated [$^3$H]NA uptake in SH-SY5Y cells. The failure of nitroprusside to inhibit salsolinol stimulated [$^3$H]NA uptake, suggests that salsolinol enhanced catecholamine uptake is not mediated through inhibition of GC. Consequently, salsolinol stimulated catecholamine uptake in SH-SY5Y cells, is not related to interactions with the second messenger systems examined here.

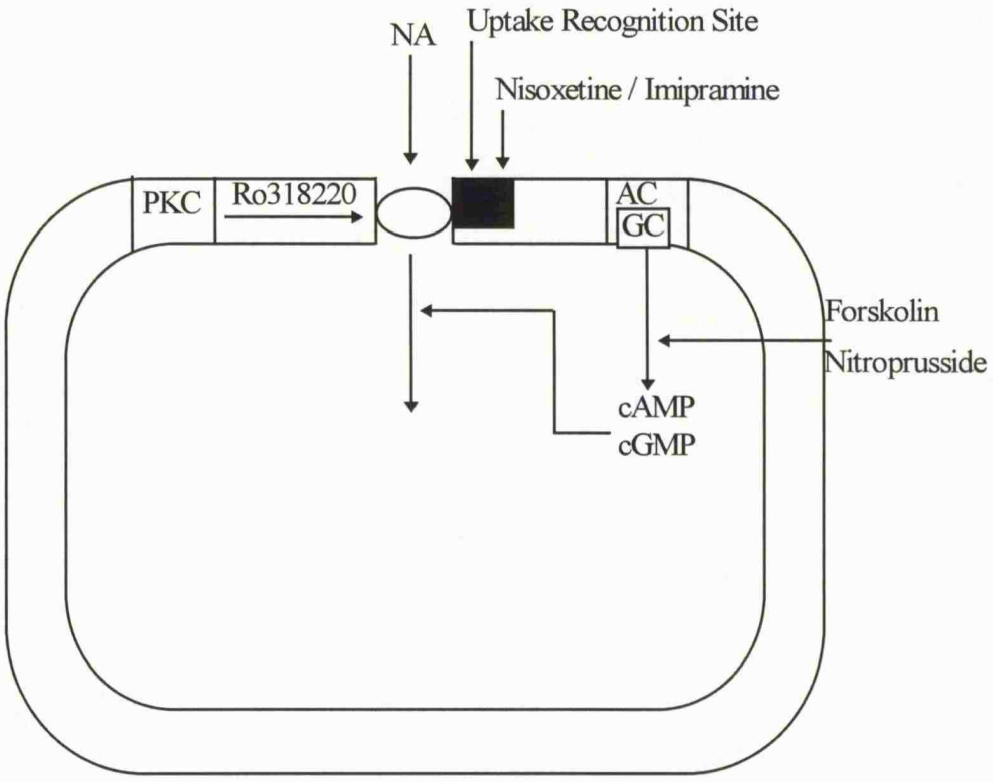



*Figure 5.8. Schematic representation of the potential interactions between second messenger systems and noradrenaline uptake in SH-SY5Y cells. Activation of PKC is*

*known to increase uptake of glutamate, therefore, inhibition of PKC by Ro 31-8220 may inhibit the stimulation of NA uptake seen with both TMIQ and salsolinol. Additionally, elevation of either cAMP or cGMP by forskolin or sodium nitroprusside, respectively, could alter NA uptake or reverse the stimulation of uptake seen with TMIQ or salsolinol.*

In contrast to previously reported data (Maruyama et al 1993a) salsolinol did not directly stimulate catecholamine release in SH-SY5Y cells. However, salsolinol (from 250μM to 1mM) significantly ($P<0.05$) inhibited potassium (100mM) and also inhibited CCH (1mM) evoked [$^3$H]NA release, in SH-SY5Y monolayers, with $IC_{50}$ values of 120μM and 500μM, respectively. The mechanism of salsolinol inhibition of potassium (100mM) evoked NA release is unclear. Salsolinol (1mM) inhibited [$^3$H]NMS binding to the muscarinic receptors sensitive to CCH by 69.9 ± 3.17 %, with $IC_{50}$ of 398μM, and $K_i$ of 215μM. Furthermore, calculation of the $K_i$ from the inhibition of CCH evoked [$^3$H]NA release data indicated that the reversal of CCH catecholamine release cannot be entirely due to muscarinic antagonism, since 5.87mM of salsolinol would be required to inhibit the observed response by 50 %. Therefore, the inhibition of CCH evoked [$^3$H]NA release from SH-SY5Y cells must be due to a different mechanism than salsolinol antagonism of CCH binding to muscarinic receptors alone. Inhibition of CCH and potassium evoked release is difficult to explain, but since the coupling mechanisms are different (Atcheson et al 1994b), an interaction with the release machinery is likely. However, as NA release is dependent upon $Ca^{2+}$, and since salsolinol inhibits both potassium and CCH evoked release, then salsolinol may interfere with both $Ca^{2+}$ entry and release from intracellular stores.

In conclusion, these data show that salsolinol is toxic to SH-SY5Y cells, but the mechanism of action of salsolinol is not clear. Salsolinol toxicity is not prevented by addition of antioxidants, or MAO inhibitors, and does not appear to involve the catecholamine uptake recognition site. In PC12h cells salsolinol is known to accumulate in the mitochondria over 3 days (Maruyama et al 1993b). Salsolinol inhibited oxygen consumption, and caused ultrastructural damage to the mitochondria of aortic endothelial cells (Melzig and Zipper 1993). These events may also occur in SH-SY5Y cells and need to be examined further. If salsolinol is cytotoxic to aortic endothelial cells (Melzig and Zipper) it may also be cytotoxic to the endothelial cells that form the blood-brain barrier. This could allow access of a variety of different compounds to neuronal cells and subsequently, lead to potential neurodegeneration.

# Chapter 6

# Studies on the Potential Neurotoxicity of 1,2,3,4-Tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ), and its effects upon catecholamine homeostasis in SH-SY5Y cells

## 6.1. Introduction.

Many potential endogenous or environmental toxins have been linked with the aetiology of neurodegenerative disorders. One such example is 1,2,3,4-tetrahydroisoquinoline (TIQ) and its derivatives. TIQ is known to produce a parkinsonian syndrome in monkeys, which is reversed after cessation of treatment (Nagatsu et al 1988). In addition, TIQ was found to occur in both normal and parkinsonian brains (Niwa et al 1987). There is also evidence that TIQ is N-methylated (Naoi et al 1989b) and metabolised by monoamine oxidase (Naoi et al 1989a) to create a compound similar to the active metabolite of the classic parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, Langston et al 1984). Many of these TIQ derivatives are also thought to occur naturally in vivo through the non-enzymic condensation of catecholamines and compounds such as aldehydes (Kohno et al 1986 and Ohta et al 1987). One potential condensation product of adrenaline, 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ) may be formed in vivo via Pictet-Spengler cyclation (Bates 1981, Deitrich and Erwin 1980). TMIQ could also be generated endogenously, since further methylation of adrenaline by phenylethanolamine N-methyl transferase occurs at 20 % of the rate constant for conversion of noradrenaline to adrenaline in vitro (Axelrod 1966, Plummer et al 1988). Furthermore, N-methyladrenaline undergoes spontaneous ring closure at physiological pH to produce TMIQ. TMIQ has structural similarity to the dopamine depleting agent 2-methylisoquinoline (Booth et al 1989), and has been shown to reduce catecholamine levels in several areas of the rat brain after intra cranial injection (Liptrot et al 1993). Moreover, in rat striatal synaptosomes TMIQ inhibited the rate limiting enzyme of catecholamine synthesis, tyrosine hydroxylase (TH) (Liptrot et al 1994). TMIQ

induced inhibition of TH can be reversed by pre-incubation with the catecholamine uptake inhibitor, nomifensine (Liptrot et al 1994). Additionally, pre-incubation with the monoamine oxidase inhibitors clorgyline and deprenyl reversed TMIQ induced TH inhibition.

Therefore, to further characterise the effects of TMIQ in a defined, isolated, cellular system, this chapter has examined the effects upon catecholamine uptake and release, and assessed the potential toxicity of TMIQ in SH-SY5Y cells.

## 6.2. Materials and Methods.

*Materials.*

TMIQ was donated by Dr. Oliver Phillipson, of Bristol University. The Roche compounds Ro 20-9747 (Trolox C) and Ro 31-8220, MAO inhibitors deprenyl and clorgyline, sodium nitroprusside and forskolin were supplied as stated previously (chapter 5). All laboratory reagents were of the best quality available and were supplied as stated previously (chapter 2).

*Cell Culture.*

Undifferentiated human SH-SY5Y neuroblastoma cells were cultured by the method described in, materials and methods, chapter 2, page 67. For experimental purposes cells were harvested, and seeded in 12 (for LDH and catecholamine studies), and 24 (for MTT studies) well multitrays.

*Toxicological Incubation Protocols.*

Confluent SH-SY5Y cells were exposed to various concentrations of TMIQ (0.3 to 100 μM) for up to 72 hours. Ascorbic acid (0.2mM) was included in all stock solutions of TMIQ to prevent autoxidation prior to addition to cell cultures. For further experiments examining the mechanism of TMIQ toxicity, relatively high concentrations (100 and 250μM) and longer incubations (48 hours) were chosen to provide a large window in which to observe any perturbation or increase in toxicity. To examine the mechanism of TMIQ toxicity, the uptake$_1$ inhibitor imipramine (1 and 10μM), MAO inhibitors (clorgyline and deprenyl) or antioxidants α-tocopherol or

Trolox C were included following the same protocols described in chapter 5, (page 128).

*LDH Release.*

Confluent SH-SY5Y cells were treated with various concentrations of TMIQ (0.3 to 500μM). After 24, 48 and 72 hours, LDH levels were assessed in both the cell media and the cell lysates. α-Tocopherol at various concentrations (2.5 to 500μM) was added in the presence or absence of TMIQ (100 or 250μM) following the protocol described previously (chapter 5).

*MTT Reduction.*

TMIQ, at various doses (0.3 to 500μM) was added to SH-SY5Y cells three days after seeding, and incubated for up to 72 hours. After 24, 48 and 72 hours MTT (0.12mM) was added and left to incubate for one hour at 37°C. The resulting dye was extracted and measured spectrophotometrically.

*Catecholamine Uptake.*

Catecholamine uptake was undertaken by the method of Atcheson et al (1993). Excluding controls to determine basal uptake, TMIQ was included during uptake at various concentrations (0.3 to 500μM). The effects of forskolin (10μM), Roche 31-8220 (1μM) and sodium nitroprusside (100μM) on TMIQ stimulated [$^3$H]NA uptake were examined following the protocols outlined in chapter 5 (page 130).

*[$^3$H]Nisoxetine Displacement Binding Studies.*

Displacement of [$^3$H]nisoxetine binding was undertaken by the method described earlier, (chapter 2, page 78). TMIQ was included in increasing concentrations between $10^{-10}$ to $10^{-3}$M.

*Catecholamine Release.*

Catecholamine release was undertaken by the method of Atcheson et al (1994a). To examine the effects of TMIQ upon catecholamine release, TMIQ was included at various concentrations (0.3μM to 1mM) in Krebs / HEPES buffer during the last of the three 15 minutes washes applied after catecholamine loading prior to either potassium (100mM) or CCH (1mM) evoked [$^3$H]NA release. The effect of TMIQ upon unstimulated [$^3$H]NA release from [$^3$H]NA loaded cells, was examined by the addition of various doses of TMIQ (0.3μM to 1mM), for 3 minutes following three 15 minute washes with Krebs / HEPES buffer, pH 7.4.

*Muscarinic Displacement Binding Studies.*

[$^3$H]NMS displacement binding studies were undertaken with the method detailed in chapter 2 (page 84). TMIQ was included at increasing concentrations from $10^{-8}$ to $10^{-3}$M.

