Neurotoxicity of Environmental Pollutants

By:-

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Synopsis

Brominated flame retardants (BFRs) and alkylphenols (APs) are pollutants commonly found within the environment and have human health concerns due to their endocrine disrupting and cytotoxic effects. BFRs are used to reduce the flammability of a variety of consumer products such as foam furnishings, whereas APs are found in plastic products used by the food industry. This study investigated the neurotoxicity of the most commonly used groups of BFRs and APs on SH-SY5Y human neuroblastoma cells. The results presented in this thesis showed (using cell viability assays) that these pollutants are toxic at low concentrations. Some compounds such as hexabromocyclododecane (HBCD) and 4-nonylphenol (4-NP) induce cell death (apoptosis) by caspases activation (Casp-8, Casp-9 and Casp-3) and cytochrome c release at low micromolar concentrations (IC_{50} ~ 4µM and 6µM, respectively). Consequently this study also showed that these compounds increased intracellular [Ca^{2+}] levels and the production of reactive oxygen species (ROS) within SH-SY5Y cells by causing Ca^{2+}-dependent depolarization of the mitochondria. In support of a Ca^{2+}-mediated mechanism, the data presented here shows that some BFRs and APs inhibit Sarcoplasmic/ Endoplasmic Ca^{2+}-ATPase (SERCA) and to corroborate this over-expressing SERCA1 improved cell viability especially in cells exposed to certain cytotoxic chemicals such as HBCD; this study is the first experiment of this type to be performed. This study also showed that some of these chemicals, at low concentrations had amyloidgenic effects causing the cleavage amyloid precursor protein (APP) into Beta-amyloid (Aβ) and could therefore be implicated in Alzheimer's disease (AD).

Dedication

This thesis is dedicated to my parents, my wife, my son and my daughter, my brother and sister and to all who encouraged and supported.

Acknowledgements

I wish to express my gratitude and appreciation to my supervisor Dr Frank Michelangeli for his sincere, kind, excellent support, advice, guidance and for being available immediately whenever needed. Without his time and infinite patience his work would never have been possible.

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CONTENTS

CHAPTER 1 INTRODUCTION Page

CHAPTER 3 Cell Toxicity Mechanism Page

CHAPTER 4 Molecular Toxicity on Ca^{2+} Transporters Page

List of Table

List of figures

Abbreviations

AchR: Acetylcholine receptor

AD: Alzheimer disease

ATP: adenosine triphosphate

APP: amyloid precursor protein

AICD: APP intracellular domain

Apaf-1: Apoptotic protease activating factor-1

Aβ: beta-amyloid

AIF: apoptosis-inducing factor

APs: alkylphenols

ARs: androgen receptors

ALT: alanine aminotransferase

Asp/ D: aspartic acid

aas: amino acids

Bcl-2: B-cell lymphoma protein 2

Bax: Bcl-2-associated X protein

BPA: bis-phenol A

BHT: 3,5-dibutyl-4-hydroxytoleuene

BHA: butylated hydroxyanisole

BHQ: 2,5-di (tert-butyl)-1,4-benzohydroquinone

BH: Bcl-2 homology

BFRs: Brominated flame retardants

BDE-47: 2,2',4,4'-Tetrabromodiphenyl ether

Bcl-2: B-cell lymphoma 2

BPA: Bis-phenol A

Ca^{2+}: calcium ions

CICR: Ca^{2+}-induced Ca^{2+} release

cAMP: 3',5'-cyclic AMP

cGMP: 3',5'-cyclic GMP

$[Ca^{2+}]_i$: intracellular concentration of calcium

CCE: capacitative Ca^{2+} entrance

CNS: central nervous system

CaM: calmodulin

CARD: caspase-recruitment domain

Cyto. c: cytochrome c

CYP: cytochrome P450

CREB: cAMP-responsive element binding protein

c-JNK: c-Jun N-terminal kinase

DAG: 1,2-diacylglycerol

DED: death effector domain

DES: diethylstilbestrol

DBDE: decabromodiphenyl ether

DBB: decabromobiphenyl

DBPE: pentabromophenyl ether

DISC: death-inducing signal complex

DNA: deoxyribonucleic acid

DHP: dihydropyridine

DD: death domain

DISC: death-inducing signal complex

DcR: decoy receptors

DMSO: dimethyl sulfoxide

ER: endoplasmic reticulum

E-P: phosphorylated SR Ca^{2+}-ATPase

FKBP: FK-506-binding proteins

FADD: Fas associated death domain

EU: Europe

EDs: Endocrine distrupters

EROD: ethoxyresorufin-O-diethylase

ERs: oestrogen receptors

EC_{50}: concentration causing 50% activation

EGTA: ethylene glycol-bis(β-amino-ethyl ether)N,N,N,N-tetra acetic acid

FRs: Flame retardants

FITC: Fluorescein-5-isothiocyanate

FADD: Fas associated death domain

FLIP: Flice-inhibitory protein

GABA: gamma-amino butyric acid

GPCRs: G protein-coupled receptors

Glu: Glutamic acid

γ -GT: L-Gamma -glutamyl-transferase

GSH: glutathione

GPCRs: G protein-coupled receptors

HBCD: hexabromocyclododecane

His: histamine

HxBB: hexabromobiphenyl

h: hour

IP_3: inositol 1,4,5-triphosphate

IP_3Rs: inositol-1,4,5-trisphosphate receptors

Ile: isoleucine

IAPs: inhibitor of apoptosis proteins

lw: lipid weight

IC_{50}: concentration causing 50% inhibition

K_d: dissociation constant

K_m: half-maximal activity constant

K_i: inhibition constant

KDa: kilodalton

Leu: leucine

Lys: lysine

LC_{50}: concentration causing 50% lethal

MCU: mitochondrial Ca^{2+} uniporter

Δψmit: mitochondrial membrane potential

MOMP: mitochondrial outer membrane permeabilization

MAP: microtubule-associated protein

MAPK: mitogen activated protein kinase

MG: myasthenia Gravis

MAP: microtubule-associated protein

MDA: malondialdehyde

min: minute

NCX: Na^+/ Ca^{2+} exchanger

NCE : Na^+/ Ca^{2+} exchanger

NMDA: N-methyl-D-aspartate

NAADP: Nicotinic acid–adenine dinucleotide phosphate

NP: 4-n-nonylphenol

NFTs: neurofibrillary tangles

OMM: mitochondrial membrane

OBDE: Octabromodiphenyl ether

OBB: Octabromobiphenyl

PIP_3: phosphatidylinositol 3,4,5-triphosphate

$PIP_{2:}$ phosphatidylinositol 4,5-triphosphate

PLC: phosolipase c

PMCAs: plasma membrane Ca^{2+}-ATPase

PLB: phospholamban

PHFs: paired helical filaments

PBBs: polybrominated biphenyls

PBDEs: polybrominated diphenyl ethers

PTP: permeability transition pore

Phe : Phenylalanine

PKC: protein kinase C

PMSF: phenylmethylsulphonyl fluoride

PD: Parkinson disease

PMCA: plasma membrane Ca^{2+} ATPase

PP1: phosphatases 1

PP2A: phosphatases 2A

PKA: protein kinase A

PKC: protein kinase C

PCD: programmed cell death

PARP: poly (ADP-ribose) polymerase

PS: presenilin

PAPS: 3′-phosphoadenosine-5′-phosphosulfate

PCBs: polychlorinated biphenyls

PAHs: polynuclear aromatic hydrocarbons

PHFs: paired helical filaments

PVC: polyvinylchloride

ROCC: receptor-operated Ca^{2+}-channels

RyRs: ryanodine receptors

ROS: reactive oxygen species

SOCC: store-operated Ca^{2+}-channels

SERCA: sarco/endo plasmic reticulum Ca^{2+} ATPase

SMOC: secondary messenger operated ion channel

STIM: stromal Interaction Molecule

SPCAs: secretory pathway Ca^{2+} ATPase

SLN: sarcolipin

SULT: sulfotransferase enzymes

SDS: sodium dodecyl sulphate

TRP: transient Receptor Potential Channels,

TM: transmembrane

Tg: thapsigargin

TBBPA: tetrabromobisphenol A

TNF: tumor necrosis factor

TNF-α: tumor necrosis factor-α

TRADD: TNFR associated death domain

TMBP: 1,1,3,3-tetramethylbutylphenol

THs: thyroid hormones

T4: 3,3',5,5'-tetraiodo-L thyronine

T3: 3,3',5-triiodo-L-thyronine

TRs: thyroid hormone receptors

TRH: thyrotropin-releasing hormone

TSH: thyrotropin

TCA: trichloroacetic acid

Thr: threonine

TTR: transport protein transthyretin

TNFRs: tumor necrosis factor (TNF) receptors

TRAIL: TNF related apoptosis inducing ligand

UDPGT: UDP-glucuronosyl transferases

VOCCs: voltage-operated Ca^{2+}-channels

VSCCs: voltage-sensitive Ca^{2+} channels

Val: valine

CHAPTER 1

Introduction

1.0-Introduction

1.1- Background

The human body contains many different organs, such as the brain, heart, lung, and kidney, with each organ performing a different function. Cells are the major structural and functional units of the body's functions **(Shuster, 2003)**. However, cells use an enormous number of signaling pathways, which are part of a complex system of communication to regulate cellular activities and coordinate cell actions **(Berridge et al., 1998)**. This ability to communicate within a cell or between cells is very significant for the correct integration, coordination and control of these cellular events, as any error in the translation of information may result in diseased conditions.