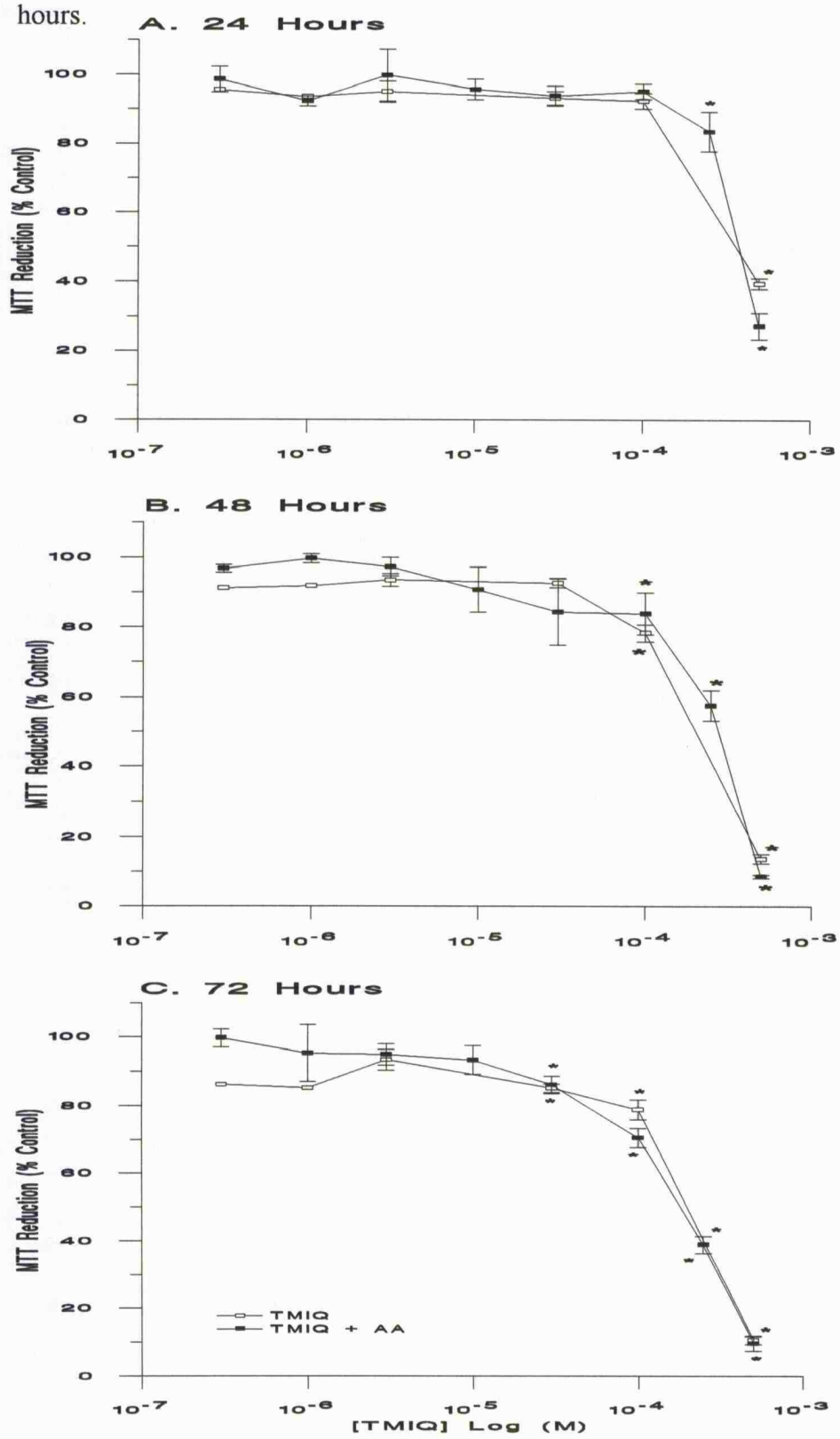
**6.3. Results.**

The adrenaline condensation derivative TMIQ induced a dose related inhibition of MTT reduction, with increasing TMIQ concentration. A significant ($P<0.05$) inhibition of MTT reduction was achieved with 500μM TMIQ at 24 hours to 72 hours of 39.5 % to 10.6 % of the untreated control, respectively (figure 6.1). TMIQ also caused a significant ($P<0.05$) inhibition of MTT reduction at 100μM from 24 hours onwards, and 30μM at 72 hours (figure 6.1). The degree of TMIQ toxicity was unaffected by the presence of ascorbate in the Krebs / HEPES buffer prior to addition of TMIQ to the incubation media (figure 6.1, table 6.1). Furthermore, TMIQ at 30μM and 100μM significantly ($P<0.05$) increased LDH release to 131.6 ± 6.13 and 157 ± 2.38 % from SH-SY5Y cells after 72 hours, respectively (figure 6.2). Blockade of $uptake_1$ sites with imipramine (1 or 10μM, an $uptake_1$ inhibitor), did not prevent TMIQ (100 or 250μM) inhibition of MTT reduction after 48 hours co-incubation (table 6.2). Pre-incubation of either clorgyline or deprenyl (0.1, 1 and 10μM) for 30 minutes followed by concurrent incubation with TMIQ (100 or 250μM) for 48 hours failed to prevent the inhibition of MTT reduction (tables 6.3, 6.4 and 6.5). In addition, pre-incubation of α-tocopherol (2.5 to 500μM) for 24 hours, did not protect SH-SY5Y cells from either TMIQ (100 or 250μM) induced LDH release or the inhibition of MTT reduction (tables 6.6 and 6.7). The concurrent incubation of the water soluble α-tocopherol analogue, Trolox-C (10 to 500μM) and TMIQ (100μM), also did not prevent TMIQ inhibition of MTT reduction over 48 hours (table 6.8).

TMIQ caused a dose related increase in both [$^3$H]NA and [$^3$H]DA uptake in monolayer cultures of SH-SY5Y cells, with $EC_{50}$ of 8μM and 54μM, respectively (figure 6.3). Maximum increase of [$^3$H]NA or [$^3$H]DA uptake occurred with 100μM and 250μM

TMIQ, respectively. Moreover, TMIQ inhibited [$^3$H]nisoxetine binding by 72.8 ± 4.7 % at 1mM, with an $IC_{50}$ of 156 ± 17μM (calculated by Graphpad) and a $K_i$ of 71 ± 8μM (figure 6.4). Pre-incubation with the PKC inhibitor, Ro 31-8220 (1μM), for 15 minutes, followed by incubation in the presence or absence of TMIQ (10 or 500μM) for one hour did not reverse TMIQ stimulated [$^3$H]NA uptake (table 6.9). Under the same conditions forskolin (10μM, adenylate cyclase activator, table 6.10), and sodium nitroprusside (100μM, guanylate cyclase activator, table 6.11) were incubated individually in the presence or absence of TMIQ (10 or 500μM). However, neither compound affected TMIQ (10 or 500μM) increased [$^3$H]NA uptake in SH-SY5Y cells. TMIQ inhibited potassium (100mM) evoked [$^3$H]NA release from SH-SY5Y cells (figure 6.4), with maximum inhibition at 1mM TMIQ (42.9 ± 9.2 % of potassium, 100mM, evoked release). Significant ($P<0.05$) inhibition of potassium evoked [$^3$H]NA release occurred at concentrations of TMIQ above 500μM (figure 6.5), and lower doses of TMIQ had no effect. TMIQ did not inhibit CCH (1mM) evoked [$^3$H]NA release from SH-SY5Y cells (table 6.12). TMIQ did not directly induce [$^3$H]NA release from SH-SY5Y cells (table 6.12). Furthermore, TMIQ (1mM) inhibited [$^3$H]NMS binding by 23.6 ± 3.73 %, and had no effect at lower doses (figure 6.6).

Figure 6.1. The effect of TMIQ upon MTT reduction in SH-SY5Y cells over 72 hours.




For legend see next page.

Legend to figure 6.1.

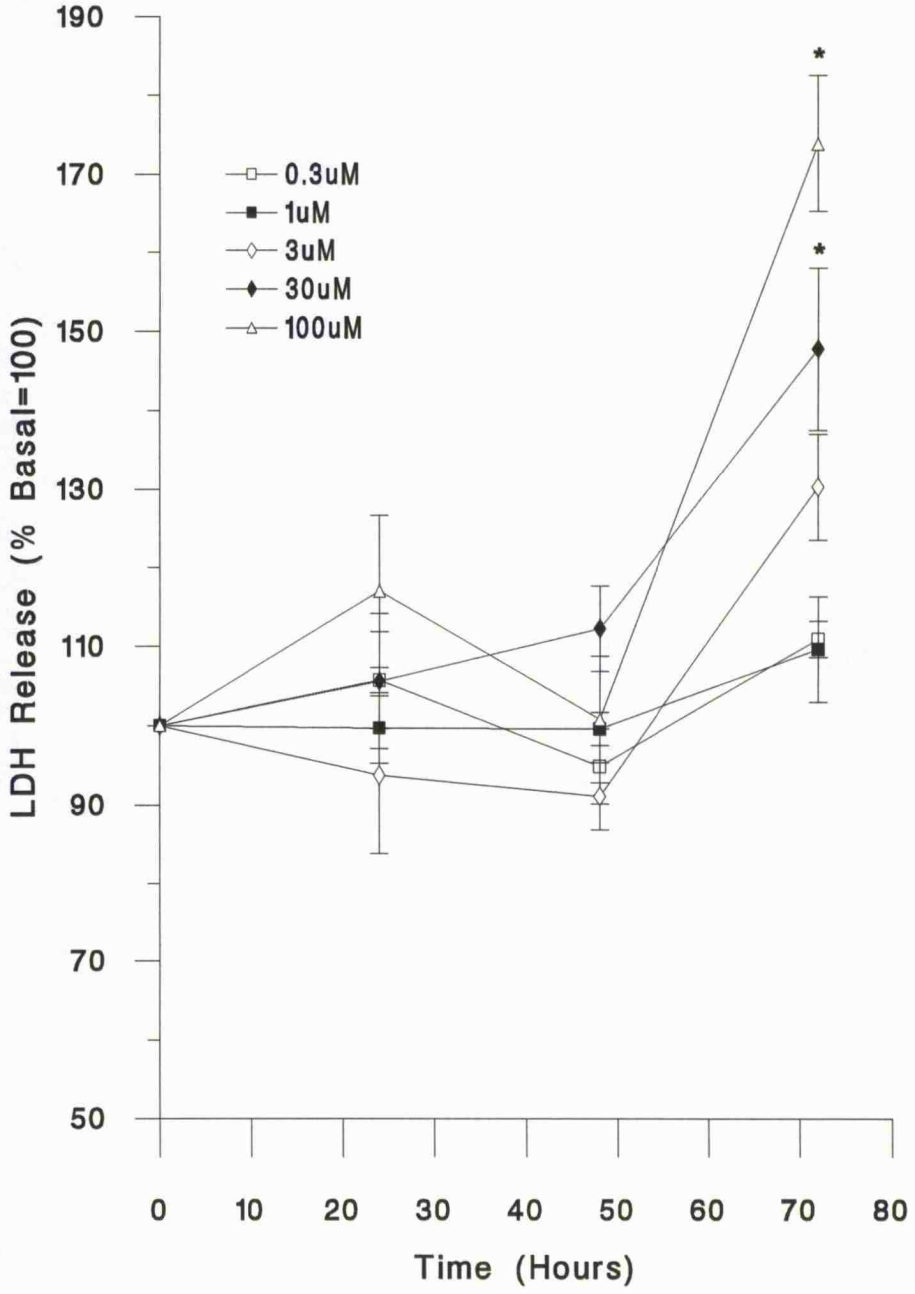
All data is expressed mean ± SEM of the percentage of the relevant untreated control (= 100), for n = 6 experiments. Toxicity was measured in the presence or absence of ascorbic acid (AA 20μM), which was used to prevent TMIQ autoxidation prior to incubation with SH-SY5Y cells. * Signifies $P<0.05$ when compared to control.

Table 6.1. Comparison of the toxicity of TMIQ in the presence or absence of ascorbic acid (AA) prior to incubation with SH-SY5Y cells.

| | Krebs / HEPES buffer | |
|---|---|---|
| Time (Hours) | No AA (n = 6) | AA (20μM) (n = 3) |
| 24 | 401 ± 47.5 | 385 ± 28.3 |
| 48 | 196 ± 12.9 | 230 ± 26.8 |
| 72 | 181 ± 6.89 | 165 ± 15.5 |

The degree of toxicity was assessed by MTT reduction. The data are expressed as the mean $IC_{50}$ value ± SEM, in the presence or absence of ascorbic acid, respectively. The final concentration of ascorbic acid (AA) was 20μM. TMIQ was added at various concentrations between 0.3 to 500μM.

Figure 6.2. The effect of TMIQ upon LDH release from SH-SY5Y cells over 72 hours.




All data are expressed as mean ± SEM of the percentage of basal (no additions = 100), for n = 4 to 7 experiments. * Signifies $P<0.05$ when compared to basal LDH release.

Table 6.2. The effect of imipramine pre-incubated for 30 minutes, followed by concurrent incubation with TMIQ for 48 hours in SH-SY5Y cells. After 48 hours toxicity was assessed by MTT reduction.

| | TMIQ (μM) | | |
|---|---|---|---|
| Imipramine (μM) | 0 | 100 | 250 |
| 0 | 100 | 76.6 ± 4.37* | 42.5 ± 5.60* |
| 1 | 105.6 ± 4.99 | 89.6 ± 1.39* | 41.1 ± 6.84* |
| 10 | 109.6 ± 10.22 | 88.0 ± 6.26* | 52.2 ± 6.77* |

The data are expressed as a percentage of the untreated control, and represented as the mean ± SEM, for n = 4 experiments. * Signifies $P<0.05$ when compared to control. The addition of imipramine had no significant effect upon salsolinol inhibition of MTT reduction.

Table 6.3. The effects of clorgyline pre-incubation, for 30 minutes, followed by concurrent incubation of TMIQ and clorgyline for 48 hours in SH-SY5Y cells. At 48 hours toxicity was assessed by MTT reduction.

| | TMIQ (μM) | | |
|---|---|---|---|
| Clorgyline (μM) | 0 | 100 | 250 |
| 0 | 100 | 80.8 ± 5.54* | 66.6 ± 8.16* |
| 0.1 | 102.4 ± 1.23 | 76.5 ± 2.25* | 56.9 ± 4.97* |
| 1.0 | 107.5 ± 8.45 | 72.4 ± 4.02* | 57.9 ± 3.35* |
| 10 | 92.40 ± 2.01 | 60.9 ± 5.50* | 35.8 ± 7.23* |

The data are expressed as a percentage of the untreated control, and are represented as the mean ± SEM for n = 4 experiments.

Table 6.4. The effects of deprenyl pre-incubation, for 30 minutes, followed by concurrent incubation of TMIQ and deprenyl for 48 hours in SH-SY5Y cells. At 48 hours toxicity was assessed by MTT reduction.

| | TMIQ (μM) | | |
|---|---|---|---|
| Deprenyl (μM) | 0 | 100 | 250 |
| 0 | 100 | 80.8 ± 5.54* | 66.6 ± 8.16* |
| 0.1 | 92.0 ± 1.23 | 84.9 ± 0.95* | 46.5 ± 2.71* |
| 1.0 | 97.8 ± 5.03 | 83.1 ± 2.02* | 40.6 ± 4.31* |
| 10 | 90.2 ± 5.24 | 86.4 ± 3.50* | 39.7 ± 5.07* |

The data are expressed as a percentage of the relevant untreated control, and are represented as the mean ± SEM for n = 4 experiments. * Signifies $P<0.05$ when compared to control.

Table 6.5. The effects of concurrent pre-incubation of clorgyline and deprenyl (10μM) for 30 minutes, followed by concurrent incubation with TMIQ in SH-SY5Y cells for 48 hours.

| Clorgyline + Deprenyl | TMIQ 100μM | TMIQ 250μM |
|---|---|---|
| 0 | 88.3 ± 3.82* | 74.7 ± 8.93* |
| C 10μM + D 10μM | 76.1 ± 3.34* | 55.7 ± 1.11* |

The data are expressed as a percentage of the relevant untreated control, and are represented as the mean ± SEM for n = 4 experiments. The degree of toxicity was measured at 48 hours by MTT reduction. * Signifies $P<0.05$ when compared to control. Clorgyline and deprenyl (10μM) had no effect upon MTT reduction in the absence of TMIQ (99.5 ± 4.36 % compared to the control = 100).

Table 6.6. The effects of α-tocopherol pre-incubation (24 hours) prior to TMIQ insult to SH-SY5Y cells. At 48 hours incubation with TMIQ, toxicity was measured by LDH release.

| α-Tocopherol (μM) | TMIQ (250μM) |
|---|---|
| 0 | 180.4 ± 31.9* |
| 2.5 | 166.2 ± 28.9* |
| 5 | 177.1 ± 16.1* |
| 10 | 172.2 ± 18.5* |
| 25 | 167.1 ± 15.2* |
| 50 | 170.9 ± 11.3* |
| 100 | 181.4 ± 18.7* |
| 250 | 174.7 ± 23.4* |
| 500 | 147.5 ± 15.2* |

The data are expressed as the percentage of the control (cells treated with the highest dose of α-tocopherol, 500μM, for 24 hours followed by no further treatment), and is represented by the mean ± SEM for n = 3 separate experiments. * Signifies $P<0.05$ when compared to control. The addition of α-tocopherol had no significant effect upon TMIQ induced LDH release.

Table 6.7. The effects of pre-incubation of α-tocopherol for 24 hours with SH-SY5Y cells prior to TMIQ incubation for a further 48 hours. At 48 hours toxicity was assessed by MTT reduction.

| | TMIQ (μM) | |
|---|---|---|
| α-Tocopherol (μM) | 100 | 250 |
| 0 | 87.3 ± 2.82* | 28.6 ± 3.00* |
| 2.5 | 80.4 ± 2.50* | 17.2 ± 1.40* |
| 5 | 88.9 ± 3.94* | 24.3 ± 3.06* |
| 25 | 84.1 ± 4.39* | 27.4 ± 3.63* |
| 50 | 85.6 ± 2.71* | 25.4 ± 3.34* |
| 100 | 82.7 ± 3.73* | 21.7 ± 3.23* |
| 250 | 84.0 ± 3.86* | 23.6 ± 3.52* |
| 500 | 83.6 ± 2.54* | 19.8 ± 0.99* |

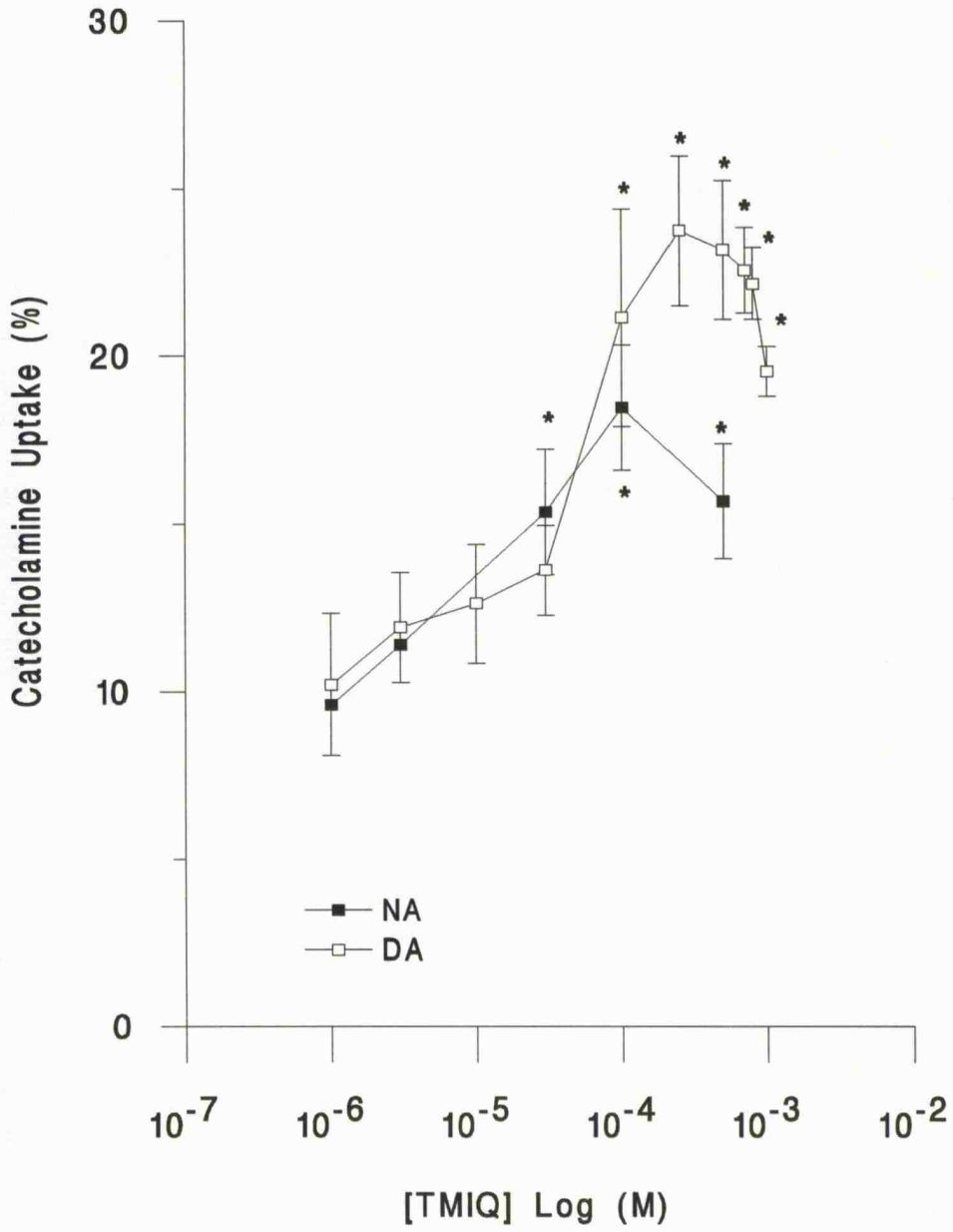
The data are expressed as a percentage of a control treated with 500μM α-tocopherol for 24 hours, then incubated in supplemented media without further additions for 48 hours. The data represent the mean ± SEM for n = 4 separate experiments. * Signifies $P<0.05$ when compared to control. The addition of α-tocopherol had no significant effect on TMIQ inhibition of MTT reduction.