1.2- Cell Communication

Some cell-to-cell communication requires direct cell-cell contact. Some cells can form gap junctions that connect their cytoplasm to the cytoplasm of adjacent cells. Cell communication occurs through both electrical and chemical signals. Communication through electrical signals is found mainly in excitable systems, particularly in the heart and brain. It is usually fast and requires the cells to be coupled together. Communication can also be by a chemical stimulus like neurotransmitters **(Kistler et al., 1982)**, hormones **(Sugden et al., 2004)** or growth factors **(Carpenter and Cohen, 1990)**, which are released by one cell and move to communicate with another cell, thus altering in the activity of the target cell **(Kistler et al., 1982)**. The latter has receptors capable of detecting the chemical signal and responds to it by producing secondary messengers which regulates many of the cellular processes, such as growth, metabolism, secretion and contraction **(Berridge et al., 1998; Li and Hristova, 2006)**.

Stages of cell signaling are divided into three parts; signal reception, signal transduction, and cellular response. The transduction stage is the translation of extracellular signaling molecules (or ligands) to cell-surface receptors that face outward from the plasma membrane and trigger events inside the cell. Most processes of signal transduction involve ordered sequences of biochemical reactions inside the cell, which are carried out by enzymes and activated by second messengers.

Secondary messengers include, calcium ions (Ca^{2+}); 3',5'-cyclic AMP (cAMP); 3',5'-cyclic GMP (cGMP); 1,2-diacylglycerol (DAG); phosphatidylinositol 3,4,5-triphosphate (PIP_3) and Inositol 1,4,5-triphosphate (IP_3) **(Berg et al., 2006; Berridge, 2008a)**.

Receptors can be divided into two major classes: intracellular receptors and cell-surface receptors. Some receptors such as an ion channel-linked **(Role, 1992; Gasic and Heinemann, 1991)** or a tyrosine kinase-linked one **(Porter and Vaillancourt, 1998)**, may not utilize secondary messengers to detect extracellular signals, although their activation can lead to a cascade of intracellular signals. The receptor type on the target cell and the intracellular trigger induced by receptor-ligand binding determines the response of a cell to external stimuli. The phosphoinositide signaling and Ca^{2+} signaling systems have been grouped together and form one major signaling pathway. The phosphoinositide pathway has been implicated in the regulation of a wide variety of cellular processes and diseases, i.e cell differentiation, apoptosis, cancer and Alzheimer's disease **(Berridge, 2008a; Bunney and Katan, 2010; Fowler, 1997)**.

1.3- Neurons

The nervous system consists of nerve cells (neurons) and non-neuronal cells (glial cells which are known as support cells for the neurons in brain) **(Azevedo et al., 2009)**. Neurons are the basic structural and functional units of the nervous system. They transmit electrochemical impulses from the brain to different parts of the body. A neuron has three fundamental parts: the cell body (soma), the dendrites, and the single axon **(fig. 1.1)**. The cell body is the main part and has all of the necessary components of the cell, such as the nucleus (contains DNA which controls cellular activity), endoplasmic reticulum and ribosomes (for building proteins) and mitochondria (for making energy) **(Berridge, 2008b)**. The dendrites are cellular extensions with many branches, and its overall shape and structure is referred to as a dendritic tree. The dendrites are short, relative to the axon. The single axon comes off the cell body and transmits information away from the cell body to other neurons. The axon terminal contains synapses, which are specialized structures where neurotransmitter chemicals are released in order to communicate with adjacent neurons.

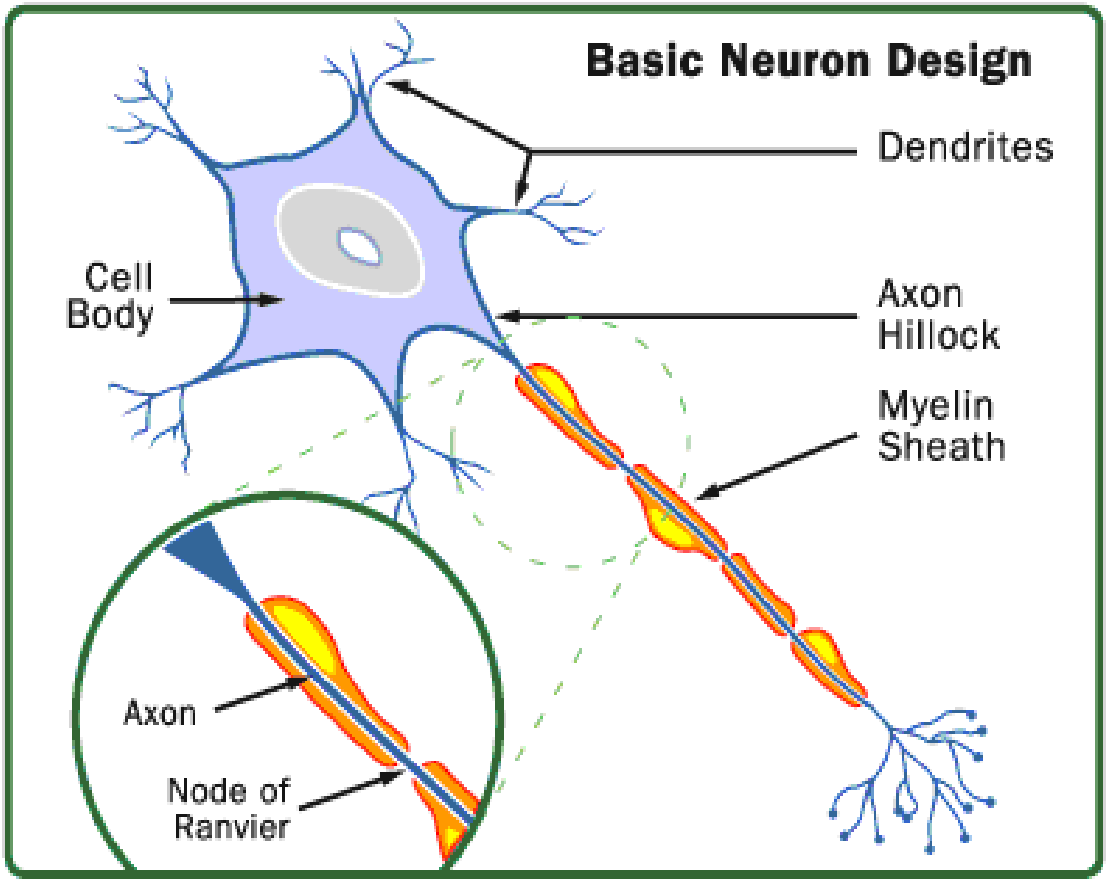

Figure 1.1: neuron structure (adapted from web site: www.health.howstuffworks.com/.../brain1.htm).

1.3.1- Signal transmission between neurons

Neurons communicate with structures called synapses through a process called synaptic transmission. The synapse consists of the two neurons, one of which is sending information to the other. The sending neuron is known as the pre-synaptic neuron while the receiving neuron is known as the post-synaptic neuron. Communication among neurons is a chemical process via synapses. The process is initiated with a wave of electrochemical excitation called an action potential, when an action potential reaches a synapse; channels in the cell membrane are opened allowing an influx of Ca^{2+} (positively charged calcium ions) into the pre-synaptic terminal. This causes a small 'packet' of a chemical neurotransmitter to be released into the small gap between the two cells, known as the synaptic cleft. The neurotransmitter diffuses across the synaptic cleft and activates receptors on the pos-tsynaptic neuron. Ca2+ is essential for this transfer process **(Berridge, 2008b)**.

1.3.2 - Types of neurotransmitters

Neurotransmitters can be divided into three classes. The first class is composed of monoamines such as norepinephrine, dopamine, serotonin and acetylcholine **(Meyers, 2000; Garraway and Hochman, 2001; Urban et al., 1989)**. The second class is composed of amino acids such as glutamic acid, glycine, gamma-amino butyric acid, (GABA), N-methyl-D-aspartate (NMDA) and aspartic acid **(Meyers, 2000)**. The third classes of neurotransmitter are peptides. For example, they are compounds that contain at least two amino acid such as neurotensin, vasopressin, somatostatin, etc **(van den Pol and Tsujimoto, 1985)**. However, once neurotransmitters have been secreted into synapses and have passed on their chemical signals, the pre-synaptic neuron clears the synapse of neurotransmitter molecules. For instance, acetylcholine is broken down by the enzyme acetylcholinesterase into choline and acetate **(Beri and Gupta; 2007)**, while, neurotransmitters such as dopamine is removed by a physical process called reuptake, causing the neurotransmitters to re-enter the pre-synaptic neuron, where they can be broken down by enzymes or re-packaged for reuse **(Arias-Carrion and Poppel; 2007)**.

Neurotransmitters can act as inhibitory or excitatory signals to the post-synaptic cell. A neurotransmitter binds and activates one or more types of receptors. These

receptors are classified into ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels that open or close through neurotransmitter binding. Metabotropic receptors (known as G protein-coupled receptors (GPCRs)), produce second messenger such as cAMP, IP_3 and DAG.

1.3.3- Roles of neurotransmitters in diseases

There are many diseases that are caused by or associated with abnormalities in neurotransmitter pathways. Major types include:-

- Alzheimer disease (AD) is known as a neurodegenerative disease characterized by reduced synthesis of the neurotransmitter; practically in acetylcholine. Patients of AD suffer from loss of intellectual capacity, disintegration of personality, mental confusion, hallucinations, and aggressive-even violent-behavior. These symptoms are the result of progressive degeneration in many types of neurons in the brain **(Alloul et al., 1998; Wenk, 2003)**.

- Parkinson disease (PD) is a degenerative disorder of the central nervous system (CNS) which is caused by deficiency of neurotransmitter dopamine. PD belongs to a group of conditions called movement disorders. The clinical features of PD include weakens in motor skills, speech, muscle rigidity, tremor, a slowing of physical movement (bradykinesia), and in extreme cases, a loss of physical movement (akinesia) **(Di- Monte et al., 2002; Jankovic, 2008)**.

- Myasthenia Gravis (MG) is a neuromuscular disease leading to muscle weakness. MG is caused by circulating antibodies that block the acetylcholine receptor (ACRs) at the post-synaptic neuromuscular junction, inhibiting the stimulatory effect of the neurotransmitter acetylcholine **(Conti-Fine et al., 2006; Lane et al., 2009)**.

1.4 - Neuronal calcium

Neurons modulate Ca^{2+} signals by regulating the influx of Ca^{2+} into the cell from the extracellular environment or by its release from internal organelles such as the endoplasmic reticulum (ER) **(Michelangeli et al., 2005)**. The ER is an essential intracellular organelle involved in calcium homeostasis, and in the folding and processing of proteins **(Baumann and Walz, 2001)**.

The concentration of cytosolic free Ca^{2+} (intracellular $[Ca^{2+}]_i$) in most neurons at rest is about 100nM. This very low resting concentration is raised rapidly to the low micromolar (0.5-1μM) range when specifically stimulated **(Burgoyne and Petersen, 1997)**. Increased $[Ca^{2+}]_i$ is vital for the effective regulation of numerous cellular functions, in particular synaptic plasticity and memory **(Berridge et al., 1998)**. It is essential for a resting cell to maintain a low cytosolic Ca^{2+} concentration or regain the resting Ca^{2+} level, immediately after an agonist-induced response; otherwise this could lead to adverse effects such as apoptosis. The level of free $[Ca^{2+}]_i$ is regulated and maintained at approximately 100 nM through the action of a number of binding proteins and transport mechanisms. Some signals can open Ca^{2+} channels in the cell membrane to allow Ca^{2+} entry **(Miller, 2001)**. The Na^+/ Ca^{2+} exchanger (NCX, also known as NCE) and the plasmalemma Ca^{2+}-ATPase, can pump Ca^{2+} out of the cell **(Carafoli, 1992)**, while the sarco/endo plasmic reticulum Ca^{2+} ATPase (SERCA) pump Ca^{2+} back into the intracellular stores **(fig. 1.2) (Rosado et al., 2004; Pozzan et al., 1994; Berridge et al., 2000)**.

The main Ca^{2+} stores in cells are in the lumen of the ER. Studies have also demonstrated that the Golgi apparatus may be a Ca^{2+} store in a variety of cells **(Hu et al., 2000; Sudbrak et al. 2000; Michelangeli et al., 2005)**, where the Ca^{2+} concentration approaches 500-1000 μM **(Mogami et al., 1998; Alvarez et al., 1999)**, which is about 10000 times higher than within the cytosol. Ca^{2+} release from the ER occurs through two types of calcium channel: ryanodine receptors (RyRs) and inositol-1,4,5-trisphosphate receptors (IP_3Rs) **(Berridge et al., 2000)**. Furthermore, SERCA Ca^{2+} pumps helps to maintain low cytosolic resting Ca^{2+} level by actively pumping Ca^{2+} from the cytosol into the ER stores **(Ogunbayo and, Michelangeli, 2007)**.

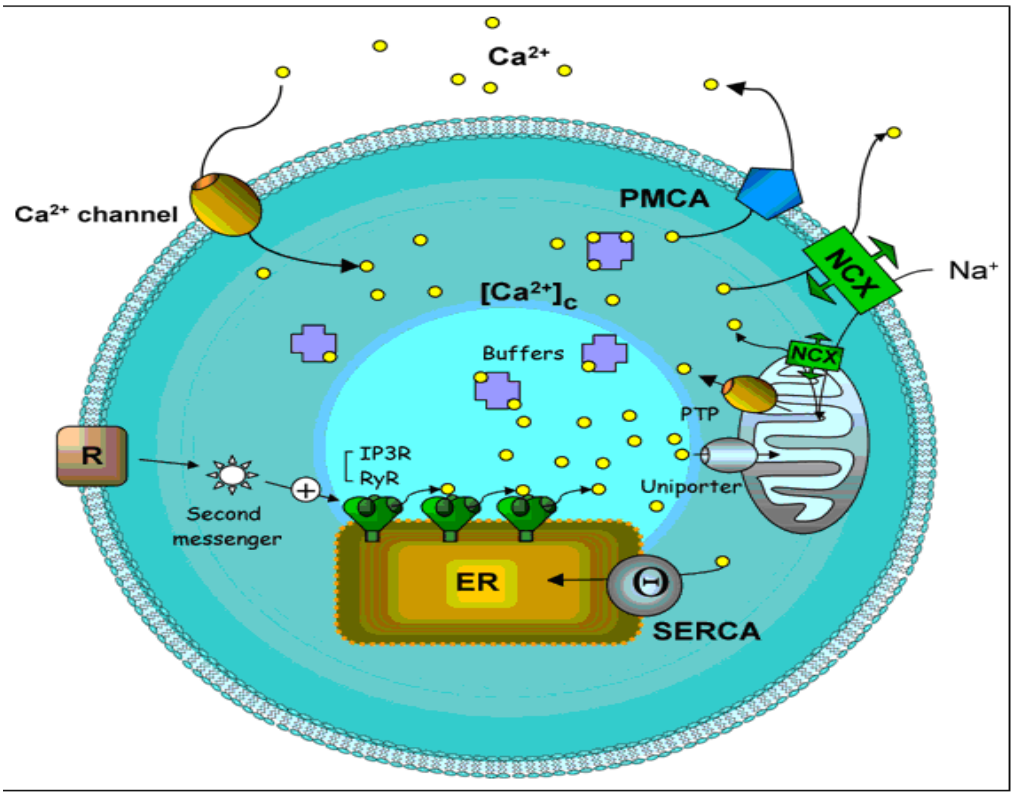

Figure 1.2: mechanisms of calcium signalling. Cell stimulation by agonists activates the formation of second messengers that induce the release of Ca^{2+} stored in the ER through IP_3R and RyR. In addition, agonists stimulate the entry of extracellular Ca^{2+} through plasma membrane channels. Most of the cytosolic Ca^{2+} is bound to to Ca^{2+} binding protein, and only a small percentage binds to effectors and activates cellular functions. Ca^{2+} removal is mediated by various pumps and exchangers, including the NCX and the plasma membrane Ca^{2+}-ATPase (PMCA) that extrude Ca^{2+} from the cytosol and the SERCA that pumps Ca^{2+} back to the ER. During the Ca^{2+} signal mitochondria sequester Ca^{2+} through an uniporter that might be then released slowly into the cytoplasm through the NCX or the permeability transition pore (PTP) **(Rosado et al., 2004)**.

1.5- Ca^{2+} entry (influx) Channels

The entry of Ca^{2+} across the plasma membrane of a cell is by activating many Ca^{2+} influx channels, which open in response to different external signals. They can be divided into groups on the basis of their activation mechanisms **(Berridge et al., 2000)**. These include:-

I- Voltage-operated Ca^{2+}-channels (VOCCs)- such as dihydropyridine (DHP)-sensitive Ca^{2+} receptor **(Lu et al., 1995; Bers, 2002; 2004)**.

II- Receptor-operated Ca^{2+} channels (ROCCs)- such as N-methyl-D-aspartate receptor (NMDAR) and glutamate receptor **(Bertolino and Llinas, 1992; Bootman et al., 2001)**.

III- Store-operated Ca^{2+}-channels (SOCCs)- such as Transient Receptor Potential (TRP) Channels, Stromal Interaction Molecule (STIM) and Orai **(Huang et al., 2006; Yeromin et al., 2006; Guo and Huang, 2008)**.

1.6- Ca^{2+} release (efflux) channels

Release of Ca^{2+} from the intracellular stores that are implanted in the ER is mediated by several different types of messenger-activated channels, and potentially by activation of the mitochondrial PTP. IP_3Rs and RyRs are the most important channels, which regulate the release of Ca^{2+}. These channels are sensitive to Ca^{2+}, and this Ca^{2+}-induced Ca^{2+} release (CICR) process contributes to the rapid rise of Ca^{2+} levels during the "on reaction" and plays a central role in the development of regenerative Ca^{2+} waves **(Bootman et al., 2001)**. Nicotinic acid–adenine dinucleotide phosphate (NAADP) has also been proposed to be involved in intracellular Ca^{2+} release from lysosomal Ca^{2+} stores **(Churchill et al., 2002; Boittin et al., 2002; Kinnear et al., 2004)**.