Table 6.8. The effects of concurrent incubation of Trolox C and TMIQ (100μM) with SH-SY5Y cells. After 48 hours incubation with TMIQ toxicity was measured by MTT reduction.

| Trolox C (μM) + TMIQ (100μM) | MTT Reduction (% Basal) |
|---|---|
| 0 | 85.5 ± 5.95* |
| 10 | 84.2 ± 6.18* |
| 30 | 88.4 ± 4.98* |
| 100 | 86.8 ± 6.53* |
| 250 | 87.6 ± 7.39* |
| 300 | 78.6 ± 8.99* |
| 500 | 66.0 ± 2.29* |
| 500 (no TMIQ) | 94.7 ± 7.23 |

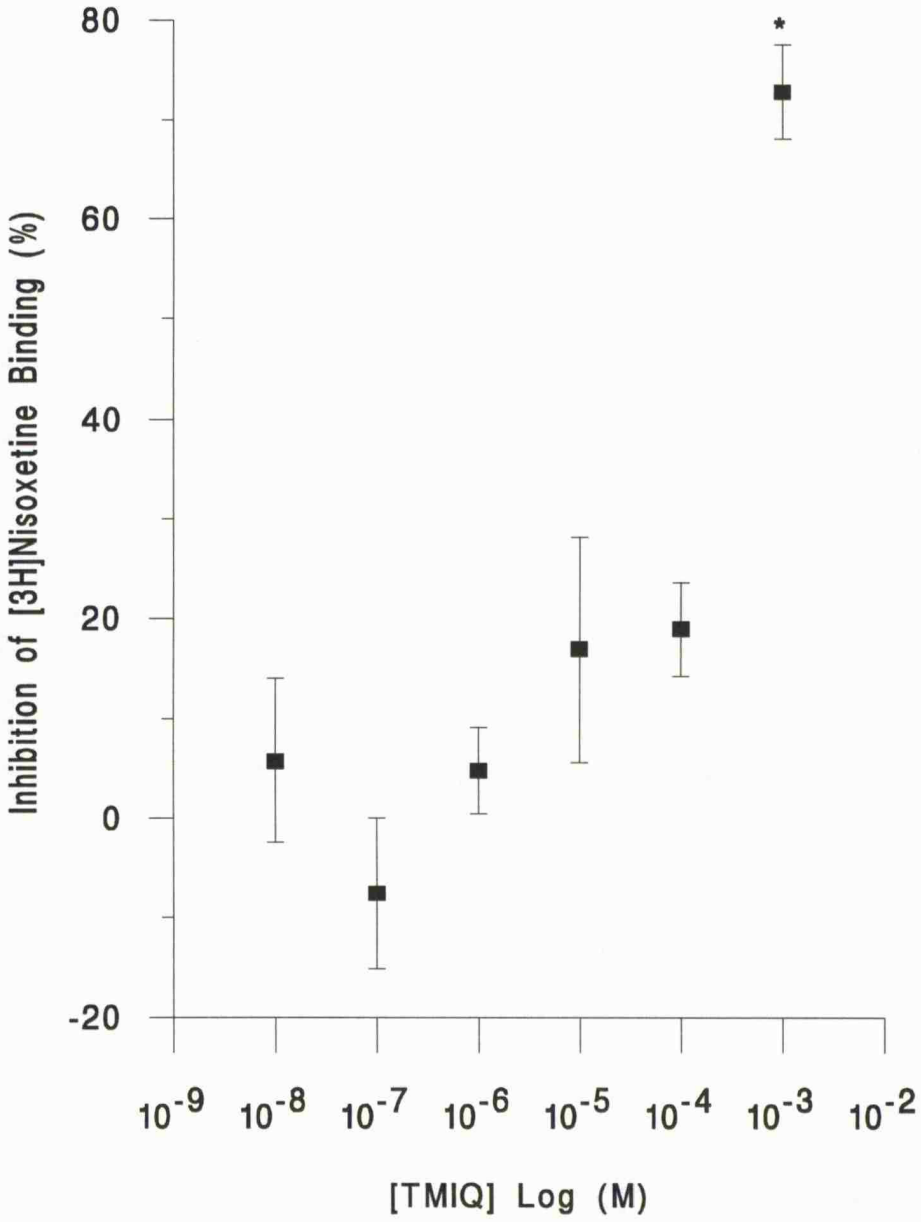
The data are expressed as the percentage of the control (untreated cells), and is represented by the mean ± SEM for n = 4 separate experiments. * Signifies $P<0.05$ when compared to control. The addition of Trolox C did not significantly prevent TMIQ inhibition of MTT reduction.

Figure 6.3. The effect of TMIQ upon catecholamine uptake in confluent SH-SY5Y cells monolayers over one hour.




All data are expressed as a percentage of the total available activity, and represented as the mean ± SEM, for n = 5 or 8 experiments for DA and NA, respectively. * Signifies $P<0.05$ when compared to basal uptake.

Figure 6.4. The effect of TMIQ upon displacement of [$^3$H]nisoxetine binding to SH-SY5Y cells.




Displacement of [$^3$H]nisoxetine binding is expressed as a percentage of the total [$^3$H]nisoxetine binding (i.e. no TMIQ), after subtraction of non-specific binding. All data are expressed as mean inhibition of [$^3$H]nisoxetine binding ± SEM, for n = 4 experiments. * Signifies $P<0.05$ when compared to total specific binding.

Table 6.9. The effect of Roche 31-8220 upon TMIQ stimulated [$^3$H]noradrenaline uptake in SH-SY5Y cells.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 5.45 ± 0.41 |
| Ro 31-8220 (1μM) | 7.08 ± 0.69 |
| 10μM TMIQ + Ro 31-8220 (1μM) | 8.36 ± 1.10 |
| 10μM TMIQ | 7.02 ± 1.01 |
| 500μM TMIQ + Ro 31-8220 (1μM) | 13.7 ± 0.43* |
| 500μM TMIQ | 14.5 ± 1.35* |

The data are expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 4 experiments. Ro 31-8220 is a PKC inhibitor (Davis et al 1989). * Signifies $P<0.05$ when compared to basal uptake.

Table 6.10. The effect of forskolin (cAMP activator), upon TMIQ stimulated [$^3$H]noradrenaline uptake in SH-SY5Y cells.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 5.10 ± 1.89 |
| Forskolin (10μM) | 5.80 ± 1.64 |
| 10μM TMIQ + Forskolin (10μM) | 9.10 ± 2.07 |
| 10μM TMIQ | 9.70 ± 1.90 |
| 500μM TMIQ + Forskolin (10μM) | 10.1 ± 1.51 |
| 500μM TMIQ | 13.3 ± 2.85* |

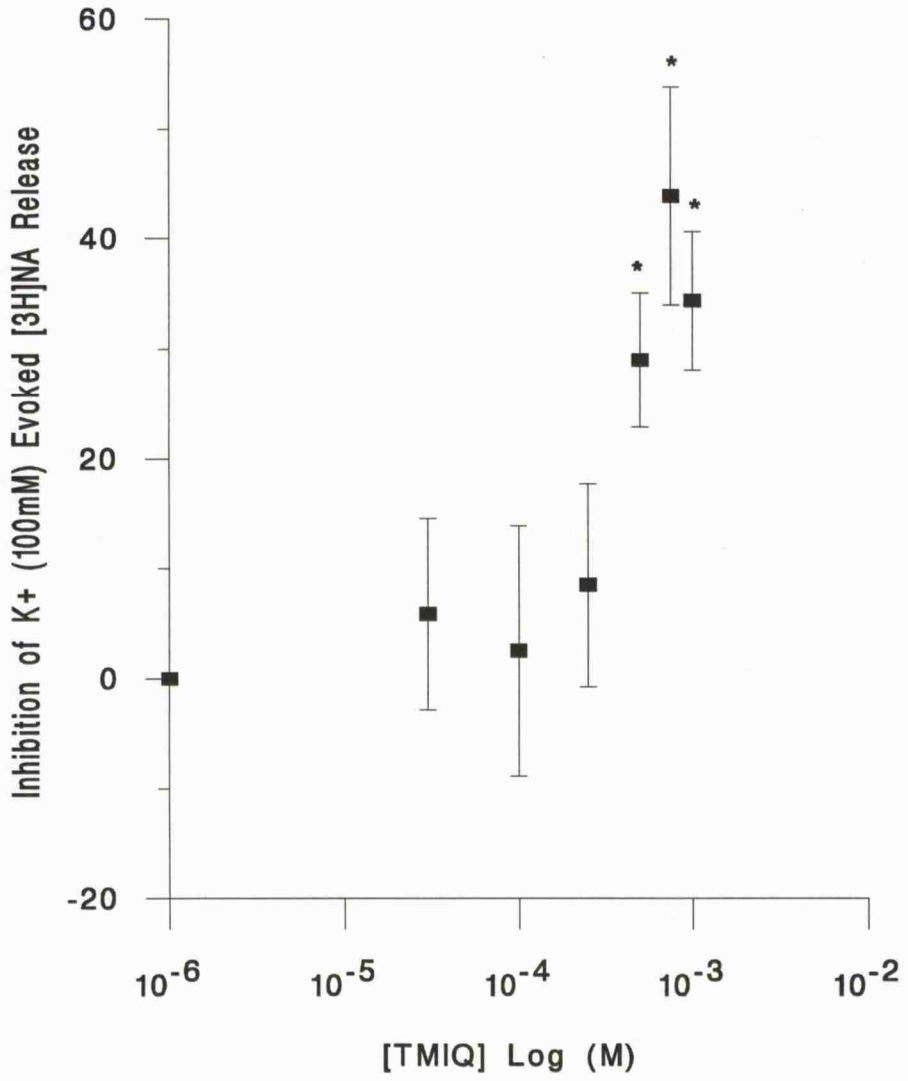
The data are expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 4 experiments. * Signifies $P<0.05$ when compared to basal uptake.

Table 6.11. The effect of sodium nitroprusside upon TMIQ stimulated [$^3$H]noradrenaline uptake.

| Sample | [$^3$H]Noradrenaline Uptake (%) |
|---|---|
| Basal | 8.00 ± 0.98 |
| Nitroprusside (100μM) | 8.10 ± 1.54 |
| 10μM TMIQ + Nitroprusside (100μM) | 10.2 ± 1.53 |
| 10μM TMIQ | 11.3 ± 1.72 |
| 500μM TMIQ + Nitroprusside (100μM) | 19.4 ± 1.61* |
| 500μM TMIQ | 19.8 ± 0.38* |

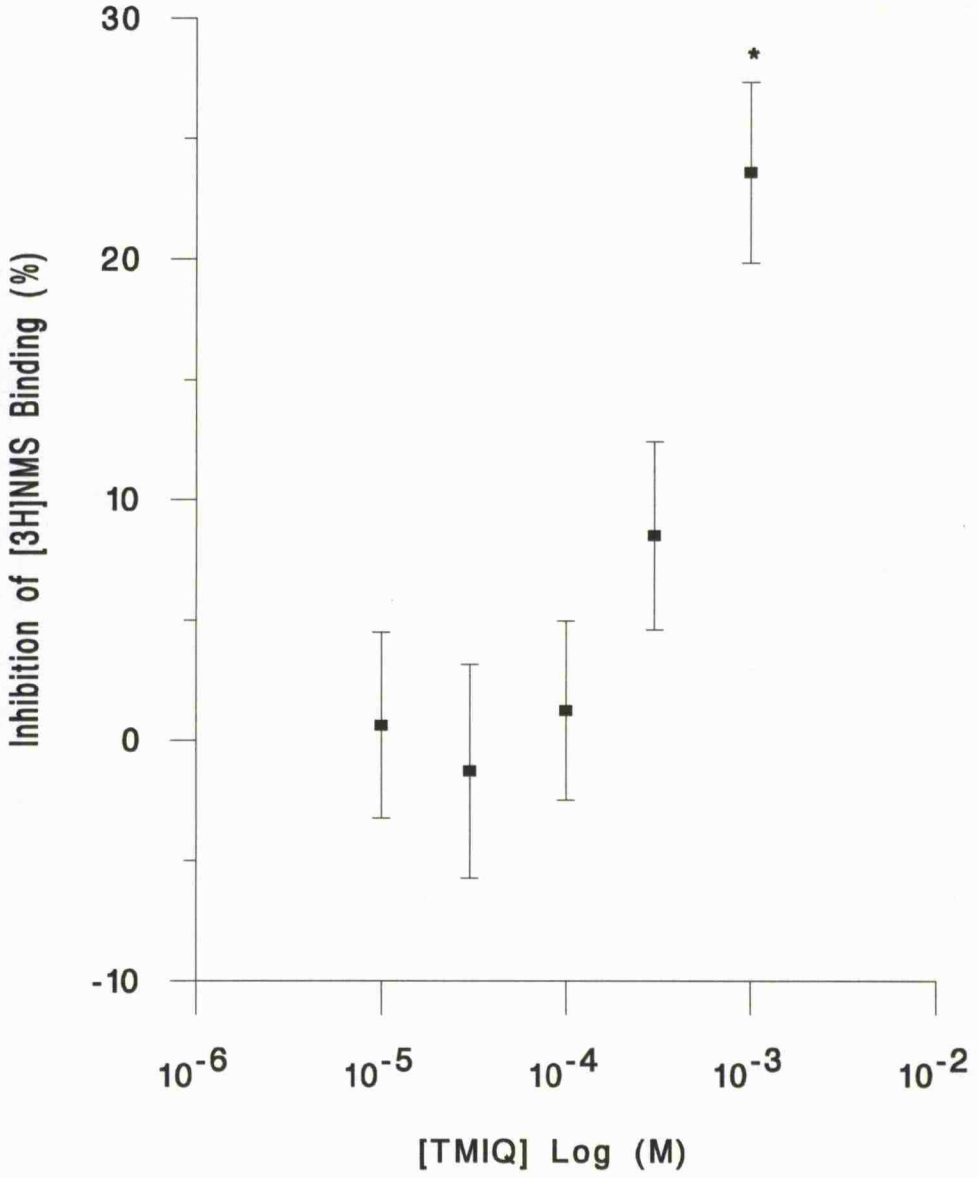
The data are expressed as the mean ± SEM of the percentage of the total available activity during uptake, for n = 4. Sodium nitroprusside is a guanylate cyclase activator (Marud et al 1978, Bohme et al 1978). * Signifies $P<0.05$ when compared to basal uptake.