1.6.1- Inositol 1,4,5-trisphosphate receptors (IP_3Rs)

The IP_3R is one of the Ca^{2+} channels found within the membrane of the ER and belongs to a group of the secondary messenger operated ion channel (SMOC), which

plays a key role in the Ca^{2+} signaling used during learning and memory **(Berridge et al., 2000)**. From molecular studies, IP_3Rs are large structures composed of four subunits (total molecular mass about 1200 kDa). Three IP_3R isoforms have been identified: IP_3R type 1 (IP_3R1), IP_3R type 2 (IP_3R2), and IP_3R type 3 (IP_3R3), which are derived from three different genes and each of them, has different affinity to IP_3 **(Patel et al., 1999**, **Yamamoto-Hino et al., 1994; Iwai et al., 2005; Dyer and Michelangeli, 2001)** Furthermore, analysis of IP_3R expression has shown that both homomeric and heteromeric IP_3Rs exist in different tissues/cells **(Taylor et al., 1999)**.

IP_3 is water soluble and produced from lipid breakdown. Elevation of cytosolic Ca^{2+} through IP_3Rs requires the activation of G proteins on the cell surface which leads to the activation of an enzyme phospholipase C (PLC), which catalyses the cleavage of phospholipids to PIP_2 to produce the intracellular messenger DAG and IP_3 **(Berridge et al., 2000)**. IP_3 diffuses into the cytosol and binds the IP_3R increasing its sensitivity to Ca^{2+} **(Kasri et al., 2004; Taylor, 2002)**, resulting in a rise in cytosolic Ca^{2+} level. After the activation of IP_3R, the IP_3 is then inactivated either by phosphorylation (to IP_4) or dephosphorylation (to IP_2) and the IP_3R is restored to the unstimulated state by an inactivation process **(Dyer and Michelangeli, 2001)**. The IP_3R can be phosphorylated by protein kinases, such as PKA, PKC, PKG, PKB and CaMKII **(Ferris et al., 1992; Wojcikiewicz and Luo, 1998; Szado et al., 2008)**. The consensus sites of phosphorylation for many different protein kinases are present in the primary sequence of all IP_3R isomers. IP_3R1 can be phosphorylated by PKA at two sites located in the regulatory domain **(Suppatapone et al., 1988)**.

IP_3R has a long N-terminal and short C-terminal with six transmembrane domains (M1-M6) located near C-terminal region **(Mikoshiba, 2007)**. The type 1 IP_3R isoform is expressed in neurons such as SH-SY5Y cells **(Mackrill et al., 1997)** and structurally divided into five different domains according to *function*: an N-terminal coupling domain (or suppressor domain), an IP_3-binding core domain, a modulatory domain (also known as an internal coupling domain), a transmembrane/ channel-forming domain, and a gatekeeper domain. The IP_3-binding signal is transmitted by the N-terminal coupling domain and the modulatory domain to the C-terminal tail (gatekeeper domain and transmembrane/ channel-forming domain), which triggers a conformational change in the activation gate (opening) **(Mikoshiba, 2007) (fig.1.3)**.

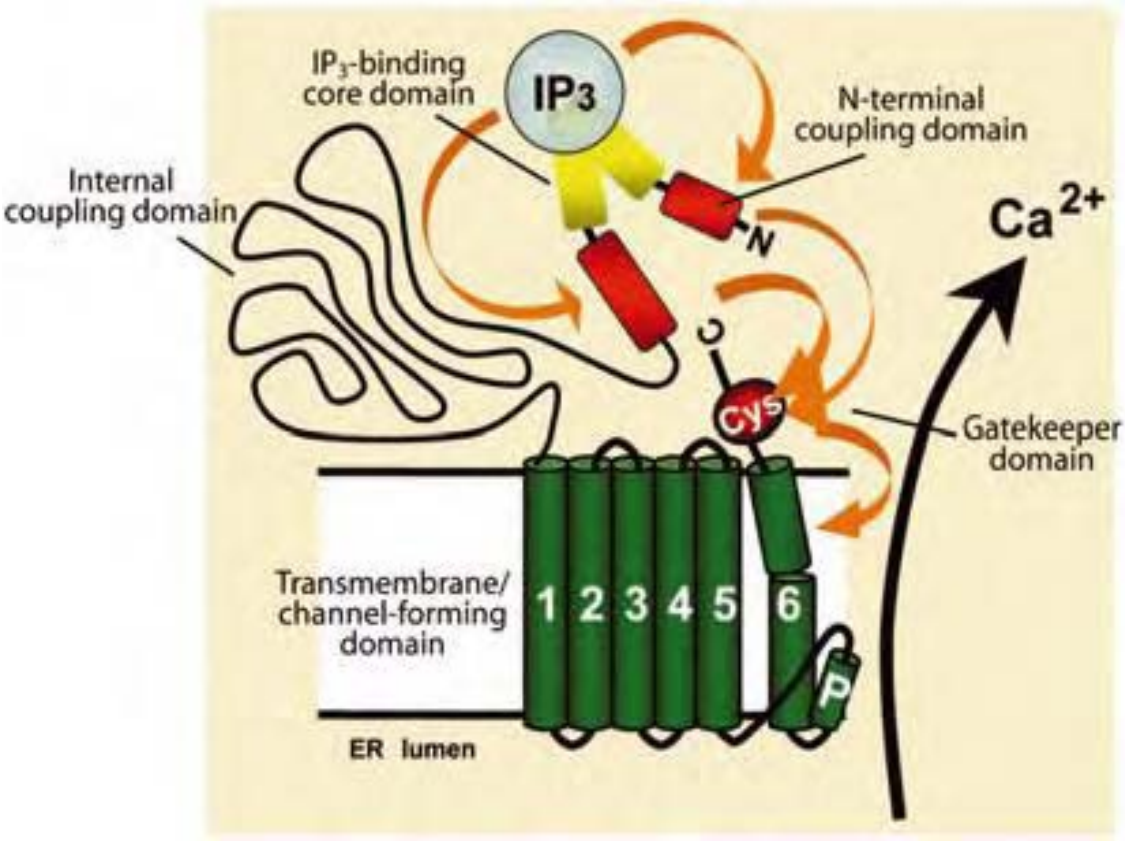

Figure 1.3: the structure of IP_3Rs

Five domains structural model of IP_3R1, which triggers a conformational change in the activation gate channels **(Mikoshiba, 2007)**.

1.6.2- Ryanodine receptors (RyRs)

RyRs is another channel by which Ca^{2+} is mobilized from intracellular stores **(Berridge et al., 2000; Carafoli, 2002; Fill and Capello, 2002)**.

RyR channels are formed by the assembly of four subunits with a large cytoplasmic assembly and a small transmembrane region. Three different isoforms of RyRs, each encoded by a different gene on different chromosomes with specific tissue distributions and distinct functions have now been identified in mammals, designated as skeletal (RyR1) **(Fleischer et al., 2008)**, RyR2 was identified in neurons (SH-SY5Y cells) **(Mackrill et al., 1997)** and cardiac cells, and most other tissues express RyR3 or a combination of all three isoforms **(Fleischer et al., 2008)**. Each channel is composed of four identical subunits that contain about 5,000 residues aas in length (~ 560 kDa) depending on the isoform **(Du et al., 2002; Fleischer et al., 2008)**. The primary domain of the RyR is present upon the cytosolic face of the membrane (known as the "foot" region) **(fig. 1.4)**.

Each subunit consists of a cytoplasmic region and a transmembrane (TM) domain **(Du et al., 2002)**. Based on sequence analysis, RyR proteins contain at least two functional domains: a large N-terminal cytoplasmic domain (about 4500 aas) and C-terminal (about 500 amino acids) region. The large cytoplasmic domain containing many of the regulatory and several binding sites for modulators such as calmodulin (CaM), FK-506-binding proteins (FKBP), high and low affinity binding sites for Ca^{2+}, as well as phosphorylation sites (protein kinase A (PKA), phosphatases 1 and 2A (PP1, PP2A) **(Wagenknecht et al., 1997; Williams et al., 2001)**. However, several models have predicted 4, 6 and 10 TM-spanning segments, but the consensus is for six **(Bers, 2004) (fig. 1.4)**.

The RyR channels activity are modulated by numerous factors, involving a number of physiological agents (e.g., Ca^{2+}, ATP, and Mg^{2+}), various cellular processes (e.g., phosphorylation, oxidation, etc.), and many pharmacological agents (e.g., Ryanodine, Caffeine, Tetracaine, and Ruthenium red) **(Franzini-Armstrong and Protasi, 1997; Shoshan-Barmatz and Ashley, 1998)**. Moreover, the three known isoforms have different sensitivities to Ca^{2+}, whereas only RyR1 isoforms is activation by Ca^{2+} at bell-shaped response **(Sarkozi et al., 2000)**.

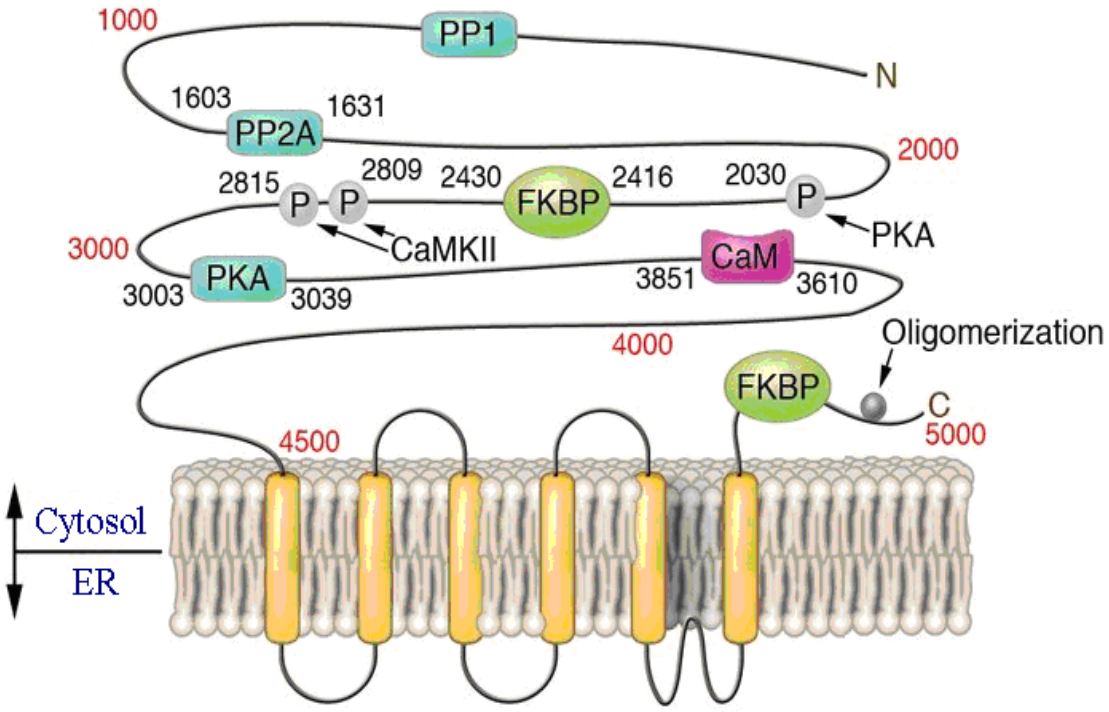

Figure 1.4: schematic showing the predicted structure of the RyR2, including the sites of interaction with ancillary proteins and the phosphorylation sites. Proteins interacting with RyR in the ER are also depicted. Protein phosphatase (PP); phosphorylation sites (P); calmodulin (CaM) **(adapted from Bers, 2004)**.

1.6.3- Permeability transition pore channels of mitochondria

Mitochondria have been known as key regulators of cell death (apoptosis) through the release of mitochondrial proteins such as cytochrome c by mitochondrial permeability transition pore (PTP) **(Bernardi et al., 1999; Halestrap et al., 1997)**. The PTP is a channel found in the outer mitochondrial membranes and is regulated by pro-apoptotic members of the Bcl-2 family (Bax and Bak) **(Isenberg and Klaunig, 2000; Tsujimoto and Shimizu, 2007)**. PTP can also release large amounts of mitochondrially stored Ca^{2+} into the cytoplasm when opened **(Bernardi et al., 1999)**.

1.7- The Ca^{2+} Pumps

Ca^{2+} transport ATPases are ion pumps which belong to a large family of membrane proteins that are responsible for the active transport of cations across biological membranes. There are at least three members of these ATPase pumps essential for cellular Ca^{2+} homeostasis: (i) the Plasma membrane Ca^{2+}-ATPase (PMCAs) **(Shull and Greeb, 1988; Strehler et al., 1990; Carafoli, 1994; Strehler and Zacharias, 2001)**; (ii) the Sarcoplasmic / Endoplasmic Ca^{2+}-ATPase (SERCA) **(MacLennan et al., 1985; Brandl et al., 1986; Burk et al., 1989)**; (iii) the Secretory pathway Ca^{2+} ATPase (SPCAs) **(Gunteski-Hamblin et al., 1992; Ton et al., 2002; Wuytack et al., 2003; Shull et al., 2003; Van Baelen et al., 2001; Wootton et al., 2004)**. These pumps depend on the auto-phosphorylation of a conserved aspartic acid residue, utilizing ATP and forming a phosphorylated intermediate during the reaction cycle **(Axelsen and Palmgren, 1998)**. PMCAs and SERCAs play a vital role in maintaining Ca^{2+} homeostasis, controlling contractility and contributing to excitably and cell signalling in smooth muscle **(Floyd and Wray, 2007)**. They fundamentally exhibit the same basic properties, such as, membrane topography and high affinity for Ca^{2+}, but are different in their functional properties for example; SERCAs are inhibited by thapsigargin, while PMCAs and the SPCAs are not. Additionally, PMCAs are stimulated by fatty acids, while SERCAs are inhibited **(Carafoli and Brini, 2000)**. As this project essentially focuses on SERCA, this will only be considered in further detail.

1.7.1- The Sarco/Endoplasmic Reticulum Ca^{2+}-ATPases (SERCA)

SERCA represent a highly conserved family of Ca^{2+}-ATPase pumps which actively transport two Ca^{2+} ions from the cytosol into the lumenal side of the SR/ER against a large concentration gradient (in exchange for 2 or 3 H^{+} per one ATP hydrolysed) **(Carafoli and Brini, 2000; Inesi, 1987)**. SERCA is an integral membrane protein with molecular mass ~110 KDa, consisting of a single polypeptide chain **(MacLennan et al., 1985)**. Three genes have been identified which code for SERCA pumps (SERCA 1, SERCA 2 and SERCA 3) In the human, these genes are located on chromosomes 16, 12 and 17, respectively and the genes products can also be expressed in a number of spliced variants **(Dode et al., 1996; MacLennan et al., 1987; Floyd and Wray, 2007)**.

1.7.1.1- Characteristics of the different isoforms of SERCA

SERCA1a and -1b are expressed in skeletal muscle (adult and neonatal, respectively), whereas SERCA2a is expressed in cardiac muscle **(Aubier and Viires, 1998; Green et al., 2009)** and SERCA2b is the major ER Ca^{2+}-ATPase in non-muscle tissues, is expressed in the heart, smooth muscle and neuronal tissues **(Lytton et al., 1989; Baba-Aissa et al., 1998)**. Moreover, SERCA 1 is exclusively expressed in fast skeletal muscles, and it is of high concentration in adults, representing up to 80 % of the total protein of the SR of striated muscle **(Michelangeli and Munkonge, 1991)**. SERCA3 has limited expression in various non-muscle tissues **(Baba-Aissa et al. 1998; Martin et al., 2002, Green et al., 2009)**. In humans, six different splice variants of SERCA3 are known (SERCA3a-f) **(Bobe et al., 2004).**

1.7.1.2- Regulation of SERCA activity

Due to the prominent importance of SERCA for the control of $[Ca^{2+}]i$ changes in many cell types, any modulation of its activity should have a considerable effect upon Ca^{2+} homeostasis, the regulation of SERCA in vivo is comparatively limited. Therefore, SERCA pump activity is regulated by two different proteins; Phospholamban (found in the heart) **(Simmerman and Jones, 1998)** and (ii) Sarcolipin (found in fast-twitch skeletal muscle) **(Odermatt et al., 1997)**.

1.7.1.3- Molecular structure of SERCA

The SERCA pump functions as an H^+ exchanger, where the translocation of Ca^{2+} ions is coupled to the liberation of protons into the cytoplasm, at the expense of ATP hydrolysis **(Inesi, 1987)**. The pump may be catalytically active as a monomer; however, **Carafoli and Brini (2000)** have shown that oligomerization is needed for transport function. The Ca^{2+} -ATPase comprises a large cytoplasmic headpiece, short lumenal loops and a transmembrane domain made of 10 transmembrane α-helices (M1-M10) (with exception of SERCA2b, which has 11) **(Toyoshima and Inesi, 2004)**. Both the C- and N-termini protrude into the cytosol (with the exception of SERCA2b). The cytoplasmic headpiece consists of three domains, which are represented as A (actuator), N (nucleotide binding) and P (phosphorylation) domains. The A domain functions as the 'actuator' of the gating mechanism that regulates the binding and release of Ca^{2+}, while the N domain contains the binding site for ATP **(Toyoshima et al., 2000) (fig. 1.5)**.

All the three cytoplasmic domains (A, P and N) of the ATPase are broadly spaced in the Ca^{2+} bound form ($E1Ca^{2+}$) and more closely associated in the Ca^{2+} free and thapsigargin (Tg)-bound structure, E2(Tg) **(Toyoshima et al., 2000; Toyoshima and Nomura, 2002)**. During the subsequent hydrolysis of E2-P to E2, the compactly organized state of the cytoplasmic domains becomes more relaxed **(Danko et al., 2001a; Danko et al., 2001b)**, and the postulated Ca^{2+} release pathway (M3-M5) **(Toyoshima et al., 2000)** and lumenal gate should be closed **(Toyoshima and Nomura, 2002)** to prevent possible Ca^{2+} leakage, and prepare for the entry of new Ca^{2+} from the cytoplasmic side to the transport sites **(Toyoshima and Nomura, 2002)**.