Figure 6.5. The effect of TMIQ upon potassium ($K^+$ 100mM, $Na^+$ adjusted) evoked noradrenaline (NA) release from SH-SY5Y cells.




Inhibition NA release is expressed as a percentage of the maximum release evoked by $K^+$ (100mM), after subtraction of basal (background) release. All data are given as the mean ± SEM, for n = 9 experiments. * Signifies $P<0.05$ when compared to maximum stimulated release.

Figure 6.6. The effect of TMIQ upon the displacement of [$^3$H]N-methyl scopolamine (NMS) to SH-SY5Y cells.




Displacement of [$^3$H]NMS binding is expressed as a percentage of the total [$^3$H]NMS binding (i.e. no TMIQ), after subtraction of non-specific binding. All data are expressed as mean inhibition of [$^3$H]NMS binding ± SEM, for n = 4 experiments. * Signifies $P<0.05$ when compared to maximum specific binding.

Table 6.12. The effect of TMIQ upon either carbachol evoked, or direct stimulation of [$^3$H]noradrenaline (NA) in SH-SY5Y cell monolayers.

| | [$^3$H]Noradrenaline Release (%) | |
|---|---|---|
| [TMIQ] (μM) | Direct | CCH (1mM) Evoked |
| 0 | 4.80 ± 0.64 | 8.30 ± 0.56* |
| 0.3 | 4.30 ± 0.51 | |
| 1 | 4.30 ± 0.33 | |
| 3 | 7.70 ± 1.46 | 9.40 ± 0.65* |
| 10 | 3.80 ± 0.24 | 9.60 ± 1.23* |
| 30 | 4.20 ± 0.32 | 8.40 ± 1.16* |
| 100 | 4.20 ± 0.43 | 8.90 ± 1.07* |
| 250 | 4.20 ± 0.58 | 8.40 ± 0.98* |
| 500 | 4.00 ± 0.38 | 8.10 ± 0.93* |
| 700 | 4.30 ± 0.51 | 7.10 ± 0.74* |
| 800 | | 7.40 ± 0.76* |
| 1000 | 4.00 ± 0.42 | 8.00 ± 0.61* |

The data are expressed as the amount [$^3$H]NA release as a percentage of the total [$^3$H]NA present, and are represented as the mean ± SEM for n = 3 experiments. The basal for the CCH evoked release experiments was 3.5 ± 0.5 %. * Signifies $P<0.05$ when compared to basal release.

**6.4. Discussion.**

This chapter has examined the potential neurotoxicity of the catecholamine derivative TMIQ (Deitrich and Erwin 1980, Bates 1980), and its effects upon catecholamine homeostasis in SH-SY5Y cells. These data show that TMIQ caused a dose and time related inhibition of MTT reduction, and increase in LDH release from SH-SY5Y cells. Therefore, TMIQ appears to be toxic to SH-SY5Y cells, at a concentration (100 μM) within the range of the classic parkinsonian neurotoxin $MPP^+$, which is reported to produce similar levels of toxicity (Sanchez-Ramos 1988, Spina et al 1992, Buckman 1991, Cobuzzi et al 1994). Both N-methylated TIQ derivatives (Naoi et al 1989a) and MPTP (Chiba et al 1984) are known to be bioactivated by monoamine oxidase (MAO) to produce a charged quaternary nitrogen, which has been linked to increased toxicity of the resultant compound (Naoi et al 1989a,b,c). In rat striatal synaptosomes inhibition of MAO, or pre-incubation of nomifensine (a dopamine uptake inhibitor), prior to the addition of TMIQ prevented the inhibition of TH activity (Liptrot et al 1994). However, in SH-SY5Y cells, inhibition of MAO, types A and B, by clorgyline (10μM) and deprenyl (10μM) did not reverse TMIQ induced toxicity. Pre-incubation with imipramine (10μM, an $uptake_1$ inhibitor), followed by concurrent incubation with TMIQ failed to prevent TMIQ induced inhibition of MTT reduction. These data indicate that TMIQ does not require MAO activation to produce cytotoxicity, and that the catecholamine uptake transporter is not involved as a site of entry of TMIQ into SH-SY5Y cells. Whilst bioactivation of TMIQ by MAO may be important in TH inhibition these data suggest that it may not be necessary for cytotoxicity.

TMIQ is related to adrenaline and other catecholamines which are very labile compounds. Therefore, one potential mechanism for toxicity may be that TMIQ may

autoxidise extracellularly, creating free radical species which may initiate cell membrane lipid peroxidation. In order to investigate this hypothesis the protection by the antioxidant and membrane stabiliser α-tocopherol was studied. Pre-incubation of α-tocopherol for 24 hours prior to TMIQ insult, did not prevent TMIQ induced decrease in MTT reduction or increased LDH release in SH-SY5Y cells. The inclusion of the water soluble α-tocopherol analogue, Trolox-C, concurrently with TMIQ did not prevent TMIQ inhibition of MTT reduction. Therefore, it appears that extracellular autoxidation is not the mechanism of TMIQ induced toxicity. In contrast to NMN, the inability of α-tocopherol to prevent TMIQ induced LDH release and the associated inhibition of MTT reduction, infers that non-specific membrane lipid peroxidation is not the mechanism of TMIQ induced toxicity in SH-SY5Y cells. These data suggest that TMIQ may have an intracellular site of action.

It has been reported that TMIQ depleted catecholamine levels in many areas of the rat brain after intracerebroventricular infusion (Liptrot et al 1993) and inhibited TH activity in rat striatal synaptosomes (Liptrot et al 1994). The study of catecholamine uptake in SH-SY5Y cells showed that in monolayer culture TMIQ induced a dose related increase in both [$^3$H]NA and [$^3$H]DA uptake. The combination of inhibition of catecholamine synthesis, and stimulation of catecholamine uptake may explain the rapid depletion of catecholamine levels in the brain after TMIQ administration (Liptrot et al 1993).

The variation observed in the absolute values of the percentages of catecholamine uptake or release for the doses of TMIQ in separate experiments (for examples see tables 6.9-11), is probably due to the variation in the number of cells present in each well (Atcheson et al 1994a). Correction for the total protein would be ideal, however,

this is not practical when dealing with monolayer multiwell culture cells, which will be radioactive after experimentation. Also, as most nerve cells in the brain are interconnected by many synapses, a culture system that mirrors this situation would be more representative than suspension culture.

The stimulation of catecholamine uptake by TMIQ could be due to many potential interactions either at the uptake recognition site, or with one or more intracellular second messengers. To investigate the potential mechanism behind TMIQ stimulated [$^3$H]NA uptake in SH-SY5Y cells, the interaction of TMIQ and [$^3$H]nisoxetine (a high affinity ligand for uptake$_1$), was examined. Only at high concentrations did TMIQ (1mM) displace [$^3$H]nisoxetine binding by 72.8 ± 17.1 % ($P<0.05$), with an estimated $IC_{50}$ of 156 and $K_i$ of 71μM, respectively. Since the stimulation of [$^3$H]NA uptake occurs at a lower concentration of TMIQ ($EC_{50}$ = 8μM), where there was no significant inhibition of nisoxetine binding, it appears unlikely that TMIQ stimulated catecholamine uptake involves the uptake recognition site. There is evidence that alteration in the balance of second messengers may alter glutamate uptake. Casado et al (1993) have demonstrated phosphorylation of the $Na^+$ dependent L-glutamate transporter in pig brain by PKC. Furthermore, glutamate uptake is inhibited after inhibition of PKC in rat spinal dorsal horn cells (Chen and Huang 1991). Stimulated [$^3$H]NA and [$^3$H]DA uptake may be due to TMIQ activation of PKC in SH-SY5Y cells. The effects of inhibiting PKC upon [$^3$H]NA uptake were examined in the presence or absence of TMIQ (10 or 500μM).

However, inhibition of PKC by addition of Ro 31-8220 had no effect upon either [$^3$H]NA uptake with or without TMIQ. In the same vein, other second messengers

may be involved in the regulation of catecholamine uptake. Activation of AC can be induced by addition of the diterpene, forskolin, to cells (Morgan et al 1993). Thus if TMIQ stimulated catecholamine uptake was modulated by to cAMP levels in cells, then enhancement of cAMP production should mirror the effect of TMIQ. Furthermore, as 10μM forskolin elicits maximal AC activation (Hirst and Lambert 1995), one may expect an enhancement of TMIQ stimulated [$^3$H]NA in the presence of both compounds. However, no increase in uptake of [$^3$H]NA was observed with forskolin alone, and raised cAMP had no affect upon TMIQ stimulated [$^3$H]NA uptake. Sodium nitroprusside is known to activate GC to produce another second messenger, cGMP (Murad et al 1978, Bohme et al 1978), which may play a role in the regulation of catecholamine uptake in SH-SY5Y cells. As was the case with forskolin, addition of sodium nitroprusside had no affect upon [$^3$H]NA uptake alone, and did not enhance TMIQ stimulated [$^3$H]NA uptake. Therefore, the increased catecholamine uptake stimulated by TMIQ appeared not to be related to the uptake transporter, nor is it linked to elevated PKC activity, or either activation of adenylate or guanylate cyclases. TMIQ stimulated catecholamine uptake may be due to inhibition of AC or GC. However, this seems unlikely since one would expect antagonism between combinations of forskolin and TMIQ, or nitroprusside and TMIQ, to effectively reduce catecholamine uptake. The precise mechanism remains to be determined.

TMIQ inhibited potassium but not CCH evoked NA release, with maximum inhibition at 750μM, and $IC_{50}$ of 490μM. Since the release coupling mechanism of CCH ($[Ca^{2+}]_i$ dependent) and potassium ($[Ca^{2+}]_i$ independent) are different in SH-SY5Y cells

(Atcheson et al 1994b), salsolinol may block $Ca^{2+}$ entry to inhibit potassium evoked release.