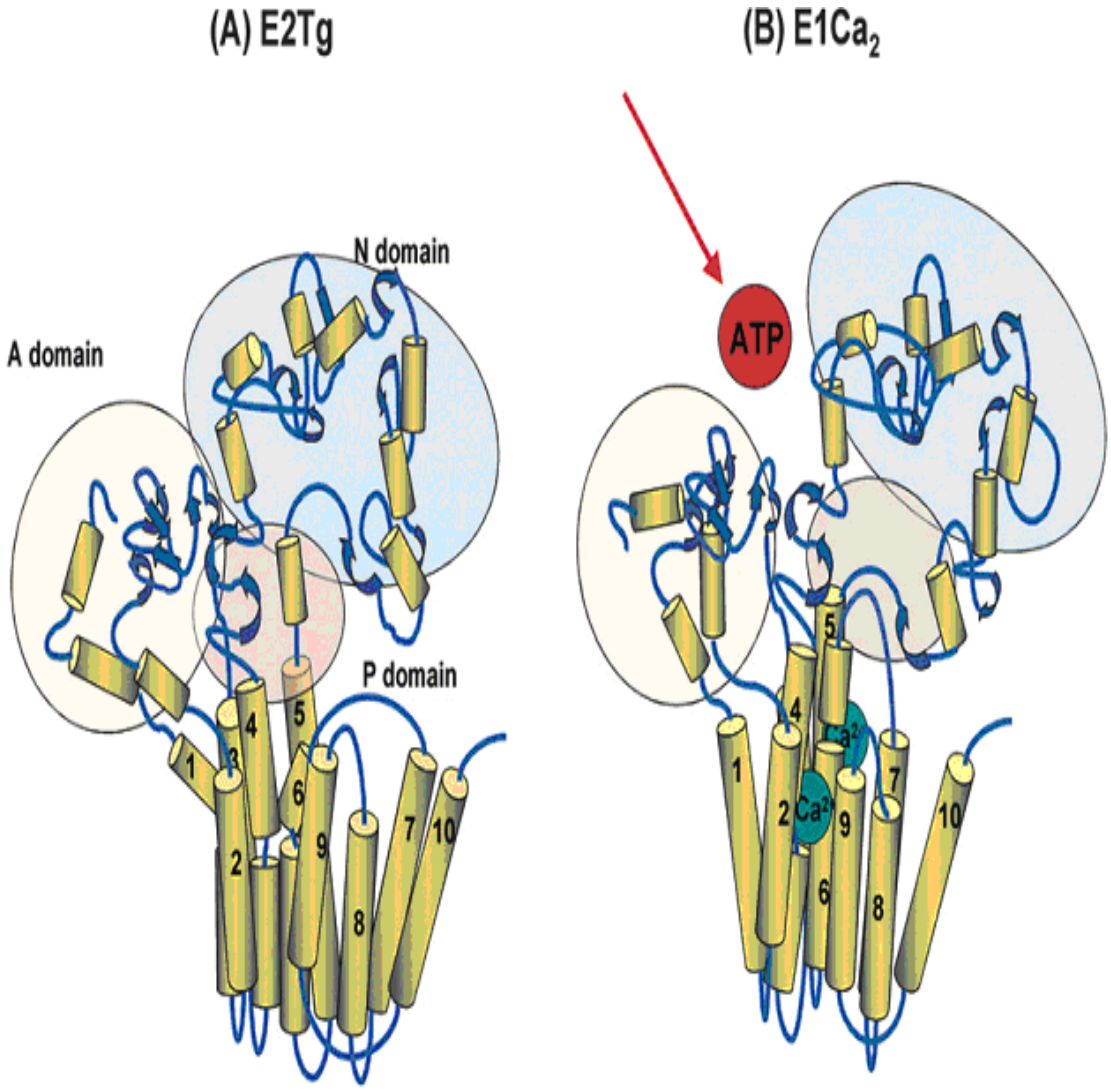

Figure 1.5 shows the crystal structure of SERCA 1a in the two conformational states (E1 and E2). The conformational change of SERCA by Ca^{2+} was established from the crystal structures of (A) the E2-thapsigargin (Tg) form and (B) the $E1Ca_2$ form of SERCA1a. There are three different domains in the cytoplasmic region (A domain, actuator; N domain, nucleotide-binding domain; P domain, phosphorylation domain) that are close to each other in the E2 Tg form. When Ca^{2+} binds to SERCA1a (E1 Ca_2 form), these three domains are separated, allowing ATP to bind to SERCA1a. At the same time, large-scale rearrangements in 6 of the 10 transmembrane helices are induced. By hydrolysis of ATP, Ca^{2+} is released into the lumen of the SR, changing conformationally to E2 form and creating a pathway for entry of new calcium ions on the cytoplasmic side. These are cartoon representations based on the two forms of the crystal structures of SERCA1a **(modified figure from Asahi et al., 2003)**.

1.7.1.4- Catalysis and transport mechanism of SERCA

The catalysis and transport mechanism of SERCA is via alternation of enzyme between two major conformational states, known as E1 and E2 **(de Meis and Vianna, 1979; de Meis, 2000; Danko et al., 2004)**. In the catalytic cycle, the ATPase is activated by the binding of two Ca^{2+} ions at high affinity transport sites (Ca_2.E1) **(fig.1.6)**. The transition between the two states (E1 and E2) is dependent on many factors, including the presence of Ca^{2+}, ATP and Mg^{2+}, in addition to pH and temperature. Phosphorylation at Asp^{351} with MgATP to form the ADP-sensitive phosphoenzyme (E1-P). Upon this E1-P formation, the two bound Ca^{2+} are occluded in the Ca^{2+} binding sites (E1-P.Ca_2 and transforms the enzyme into an E2 conformation. During this conformational change, Ca^{2+} is then released from the binding site as a result of a decrease in Ca^{2+} affinity associated with the transformation of E1 to E2 state and Ca^{2+} passes through the ATPase into the lumen. Finally, Asp^{351}-acylphosphate in E2-P is hydrolyzed to form the Ca^{2+}-unbound inactive E2 state. Mg^{2+} bound at the catalytic site is required as a physiological catalytic cofactor in phosphorylation and dephosphorylation and consequently for the transport cycle. **(MacLennan et al., 1997; McIntosh et al., 1998; Wuytack et al., 2002; Toyoshima and Inesi, 2004; Toyoshima, 2008; 2009)**.

1.7.1.5- ATP binding and phosphorylation

Binding of ATP to the Ca^{2+}-ATPase in the presence of Ca^{2+} leads to closer rearrangements of the N and P domains, so that the terminal phosphate of ATP bound to the N domain come into contact to Asp^{351} (D351), which is the residue of phosphorylation in the P domain **(Hua et al., 2002)**. Further, it is hypothesized that Ca^{2+} binding somehow triggers movement within the transmembrane domains to allow movement in the A domain. This domain in turn, acts on the N domain by bringing it closer to the P domain **(fig. 1.7) (Toyoshima et al., 2000)**. The movement of the A domain upon Ca^{2+} binding is quite substantial.

The N domain contains the adenosine binding pocket formed by residues including Phe^{487}, Lys^{492}, and Lys^{515}. Lys^{515} is actually located in the depth of the ATP binding packet **(fig. 1.7) (Toyoshima et al., 2000)**, and not over the mouth of it, as previously suggested **(Pick, 1981)**.

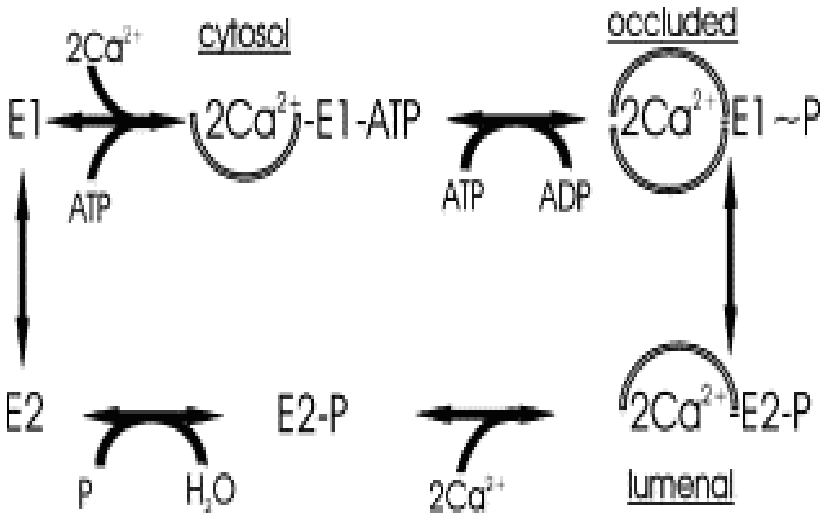

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Figure 1.6: scheme of Ca^{2+}transport cycle of SERCA **(Wuytack et al., 2002)**.