A rough estimate of the concentration of TMIQ after intracerebroventricular infusion can be obtained if the following are taken into account (Cserr and Patlak 1992). Firstly, complete mixing time in cerebral spinal fluid (CSF) is 20 minutes. Secondly, the average CSF volume in the adult rat is 400μL. Thirdly, the half life of CSF is 100 minutes. As the maximum dose of TMIQ infused into rat brain was 300nmole /μL / hour over three hours. One would expect to loose 25 % over the first hour, 50 % over the second hour, and 33 % during the final hour. This would leave a total applied dose of 525nmoles. If one assumes that the extracellular volume, including the interstitial fluid (ISF approximately 15-20 % of the total brain volume of 2000μL), then the extracellular concentration of TMIQ would be approximately 625μM. If the distribution volume were to include even distribution of TMIQ across the whole brain then the added concentration of TMIQ drops to 263μM. The lowest infusion of TMIQ was 100nmoles / μL / hour. If the same calculations are applied to this infusion the concentration of TMIQ in the extracellular fluid would be 219μM and across the whole brain 88μM. It is interesting to note that these rough calculations of rat brain TMIQ concentrations are much greater than the $EC_{50}$ values for catecholamine uptake (8μM and 54μM for Na and DA respectively) in SH-SY5Y cells, and the $IC_{50}$ for TH inhibition in striatal synaptosomes (Liptrot et al 1994). Similarly, rat brain concentrations of TMIQ initially are greater than those required to induce cytotoxic to SH-SY5Y (500μM, after 24 hours), and are well above those found to be toxic after 72 hours (i.e. 30μM).

In conclusion, TMIQ is cytotoxic to human SH-SY5Y cells and enhanced the uptake of catecholamines in to SH-SY5Y cells, in monolayer culture. The combination of stimulated catecholamine uptake and inhibition of TH activity may explain the catecholamine depleting properties of TMIQ in rat brain. Additionally, TMIQ inhibits mitochondrial complex I respiration with sub-mitochondrial particle preparations to a greater degree than MPTP (Phillipson et al 1994, Bristol, personal communication). Therefore, it appears that TMIQ could induce cytotoxicity by inhibition of energy metabolism in SH-SY5Y cells, although this requires further study.

# Chapter 7

# GENERAL DISCUSSION

**7.1. Comparison and summary of the effects of NMN, TMIQ and salsolinol.**

The nicotinamide derivative, NMN, and the catecholamine condensation products TMIQ and salsolinol all induce cytotoxicity in SH-SY5Y cells (table 7.1). NMN is also cytotoxic to B65 cells. However, the structural analogue control for NMN, nicotinamide, is not toxic to SH-SY5Y or B65 cells. Comparison of the individual concentration of NMN (10mM), TMIQ ($IC_{50}$ = 181μM, after 72 hours, measured by MTT reduction) and salsolinol ($IC_{50}$ = 54μM, after 72 hours, measured by MTT reduction), required to induce cytotoxicity shows that TMIQ and salsolinol are far more potent than NMN. TMIQ and salsolinol induce cytotoxicity at a concentration within the range of the potent PD toxin $MPP^+$ (see table 7.4), in vitro. In SH-SY5Y cells, both salsolinol and TMIQ induced a dose dependent increase in LDH release, and inhibition of MTT reduction, which was exacerbated with increased incubation up to 72 hours. In contrast, NMN (10mM) induced maximum release of LDH after 24 hours, combined with gradual decrease in [$^3$H]thymidine incorporation over time. The addition of the antioxidant α-tocopherol produced a dose related inhibition of NMN (10mM) induced LDH release. Conversely, the antioxidants α-tocopherol and Trolox C, had no effect on TMIQ and salsolinol induced LDH release or inhibition of MTT reduction. NMN does not alter membrane fluidity measured in erythrocyte ghosts (B.A. Appadu 1994, personal communication). Therefore, as α-tocopherol acts as both an antioxidant and membrane stabiliser, one may conclude that NMN toxicity may be directed towards the cell membrane, possibly through the induction of free radical damage.

The mechanism of salsolinol and TMIQ toxicity was examined by the inclusion of the MAO inhibitors clorgyline and deprenyl. However, neither clorgyline or deprenyl,

individually, or in combination, prevented the inhibition of MTT reduction induced by salsolinol and TMIQ. Addition of the catecholamine uptake$_1$ inhibitor, imipramine, also failed to prevent salsolinol and TMIQ induced inhibition of MTT reduction.

*Table 7.1. Summary of the cytotoxic effects of NMN, TMIQ and salsolinol in SH-SY5Y.*

| Toxicity | NMN | TMIQ | Salsolinol |
|---|---|---|---|
| LDH release | ↑ | ↑ | ↑ |
| + α-tocopherol | inhibition of release | ND | ND |
| MTT reduction | | ↓ | ↓ |
| + α-tocopherol | | ND | ND |
| + Trolox C | | | ND |
| + Imipramine | | ND | ND |
| + MAO Inhibitors | | ND | ND |
| [$^3$H]Thymidine incorpor. | ↓ | | |
| + α-tocopherol | ND | | |

*The above table indicates the effects of NMN, TMIQ and salsolinol on SH-SY5Y cytotoxicity measured by LDH release, MTT reduction and [$^3$H]thymidine incorporation. The affects of antioxidants (α-tocopherol and Trolox C), imipramine (uptake$_1$ blocker), and MAO inhibitors (clorgyline and deprenyl) are also shown. An increased response (compared to the relevant control) is shown as ↑, and a decreased response as ↓. ND indicates where no difference in response was observed in the*

*presence or absence of antioxidants, imipramine, or MAO inhibitors. NMN cytotoxicity was not assessed using MTT reduction.*

Examination of the effects of the potential toxins, NMN, TMIQ and salsolinol, on catecholamine homeostasis, in SH-SY5Y cells, showed that TMIQ and salsolinol stimulated catecholamine uptake, whilst NMN had little effect (table 7.2). Although NMN (10mM) produced a small decrease in both DA and NA uptake compared to nicotinamide (10mM), at lower doses NMN had no effect. In SH-SY5Y cell monolayer cultures, both salsolinol and TMIQ stimulated catecholamine uptake. TMIQ stimulated uptake with $EC_{50}$ values of 8μM and 54μM, for NA and DA, respectively. Whilst salsolinol stimulated catecholamine uptake below 100μM with $EC_{50}$ values of 17μM and 11μM, for NA and DA, respectively, followed by inhibition above 100μM with $IC_{50}$ values of 411μM and 379μM, for NA and DA, respectively. Investigation of TMIQ and salsolinol stimulated NA uptake indicated that both compounds interact with the uptake recognition site, displacing [$^3$H]nisoxetine binding, with $K_i$ values of 71μM and 353μM, for TMIQ and salsolinol, respectively. However, as salsolinol and TMIQ stimulate catecholamine uptake with lower $EC_{50}$ values than $K_i$ it would seem that the stimulation of uptake does not involve the uptake recognition site. Salsolinol induced inhibition of catecholamine uptake appears to involve the catecholamine binding recognition site, since the $IC_{50}$ values (411μM and 379μM) are similar to the $K_i$ value (353μM) for displacement of nisoxetine binding.
Forskolin was used to increase intracellular cAMP and sodium nitroprusside to increase intracellular cGMP concentrations. However, neither increased cAMP or cGMP concentrations had any effect on NA uptake. In addition neither increased

cAMP or cGMP affected salsolinol or TMIQ stimulated NA uptake. Inhibition of PKC has been shown to decrease glutamate uptake in the dorsal horn cells of the rat spinal cord (Chen and Huang 1991), and PKC can phosphorylate the L-glutamate uptake transporter in pig brain (Casado et al 1993). Despite subsequent inhibition of PKC, with Ro 31-8220 during uptake, no alteration in TMIQ or salsolinol stimulated NA uptake was observed. The effects of NMN on nisoxetine binding were not investigated as only a weak inhibition of catecholamine uptake was observed at 10mM.

*Table 7.2. Summary of the effects of NMN, TMIQ and salsolinol on catecholamine uptake in SH-SY5Y cells.*

| Catecholamine Uptake | NMN | TMIQ | Salsolinol |
|---|---|---|---|
| Monolayer culture | ↓ at 10mM | ↑ | ↑ below 100μM<br>↓ above 250μM |
| + Forskolin | | ND | ND |
| + Nitroprusside | | ND | ND |
| + Ro 31-8220 | | ND | ND |
| [$^3$H]Nisoxetine Binding | | ↓ at 1mM | ↓ at 1mM |

*The above table shows the effects of NMN, TMIQ and salsolinol on catecholamine uptake in SH-SY5Y cells. ↓ Indicates a decrease in catecholamine uptake, and ↑ indicates an increase in catecholamine uptake over a one hour incubation in confluent*

*monolayer cultures. ND indicates no difference in the response was observed in the presence of forskolin, nitroprusside or Ro 31-8220.*

The effects of NMN, TMIQ and salsolinol on NA release from SH-SY5Y cells were also examined. None of the three compounds directly stimulated or inhibited NA release. Both salsolinol ($IC_{50}$ = 120μM) and TMIQ ($IC_{50}$ = 490μM) inhibited $K^+$ (100mM) evoked NA release. Salsolinol ($IC_{50}$ = 500μM) but not TMIQ inhibited CHH (1mM) evoked NA release. Salsolinol can displace the binding of N-methyl scopolamine (NMS, a muscarinic radioligand), with $K_i$ of 215μM. However, salsolinol inhibition of CCH evoked NA release cannot be entirely due to muscarinic antagonism since working from a $K_i$ of 215μM for NMS binding, 5.87mM salsolinol would be required to displace CCH binding by 50 %. In addition TMIQ inhibited NMS binding weakly at 1mM and had no effect at lower concentrations. The effects of NMN on NMS binding were not investigated since NMN had no effect on NA release.

*Table 7.3. Summary of the effects of NMN, TMIQ and salsolinol on [$^3$H]noradrenaline release from SH-SY5Y cells.*

| NA Release | NMN | TMIQ | Salsolinol |
|---|---|---|---|
| Direct stimulus | ND | ND | ND |
| CCH (1mM) | | ND | ↓ above 100μM |
| $K^+$ (100mM) | ND | ↓ above 500μM | ↓ above 100μM |
| [$^3$H]NMS Binding | | weak ↓ at 1mM | ↓ above 100μM |

*An inhibition of CCH (1mM) or $K^+$ (100mM, $Na^+$ adjusted buffer) evoked [$^3$H]NA release from SH-SY5Y cells is shown as ↓. ND indicates where there was no difference in NA release in the presence or absence of toxin.*

### 7.2. Comparison of the toxicity of NMN, TMIQ and salsolinol with MPTP and $MPP^+$.

The majority of work with either MPTP or $MPP^+$ has been undertaken in animal models. However, some studies have shown toxicity of MPTP and $MPP^+$ to isolated cell systems, a summary of which can be seen below:

*Table 7.4. Comparison of the relative toxicity of MPTP and $MPP^+$ to various catecholaminergic cell lines.*

| Cell Type | Incubation Time (Hours) | Concentration of Toxin (μM) | Toxin | Index of Toxicity (%) | Assessment Method |
|---|---|---|---|---|---|
| SH-SY5Y° | 24 | 10 | $MPP^+$ | 95 | TB |
| SKNSH# | 24 | 200 | MPTP | 50 | TB |
| PC12* | 48 | 250 | $MPP^+$ | 150 | LDH |
| | 48 | 500 | $MPP^+$ | 200 | LDH |
| 140-3^ | 24 | 50 | $MPP^+$ | 80 | % VC |
| | 48 | 50 | $MPP^+$ | 55 | % VC |
| | 72 | 50 | $MPP^+$ | 58 | % VC |
| | 120 | 50 | $MPP^+$ | 20 | % VC |
| NCB-20^ | 120 | 50 | $MPP^+$ | 38 | % VC |
| SH-SY5Y$ | 24 | 500 | TMIQ | 50 | MTT |
| | 72 | 100 | TMIQ | 79 | MTT |
| | 24 | 250 | SAL | 186 | LDH |
| | 24 | 250 | SAL | 53 | MTT |
| | 72 | 3 | SAL | 74 | MTT |