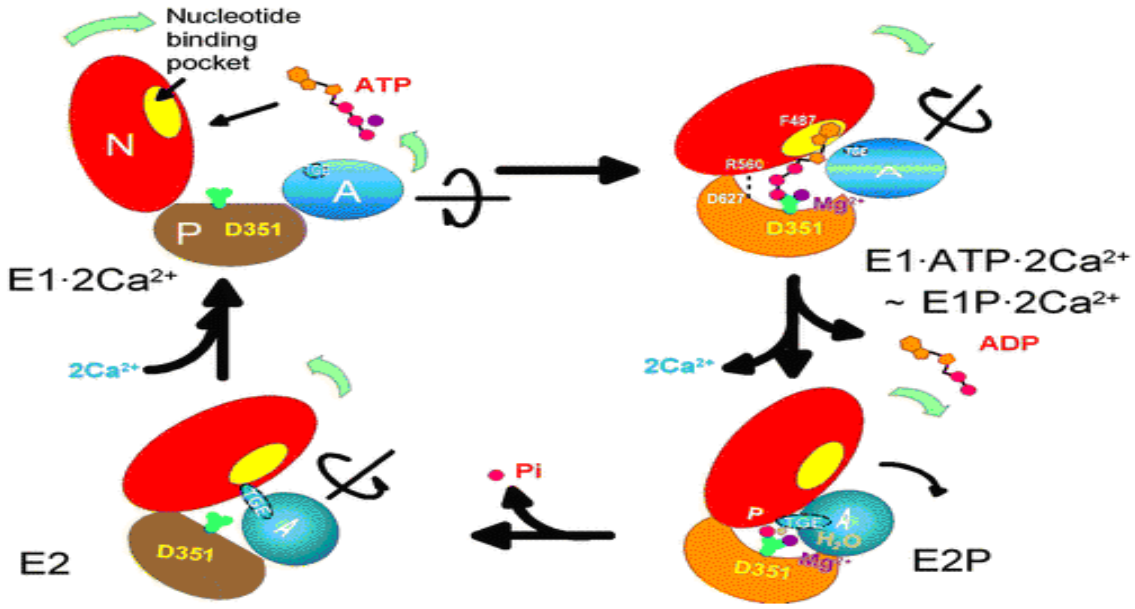


Figure 1.7: schematic models for four distinct arrangements of the three cytoplasmic domains in the reaction cycle of Ca^{2+}-ATPase. D351 (Asp^{351}) is the residue of phosphorylation. Transfer of "occluded" Ca^{2+} to the lumenal side is thought to take place between E1P and E2P **(Toyoshima and Inesi, 2004)**.

Because of the high resolution of SERCA structures to 1.9Å, **Toyoshima and Inesi (2004)** assumed that since the position of the A domain is affected by the N and P domains, the interfaces between them must be critical and should be altered during ATPase reaction cycle. These researchers elucidated that phosphorylation or the binding of the γ-phosphate (which accompanies the dissociation of Mg^{2+} from ATP and the binding of Mg^{2+} to the P domain around Asp^{703}) makes the position of the hinge between the N and the P domains substantially higher and also alters the direction of the inclination of the N domain. They suggested that this Mg^{2+} bound to the P domain directly alters the position of the A domain in E2-P and therefore, one of the roles of phosphorylation is to change the interface between the cytoplasmic domains.

Although ATP can bind to the ATPase in the absence of Ca^{2+}, the reaction cycle cannot proceed without Ca^{2+}. With the arrangement of the cytoplasmic domains in the E2, the γ-phosphate of ATP comes close to, but cannot reach the phosphorylation residue, Asp^{351}. However, in the presence of Ca^{2+}, the transmembrane helices will shift into different positions and will prevent the tilting of the P domain to form a close configuration, thus, resulting in a deeper inclination of the N domain or the rotation of the A domain which will consequently release the N domain **(Toyoshima and Nomura, 2002)**. Interestingly, the rotation of the A domain occurs during the transition of E1-P to E2-P **(Danko et al., 2001a)**, and the enzymatic cleavage of the link between this domain and the M3 helix, results in the complete inhibition of the ATPase activity **(Juul and Moller, 2003; Jorgensen and Collins, 1986)**. Since the position of the A domain regulates the gates from and to the binding sites, it has to be fixed in the E2 conformation by the N and P domain **(Toyoshima and Inesi, 2004)**. The closed configuration of the cytoplasmic domains in the E2 conformation will thus limit thermal movements of the transmembrane helices and therefore the leakage.

1.7.1.6- Beta-amyloid production is regulated by SERCA activity

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia in the elderly **(Yamin et al., 1999; Wang et al., 2002)**. The AD is associated with accumulation of Beta-amyloid (Aβ) deposits in senile

plaques and neurofibrillary tangles (NFTs) lesions in specific areas of brain **(Jang and Surh, 2003; Kayed et al., 2003)**. Aβ is a peptide composed of 38-43 amino acids and a proteolytic product of a large precursor protein (amyloid precursor protein; APP) through the α-, β- and γ-secretase **(Selkoe, 1994; Checler, 1995)**. β-secretase (called BACE) works as a single protein, while γ-secretase is formed by at least four proteins: presenilin (PS), nicastrin, anterior pharynx (Aph-1) and presenilin enhancer 2 (Pen-2). Presenilins (PS1 and PS2) are of exceptional pathophysiological importance because association with other protein forms the γ-secretase complex **(Imbimbo, 2008)**. Further, the presenilins are an integral part of the γ-secretase complex that is responsible for cleavage of the C99 fragment of APP to form Aβ and the APP intracellular domain (AICD) **(Green et al., 2008)**. Also, PS and APP mutations are the primary cause of familial AD **(Hwang et al., 2002; Patel and Brewer, 2003)**.

Several studies have shown that the ER stress has been implicated in many diseases, including neurodegenerative conditions such as AD **(Nakagawa et al., 2000; Reddy, 2006)** also, increasing intracellular Ca^{2+} will invariably alter the activity of SERCA **(Yano et al., 2004)**.

A recent study by **Green et al. (2008)** found that modulation of SERCA (SERCA2b) affected Aβ production. Reduced SERCA pump activity lead to decreased Aβ production and increased SERCA activity lead to increased formation of Aβ. This observation suggests that activity of SERCA has a modulatory role in γ-secretase function or can increase in cleavage of APP, which then leads to more generation of the Aβ peptide. However, it has been showed that both stimulation **(Pierrot et al., 2004)** and inhibition **(Yoo et al., 2000)** of capacitative Ca^{2+} entrance (CCE) via SOCCs also caused increased production of Aβ (Aβ 1-42).

1.7.1.7- Pharmacological modulators of SERCA Ca^{2+} pumps

Many pharmacological agents have been used to study the regulation of the SERCA Ca^{2+} pumps, most of which act as inhibitors. These inhibitors have proved invaluable in helping to elucidate mechanistic steps within the Ca^{2+} transport process **(Wictome et al., 1992a; Longland et al., 1999; Wootton and Michelangeli, 2006)**.

A- Thapsigargin

The best known inhibitor of the SERCA Ca^{2+} pump is Thapsigargin (Tg), a potent and specific inhibitor of all SERCA isoforms **(**Lytton et al., 1991**)**. Tg was first shown to increase free cytosolic Ca^{2+} concentration in platelets **(Ali et al., 1985)** and later shown to inhibit the Ca^{2+}ATPase activity **(Sagara and Inesi, 1991)**, indicating that it triggers Ca^{2+} release from the intracellular stores as a result of leakage, rather than through a specific Ca^{2+} channel **(Thastrup et al., 1990)**. Tg has been shown to be a specific inhibitor of the SERCA pumps, having little effect on the plasma membrane Ca^{2+}-ATPase or any ATP and Ca^{2+} -dependent ion channel **(Sagara and Inesi et al., 1991)**. Tg has been observed to reduce the affinity of ATPase for Ca^{2+} binding in a non-cooperative manner, since an increase in concentration of Tg beyond a 1:1 ratio with the ATPase did not cause any further decrease in the Ca^{2+} binding affinity **(Wictome et al., 1995)**.

B- Estrogenic compounds

The majority of estrogenic compounds contain phenolic groups which have the pathobiological effects on human cells **(Chang et al., 2000)**. Further, some of these chemicals can act as xenoestrogens (pollutants, which mimic estrogens or antiestrogens but have toxic properties on humans and animals). Estrogens are hydrophobic steroid hormones that control cell proliferation and differentiation **(Olea et al., 1998)**. Estrogenic alkylphenols have also been shown to inhibit SERCA pump activity **(Hughes et al., 2000; Sokolove et al., 1986; Michelangeli et al., 2008)**. Consequently, such disruption in the tight regulation of cellular Ca^{2+} can lead to deleterious effects, such as, apoptosis **(Hughes et al., 2000; Michelangeli et al., 2008)**. Therefore, these compounds affect other cell signaling pathways in addition to affecting steroid-signaling pathways. There are however other known inhibitors of the SERCA family such as 4-n-nonylphenol (NP), bis-phenol A (BPA), 3,5-dibutyl-4-hydroxytoleuene (BHT), butylated hydroxyanisole (BHA) **(Sokolove et al., 1986)**, quercetin **(Shoshan and MacLennan, 1981)**, 1,1,3,3-tetramethylbutylphenol, diethystibestrol (DES) **(Khan, 2001)** and BFRs (TBBPA) **(Ogunbayo et al., 2007)**. Another compound 2,5-di (tert-butyl)-1,4-benzohydroquinone (BHQ) has also been shown to be a potent inhibitor of the ATPase (IC_{50} was 0.4 μM) **(Wictome et al.,**

1992b). However, BHQ, though having a similar inhibitory effect as Tg, does not bind to the same site on the ATPase **(Khan et al., 1995; Obara et al., 2005; Ogunbayo and Michelangeli, 2007)**.