*The data is expressed in different formats for each experiment. The SH-SY5Y data is taken from a study by °Spina et al (1992), cytotoxicity was measured by trypan blue exclusion (TB). The SKNSH data is from a study by #Lai et al (1993), cytotoxicity was*

*measured by TB exclusion. The data is expressed as the mean of dead cells from the total counted. The cytotoxicity of PC12 cells was undertaken by *Cobuzzi et al (1994) and is expressed as a percentage of the LDH activity released compared to an untreated control. Data on both the 140-3 and NCB-20, two neuroblastoma hybrids that express MAO-B, is from a study by ^Buckman (1991). The data is expressed as a percentage of the number of viable cells (% VC) present after incubation of the toxin. The data indicating TMIQ and salsolinol toxicity in SH-SY5Y$ cells is from this study, where LDH data is expressed as a percentage of basal release (= 100), and MTT reduction is expressed as a percentage of the control (= 100).*

Despite the differing cell lines, incubation protocols and the methods used to assess cytotoxicity, there is similarity between the concentrations of $MPP^+$, TMIQ and salsolinol used to induce similar levels of cytotoxicity. For example, in SH-SY5Y cells $MPP^+$ (10μM) induced 95 % cell death after 24 hours incubation, measured by trypan blue exclusion. In PC12 cells, $MPP^+$ (250μM) induced 150 % LDH release after 48 hours incubation (i.e. 50 % greater than the control, Cobuzzi et al 1994). The level of LDH release is similar to that released by both salsolinol (100μM) and TMIQ (100μM), after 48 and 72 hours, respectively. Cobuzzi and colleagues report LDH release of 200 % above the control in PC12 cells, after 48 hours incubation with $MPP^+$ (500μM), which is lower than that induced by salsolinol (500μM). Conversely, Buckman (1991) showed that approximately 50 % of 140-3 cells died after 48 to 72 hours incubation with $MPP^+$ (50μM). Indeed, in NCB-20 and 140-3 cells, $MPP^+$ appeared to be toxic at lower doses than 50μM. However, the incubation period was increased to 120 hours. These data would suggest that $MPP^+$ is more potent than

salsolinol or TMIQ. However, after 72 hours TMIQ (30μM) produces significantly increased ($P<0.05$) LDH release and significantly inhibited MTT reduction. Furthermore, salsolinol (10μM) induced a significant ($P<0.05$) increase in LDH release, and 3μM salsolinol significantly ($P<0.05$) inhibited MTT reduction after 72 hours. The small differences between the concentration of $MPP^+$ required to induce similar levels of cytotoxicity may be due to several variations. These may include differences between individual cell susceptibility and variation in the sensitivity of the technique used to assess cytotoxicity. Data from Spina et al (1992) indicated that $MPP^+$ (10μM) induced 90 % cell death (by trypan blue exclusion), after 24 hours incubation. This data allows direct comparison of the potencies of TMIQ, salsolinol and $MPP^+$. Thus, over 24 hours incubation, salsolinol is 25 fold and TMIQ 50 fold less potent than $MPP^+$, in SH-SY5Y cells. This difference decreases over time and may be related to the relevant rates of accumulation of each compound in SH-SY5Y cells.

Since NMN may have induced similar levels of toxicity as $MPP^+$ in vitro one could suggest that the compound is cytotoxic. However, 1000 fold more NMN is required to induce cytotoxicity to the levels seen with $MPP^+$.

**7.3. Is the Bioactivation of Potential Toxins Required to induced Cytotoxicity ?**

Current opinion agrees that the classical PD toxin MPTP is bioactivated to produce a toxic metabolite, $MPP^+$. Bioactivation occurs via the action of MAO-B in glial cells prior to $MPP^+$ uptake into dopaminergic neurones (Salach et al 1984, Westlund 1985, Levitt 1982). The structural alteration of MPTP to $MPP^+$, enables $MPP^+$ to become a substrate for the high affinity DA uptake transporter (Chiba et al 1985, Jarvitch et al 1985). Additionally, blockade of DA re-uptake sites with DA uptake inhibitors will prevent $MPP^+$ toxicity (Jarvitch et al 1985). Given the importance of bioactivation in MPTP toxicity, many researchers have attempted to fit the same bioactivation pathway to potential neurotoxins. For example, the neurotoxin, salsolinol can be N-methylated, during in vivo microdiaylsis (Maruyama 1992) to produce a compound with similar structure to MPTP. Furthermore, N-methylated derivatives of salsolinol have been detected in rat brain (Niwa et al 1991). In a single cell culture, the order of bioactivation would be reversed, requiring uptake followed by MAO bioactivation. As demonstrated in SH-SY5Y cells, salsolinol cytotoxicity is not inhibited by the pre-incubation of MAO with inhibitors (type A and B). Salsolinol is a competitive inhibitor of MAO-A (Naoi 1993), and appears not to be a substrate for this enzyme. This evidence suggests that MAO activation of salsolinol is not required for cytotoxicity. The adrenaline derivative TMIQ is already N-methylated, and as such would only require MAO activation to produce a compound structurally similar to $MPP^+$. However, despite preincubation of MAO inhibitors, TMIQ is cytotoxic to SH-SY5Y cells, indicating that MAO activation of TMIQ is not required to facilitate toxicity. Since bioactivation appears not to be required for salsolinol and TMIQ, and to exert a cytotoxic effect, a charged quaternary nitrogen may be required for uptake

into catecholaminergic neurones, one might doubt whether salsolinol and TMIQ are transported by catecholamine re-uptake systems. NMN appears to resemble the end product of bioactivation, containing a charged quaternary nitrogen. Despite this NMN is neither cytotoxic nor a modulator of catecholamine uptake, except at high concentration (10mM). These data indicate that more than structural similarity to $MPP^+$ is required for uptake and toxicity. Blockade of uptake recognition sites with imipramine failed to inhibit either TMIQ or salsolinol induced toxicity in SH-SY5Y cells. Salsolinol is also toxic to aortic endothelial cells, in vitro, with a similar $IC_{50}$ (38 μM), to that of salsolinol in SH-SY5Y cells (54μM). Thus the presence of a high affinity uptake system appears not to be necessary to facilitate salsolinol toxicity. Consequently, bioactivation of either salsolinol or TMIQ appears not to be an essential process in their toxicity. In addition during catecholamine uptake and release experiments pargyline, a MAO inhibitor, was included primarily to prevent degradation of catecholamines prior to release. Hence, as MAO is inhibited by the action of pargyline, it is clear that bioactivation of salsolinol or TMIQ is not possible. Thus, bioactivation is not required for the stimulation of uptake and inhibition of release seen with both compounds.

**7.4. Do the toxins studied here have intracellular or extracellular sites of action ?**

The fact that neither salsolinol nor TMIQ interact with the catecholamine uptake recognition sites, except at concentrations higher than the $EC_{50}$ values for stimulation of catecholamine uptake, combined with the lack of cytotoxic protection afforded by blockade of $uptake_1$ sites, may suggest that salsolinol or TMIQ do not utilise $uptake_1$ to facilitate cellular entry. Thus one may infer that salsolinol and TMIQ do not enter SH-

SY5Y cells via uptake$_1$. If salsolinol and TMIQ had an extracellular site of action then as both compounds are labile, one may expect cell damage through extracellular autoxidation. However, the addition of the antioxidants α-tocopherol and Trolox C did not inhibit either TMIQ or salsolinol induced toxicity. Therefore, if TMIQ or salsolinol toxicity is extracellular it is not mediated through autoxidation or induction of membrane lipid peroxidation. The high concentration of NMN (10mM) required to induce cytotoxicity, and the lack of interaction of NMN with cellular catecholamine uptake and release infer that NMN may have an extracellular site of action. NMN cytotoxicity is inhibited in SH-SY5Y cells by the inclusion of α-tocopherol. Thus NMN cytotoxicity in these cells is probably induced through non-specific membrane oxidation.

TMIQ and salsolinol stimulate catecholamine uptake with $EC_{50}$ values well below their respective $K_i$ values calculated from the displacement of nisoxetine from the uptake recognition sites. This evidence points to an intracellular site of action. However, the stimulation of catecholamine uptake is not related to alteration of cAMP, cGMP or PKC. Salsolinol inhibited both potassium and CCH evoked NA release, whilst TMIQ inhibited only potassium evoked NA release. Since the mechanisms that couple potassium and CCH evoked release are different in SH-SY5Y cells (Atcheson et al 1994b), one could infer that salsolinol may interfere with either $IP_3$ formation or release of $Ca^{2+}$ from intracellular stores to inhibit CCH evoked NA release.

Data from PC12h cells indicates that salsolinol is accumulated in mitochondria (Maruyama et al 1993b). Indeed PC12h cells have a similar catecholaminergic uptake system to that found in SH-SY5Y cells, allowing the uptake of both DA and NA

(Takahashi et al 1987). In SH-SY5Y cells the catecholamine uptake transporter allows the accumulation of $MPP^+$ (Spina et al 1992) to facilitate cytotoxicity. The involvement of the catecholamine uptake system in salsolinol induced cytotoxicity has not been studied in PC12h cells, although it does facilitate $MPP^+$ toxicity (Takahashi et al 1987). TMIQ is known to inhibit TH in synaptosomal preparations, and this inhibition is blocked by uptake inhibitors (Liptrot et al 1994). Therefore, it seems likely that both TMIQ and salsolinol, but not NMN have intracellular sites of action, although their site of entry appears not to be through $uptake_1$.

**7.5. Effects of salsolinol, TMIQ and NMN on catecholamine homeostasis: comparison of in vitro findings and extrapolation to the aetiology of PD.**

The main feature of PD is the loss the of dopaminergic neurones of substantia nigra leading to the subsequent loss of DA. This study has shown that both salsolinol and TMIQ stimulate the uptake of catecholamines into SH-SY5Y cells. Furthermore, salsolinol and TMIQ inhibit potassium depolarization-mediated, and salsolinol inhibits muscarinic receptor-mediated NA release. Stimulation of catecholamine uptake occurs at low concentrations of both TMIQ and salsolinol, with $EC_{50}$ values below 20μM. Therefore, both compounds will decrease the stimulation of DA release and promote the rapid re-uptake of DA into neurones. If this situation were to occur in vivo the effect would mimic the depletion of dopaminergic cells, since DA release would be inhibited and re-uptake stimulated. The overall effect may be to induce PD like symptoms due to the reduction of synaptic catecholamine concentrations. Furthermore, TMIQ is known to inhibit TH activity in substantia nigra synaptosomal preparations (Liptrot et al 1994). These findings may explain why TMIQ depletes

catecholamine levels in several areas of the rat brain, including the substantia nigra (Liptrot et al 1993). NMN has only limited effects on catecholamine homeostasis in SH-SY5Y cells at very high concentrations. Thus, despite the specific peripheral accumulation of NMN in PD patients, the lack of effects of NMN on DA homeostasis, suggest that it may be unlikely to induce cell death seen in PD.

### 7.6. Potential mechanisms involved in TMIQ, salsolinol and NMN induced toxicity.

*7.6.i. Catecholamine turnover and oxidative stress.*

In PD the gradual decrease in the number of dopamine containing cells produces a subsequent decrease in DA. The fact that clinical symptoms are not observed until up to 80 % of the DA containing cells of the substantia nigra are lost, is due primarily to the rapid turnover of DA in the nerve terminals (Berheimer et al 1961, Creese and Snyder 1979, Schultz 1982). This action decreases the intracellular concentration of DA to relieve the feedback inhibition of TH by DA. Thus, DA synthesis is promoted. However, the rapid turnover of DA, by the action of MAO, may lead to the formation of $H_2O_2$. As $H_2O_2$ can cause oxidative damage to cells, a permanent state of oxidative stress will be present in PD. In addition the high levels of $Fe^{2+}$ may interact with $H_2O_2$ to produce hydroxyl radicals, thus exacerbating the potential for oxidative damage (Cohen 1988, Hirsch et al 1988, Dexter et al 1989, Youdim et al 1989, Jenner et al 1990, Ben-Shacher et al 1991). These events may be responsible for the reports of decreased antioxidants such as glutathione in PD patients (Poirier et al 1986, Riederer et al 1989, Sofic et al 1992). Consequently, any potential toxin that could promote catecholamine turnover may increase oxidative stress in neurones (Spina et al 1989, De

Erausquin et al 1994). Both TMIQ and salsolinol promote the uptake of DA and NA in SH-SY5Y cells, and TMIQ is known to deplete catecholamine levels in rat brain, possibly through the combination of TH inhibition and stimulation of catecholamine uptake. Salsolinol inhibits receptor-mediated NA release, whereas both TMIQ and salsolinol inhibit depolarization-mediated NA release. The combination of these effects would effectively reduce the turnover of catecholamines in vivo. Therefore, TMIQ and salsolinol toxicity is probably not mediated through increased catecholamine turnover and increased oxidative stress.

*7.6.ii. Inhibition of mitochondrial function.*

Mitochondria are central to neuronal energy requirements as neurones rely primarily upon glucose metabolism, being unable to utilise many other potential sources of energy, such as lipids. Additionally, mitochondria are vital for the synthesis of ACh, supplying acetyl CoA. The relationship between energy levels and ACh synthesis is integrally linked, since bout s of mild hypoglycemia are sufficient to deplete ACh synthesis (Gibson et al 1991). Mitochondria also contain MAO, the enzyme that catabolises the breakdown of both DA and NA. Correct mitochondrial function is essential to provide energy, in the form of ATP, via the oxidation of pyruvate. This process also involves the reduction of oxygen to water, during which reactive oxygen species and radicals can be formed (see introduction section 1.7*iv*). The reduction of oxygen is normally tightly controlled to prevent the escape of these cytotoxic molecules from mitochondria. There are specific antioxidant systems in the mitochondria and the cell to intercept any reactive oxygen species, such as glutathione, catalase and superoxide dismutase (Weisinger and Fridovich 1973, Fridovich 1978,

Flohe 1982, Donaldson and Barbeau 1985). Any disturbance of the correct functioning of mitochondria may lead to severe consequences in the cell.

Salsolinol and TMIQ are known to affect mitochondrial integrity. TMIQ is a more potent inhibitor of mitochondrial complex I than MPTP in submitochondrial particles (Phillipson et al 1994, Bristol, personal communication). Whilst salsolinol (40μM) caused major structural damage to mitochondria in aortic endothelial cells (Melzig and Zipper 1993). The accumulation of salsolinol has been reported in PC12h cells (Maruyama et al 1993b). However, it is unclear whether salsolinol directly inhibits mitochondrial respiration (Suzuki et al 1990).

Inhibition of mitochondrial oxidative phosphorylation can induce the production of free radicals. For example inhibition of cytochrome $bc_1$ complex by antimycin can induce superoxide formation (Ksenzenko et al 1983). Furthermore, $MPP^+$ inhibition of mitochondrial complex I induces hydroxyl radical formation detected by electron spin resonance (Rossetti et al 1988). In mitochondria the main protection against free radical damage is provided by glutathione (Donaldson and Barbeau 1985). This protein is used by enzymes, such as glutathione peroxidase to detoxify free radical species, thus preventing oxidative damage. However, in some neurodegenerative diseases, such as PD glutathione levels are depleted (Ambani et al 1975, Kish et al 1985, Perry et al 1982, Riederer et al 1989). Glutathione depletion occurs as a consequence of increased oxidative stress promoted by the increase in dopamine turnover in PD (Hornykiewicz and Kish 1986). Mitochondrial glutathione comprises approximately 15 % of the total cell glutathione (Jocelyn and Kamminga 1974, Jocelyn 1975, Wahllander et al 1979). Indeed, mitochondria lack the enzymes to synthesise glutathione, and rely on uptake from the cytosolic glutathione pool (Griffith and

Meister 1985, Martensson et al 1990, Kurosawa et al 1990). Specific agents that deplete mitochondrial glutathione are known to potentiate the cytotoxicity of xenobiotics, that produce free radical damage (Shan et al 1993). Therefore, a combination of free radical generation, from inhibition of respiratory complex I, and depletion of glutathione during neurodegeneration, may lead to increased susceptibility of mitochondria to free radical damage. Due to their highly reactive nature and very short life span, the damage caused by free radical species is usually specific to their site of generation. This may explain why the addition of α-tocopherol, which incorporates into membranes, was not sufficient to protect the cells from TMIQ or salsolinol toxicity. However, although TMIQ is a more potent inhibitor of mitochondrial complex I than MPTP in submitochondrial particles, the accumulation of TMIQ in intact mitochondria needs to be examined. Furthermore, salsolinol may accumulate in mitochondria (Maruyama et al 1993b), but inhibition of complex I is yet to be demonstrated.

Disturbance of mitochondrial energy production may lead to many other potentially cytotoxic consequences as the energy levels within the cell are depleted. In neuronal cells the membrane potential is generated by the active extrusion of $Na^+$ ions. $Ca^{2+}$ is also removed from the cytoplasm, via active extrusion and active uptake into the endoplasmic reticulum and other organelles. Reduction of cellular energy content will lead to increased $Ca^{2+}$ concentrations in the cytoplasm. This may occur directly due to dysfunction of active transport systems, and through the reversal of the $Na^+$ / $Ca^{2+}$ antiporter. When the neurone is not metabolically compromised the membrane potential is maintained. In such conditions $Ca^{2+}$ is swapped for $Na^+$ in the ratio of 1:3. However, when $Na^+$ concentrations within the neurone are high the antiporter reverses

to extrude $Na^{+}$ in return for $Ca^{2+}$. Since $Ca^{2+}$ controls the activity of numerous enzymes that affect many cellular responses, disturbance of $Ca^{2+}$ homeostasis can lead to cell dysfunction and potential cytotoxic effects (Orenius et al 1989, 1993, Garthwaite and Garthwaite 1990).

### 7.7. Summary and Future Investigations.

This thesis has shown that TMIQ and salsolinol are cytotoxic to human SH-SY5Y neuroblastoma cells, at concentrations within the range of the potent neurotoxin $MPP^+$ (Sanchez-Ramos et al 1988, Buckman 1991, Spina et al 1992, Cobuzzi et al 1994). Salsolinol also appears to be more toxic to SH-SY5Y cells than PC12 cells (Maruyama et al 1993b). In SH-SY5Y cells, salsolinol and TMIQ cytotoxicity is not mediated through MAO bioactivation, and is not prevented by $uptake_1$ inhibitors. Although salsolinol is reported as a MAO inhibitor (Minami et al 1992, Naoi et al 1989c), further investigation of any interaction of TMIQ and MAO in a cell free system, would be required to determine whether bioactivation of TMIQ is possible. Moreover, cell death appears not to be related to oxidative damage directed towards the cell membrane, and autoxidation of salsolinol or TMIQ to instigate oxidative damage, appears unlikely. However, the exact mechanism of TMIQ or salsolinol toxicity remains to be elucidated. Therefore, it would be important to ascertain whether, and to what extent salsolinol and TMIQ accumulate in SH-SY5Y cells. It would also be useful to examine the subcellular distribution of both compounds.

1) Brain derived growth factor (BDNF) is known to protect SH-SY5Y cells from oxidative (6-OH dopamine) and $MPP^+$ damage, through prevention of glutathione oxidation via stimulation of glutathione reductase activity (Spina et al 1992). As both TMIQ and salsolinol are reported to affect mitochondrial function (Suzuki et al 1989, Melzig and Zipper 1993, Phillipson 1994, personal communication), inclusion of BDNF, which prevented the depletion of intracellular glutathione concentrations would be interesting in an attempt to prevent toxicity.

2) Salsolinol and TMIQ may also alter $Ca^{2+}$ homeostasis in SH-SY5Y cells, or may induce excitotoxicity, either directly, or via depletion of cellular energy balance. To investigate this possibility one could include calcium channel blockers, or measure any changes in intracellular $Ca^{2+}$ in the presence of salsolinol and TMIQ. However, initial studies (not shown) have shown that TMIQ fluoresces over the range of all known $Ca^{2+}$ fluorescent dyes. Thus further investigation would require the use of $^{45}Ca$.

3) Despite the examination of salsolinol (Suzuki et al 1989, Melzig and Zipper 1993) and TMIQ (Phillipson et al 1994, personal communication) interaction with mitochondrial respiration in previous studies, further examination would be helpful for clarification of potential mechanisms of toxicity.

5) In our laboratory (Thomas et al 1994, personal communication) $MPP^+$ has been shown to induce oxidative damage to DNA in SH-SY5Y cells. Thus due to the similarities between TMIQ, salsolinol and $MPP^+$, examination of the potential of salsolinol and TMIQ to induce oxidative DNA damage would be interesting. Especially as oxidative DNA damage can induce cytotoxicity if the repair capacity of the cell is exceeded.

6) Both TMIQ and salsolinol stimulate catecholamine uptake in SH-SY5Y cells. In addition at concentrations greater than 250μM, inhibited catecholamine uptake, which correlated to the increasing interaction of salsolinol with the catecholamine uptake recognition site. Stimulation of catecholamine uptake appears not to involve elevation of cAMP or cGMP, or inhibition of PKC. Therefore, further investigation of any second messenger interaction (e.g. $IP_3$, nitric oxide, phospholipase A or phopholipase D) is required to ascertain the mechanism behind TMIQ and salsolinol stimulated catecholamine uptake.

6) Salsolinol and TMIQ had no effect upon basal NA release from SH-SY5Y cells, but inhibited $K^+$ evoked release. The mechanism of this inhibition is unclear and requires further investigation. In addition, salsolinol but not TMIQ inhibited CCH evoked NA release. However, although the mechanism of CCH release may involve some muscarinic receptor antagonism by salsolinol, a much greater concentration would be required to explain salsolinol inhibition of CCH evoked release. Therefore, determination of the site of salsolinol action would be necessary. For example CCH evoked NA release is mediated through $IP_3$ formation and release of $Ca^{2+}$ from intracellular stores. Therefore the effects of salsolinol on $IP_3$ formation may provide further information to explain how salsolinol inhibits CCH evoked NA release.

# Chapter 8

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## 9. PUBLICATIONS ARISING FROM THIS THESIS.

Full Papers.

1. Studies on the neurotoxicity of 6,7-dihydroxy-1-methyl-1,2,3,4-tetrahydroisoquinoline (salsolinol), in SH-SY5Y cells. Accepted for publication in the European Journal of Pharmacology - Environmental Toxicology and Pharmacology section.

2. Studies on the neurotoxicity of 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (TMIQ). In preparation.

Abstracts

A1. Willets J.M., Lunec J., Williams A.C. and Griffiths H.R. Neurotoxicity of nicotinamide derivatives; Their role in the aetiology of Parkinson's disease. Biochemical Society Transactions, 1993; 21: 299S.

A2. Willets J.M., Lambert D.G. and Griffiths H.R. Suitability of B65 and SH-SY5Y neuroblastoma cells as models for 'in vitro' neurotoxicity testing. Biochemical Society Transactions, 1993; 21: 452S.