1.8- Apoptosis and Caspases

There are two essential types of cell death; apoptosis and necrosis **(Geske and Gerschenson, 2001; Kiechle and Zhang, 2002)**. Cellular necrosis occurs as a result of cell injury or exposure to certain cytotoxic chemicals, is distinct from apoptosis in both morphological and biochemical characteristics. Necrotic cell death begins with swelling of cell and mitochondrial contents, followed by rupture of the cell membrane **(Manjo and Joris, 1995; Kanduc et al., 2002)**, whereas apoptosis (programmed cell death (PCD)) is a normal part of organismal development or a response to stressful environmental conditions and that usually is adaptive **(White, 1996; Gray, 2004; Potten and Wilson, 2004)**. Further, apoptosis is a form of cellular suicide that involves DNA fragmentation, membrane blebbing, cell shrinkage and disassembly into membrane-enclosed vesicles and is also under strict control. Excessive apoptosis can cause number of clinical disorders such as neurodegenerative diseases and ischemic damage (ischemia occurs because of a lack of oxygen and nutrients), whereas disorders such as cancer and autoimmune diseases can be caused by insufficient apoptosis **(Earnshaw et al., 1999)**.

Apoptosis is classically divided into three sequential phases: the induction phase, the commitment phase, and the execution phase. During the execution phase, all the morphological characteristics of apoptosis occur, and the cell loses viability. Once this phase begins, death becomes irreversible **(McCarthy et al., 1997)**. This phase is initiated by the activation of several subsets of proteases, called caspases. The name caspase derives from cysteine-dependent aspartate specific protease **(Alnemri et al., 1996)**. Caspases are essential in cells for apoptosis, and development. In most mammalian cells, there are fourteen caspases of which only eleven are found in humans **(Wolf and Green, 1999; Stegh and Peter, 2002)**. Caspase-1 is the founding member of the caspases family, which was initially identified based on its ability to cleave pro-interleukin-1b to the mature cytokine. Other caspases are numbered in order of identification with caspase -3, -8 and -9 being most important in this study.

1.8.1- Structure of caspases

All caspases are found as inactive pro-forms or zymogens (procaspases) within the cell cytoplasm **(Nicholson and Thornberry, 1997)**. All procaspases contain a highly homologous protease domain. This domain can be further subdivided into two subunits, a large subunit of approximately 20 kDa and a small subunit of approximately 10 kDa **(Chang et al., 2000; Fuentes-Prior and Salvesen, 2004)**. Furthermore, x-ray crystal structure and peptide sequencing studies have shown that procaspases can be divided into three major parts: a 17-21 kDa central large internal domain (p20) containing a large catalytic subunit (active site), a 10-13 kDa small C-terminal domain (p10) also called small catalytic subunit, and a 3-24 kDa N-terminal prodomain which can bind to death domain (DD). In most of procaspases, the p20 and p10 subunits are sometimes separated by a small linker sequence. The large N-terminal prodomains of procaspases contain 80-100 residue long structural motifs, called death domain superfamily, are involved in the transduction of apoptotic signals **(Martinon et al., 2001; Weber and Vincenz, 2001; Kumar, 2007)**. The procaspases prodomains have three subdomains, DD, the death effector domain (DED) and the caspase-recruitment domain (CARD) **(Fesik, 2000; Kumar, 2007)**. Also, caspases can be subdivided into three groups based on protein structure and putative function: the first group is classified as cytokine activators, this includes members of the caspase-1 subfamily with large prodomains (which also include caspase-1, -4, -5, -12, and -13); they are involved in the inflammatory process and called group-I caspases or inflammatory caspases **(Deveraux et al., 1998)**. Second group of caspases with a long prodomain, include caspase -2, -8, -9, and -10; this group contains either DED domains (example include caspase -8 and -10) or CARD (example include caspase -2 and -9) and are also called upstream initiator of apoptosis caspases or group-II **(Zou et al., 1997)**. A third group contain short prodomains (20-30 amino acids) are called downstream executioners of apoptosis or group-III caspases, which include the caspases-3 subfamily (i.e. caspase -3, -6, and -7) **(Deveraux et al., 1997, 1998; Xu et al., 2001)**. Caspase-14 has role in supporting the terminal differentiation of keratinocytes in the epidermis and cornea without promoting the characteristic features of apoptosis **(Kuechle et al., 2001; Sanchez Mejia et al., 2001)**. Two tandem copies of DEDs are found in prodomains of procaspases-8 and -10 **(Muzio et al., 1996; Sprick et al., 2002)**, whereas, the CARD is establish in procaspases-1, -2, -

4, -5, -9, -11, and -12 **(Fuentes-Prior and Salvesen, 2004; Lamkanfi et al., 2005)**. Further, DED and CARD are responsible for the recruitment of initiator caspases into death or inflammation-inducing signaling complexes, resulting in proteolytic autoactivation of caspases that afterward initiates apoptosis **(Eckhart et al., 2005; Kumar, 2007)**.

1.8.2- Substrate specificity of caspases

Caspases commonly recognize a four amino acid sequence on their substrates and *cleave* after aspartic acid residues **(Nicholson, 1999; Kelly et al., 2001)**. The tetra peptides (X-X-X-D) are subdivided into the following *three categories* based on their specific substrates: Group I (caspase-1, -4, and -5; choose WEHD or YVAD substrate), Group II (caspase-2, -3, -7, and -10; DEXD) and Group III (caspase-6, -8, and -9; (I/ V/ L- EXD) **(Alnemri et al., 1996; Thornberry et al., 1997; Chowdhury et al., 2008)**. Activated caspases have also been shown to cleave nuclear enzyme poly (ADP-ribose) polymerase (PARP) at the carboxyl-terminus. The sequence of the cleavage site in PARP is DEVD (Asp-Glu-Val-Asp); for caspases -3, IETD (Ile-Glu-Thr-Asp); for caspases -8, and LEHD (Leu-Glu-His-Asp); for caspase-9 **(Thornberry et al., 1997; Chowdhury et al., 2008)**. These peptides have been incorporated into caspases-specific inhibitors and fluorescent substrates, which have been used in this thesis.

1.8.3- Apoptosis pathways (Caspases activation)

The initiation of apoptosis leads to caspase activation by two main pathways, the extrinsic pathway, which is characterised by the death-ligand-mediated receptor activation and the intrinsic pathway also known as the Bcl-2 family-regulated or mitochondrial pathway, and which is characterized by the activation of the mitochondria-apoptosome system **(Kohlhaa et al., 2007)**. These pathways can be activated separately; for instance, caspase-9 plays a vital role in the intrinsic pathway which is activated after perturbation of mitochondria resulting from cellular stress, growth factor withdrawal, or cytotoxic insults **(Reis et al., 2007)**. Caspase-8 is the essential caspase in the extrinsic pathway **(Oliver and Vallette, 2005)**. There is also

some evidence to indicate that in some cells the extrinsic pathway can activate both caspase-9 and caspase-8 **(Racke et al., 2002; Suzuki et al., 2008)**.

Caspase cascade can result from activation of extrinsic (death receptor-mediated) and intrinsic (mitochondria mediated) pathways **(Scaffidi et al., 1998; Wang et al., 2005; Chowdhury et al., 2006) (Fig.1.8)**.

1.8.3.1- Death receptor signaling (extrinsic) pathways

Most cells have death receptors in plasma membrane; death receptor signaling pathways plays an important role in the caspase activation **(French and Tschopp, 2003)**. The death receptors are a subfamily of the tumor necrosis factor (TNF) receptors (TNFRs) **(Krammer et al., 1999)**. TNFRs family members include TNF-related apoptosis-inducing ligand receptor 1 (TNFR1; also named as DR1, CD120a, p55, or p60) **(Li and Yuan, *2008)***, CD95 (also called Fas, APO-1, or DR2), DR3 (also known as APO-3, LARD, TRAMP, or WSL1), TRAIL-R1 (known as DR4 or APO-2), TRAIL-R2 (also named DR5, KILLER, or TRICK2), and DR6, Ectodysplasin A receptor (EDAR) and Nerve growth factor receptor (NGFR) **(French and Tschopp, 2003; Wajant, 2003; Gloire et al., 2008)**. Most of these are characterized by the presence of a DD motif within their intracellular domain. They play a significant role in apoptosis and can activate a caspase cascade within seconds of ligand binding **(Curtin and Cotter, 2003; Lavrik et al., 2005)**. Several studies have shown that the receptors of the three main death receptors pathways (TNF-α, CD95, and TRAIL) may act as the main participant in the initiation of apoptosis by the extrinsic pathway **(Sheard et al., 1997; Luce et al., 2009)**.

1.8.3.1.1- Signalling by Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α) is a pro-inflammatory cytokine with a broad variety of functions in many cell types. TNF-α exerts its biological activity by binding to receptors TNFR-1 and TNFR-2 and activating several signaling pathways **(Ashkanazi and Dixit, 1998; Darnay and Aggarwal, 1997; Screaton and Xu, 2000)**. The interaction of TNFα with its receptor, leads to trimerization and clustering of intracellular death domain. This allows the binding of intracellular adapter

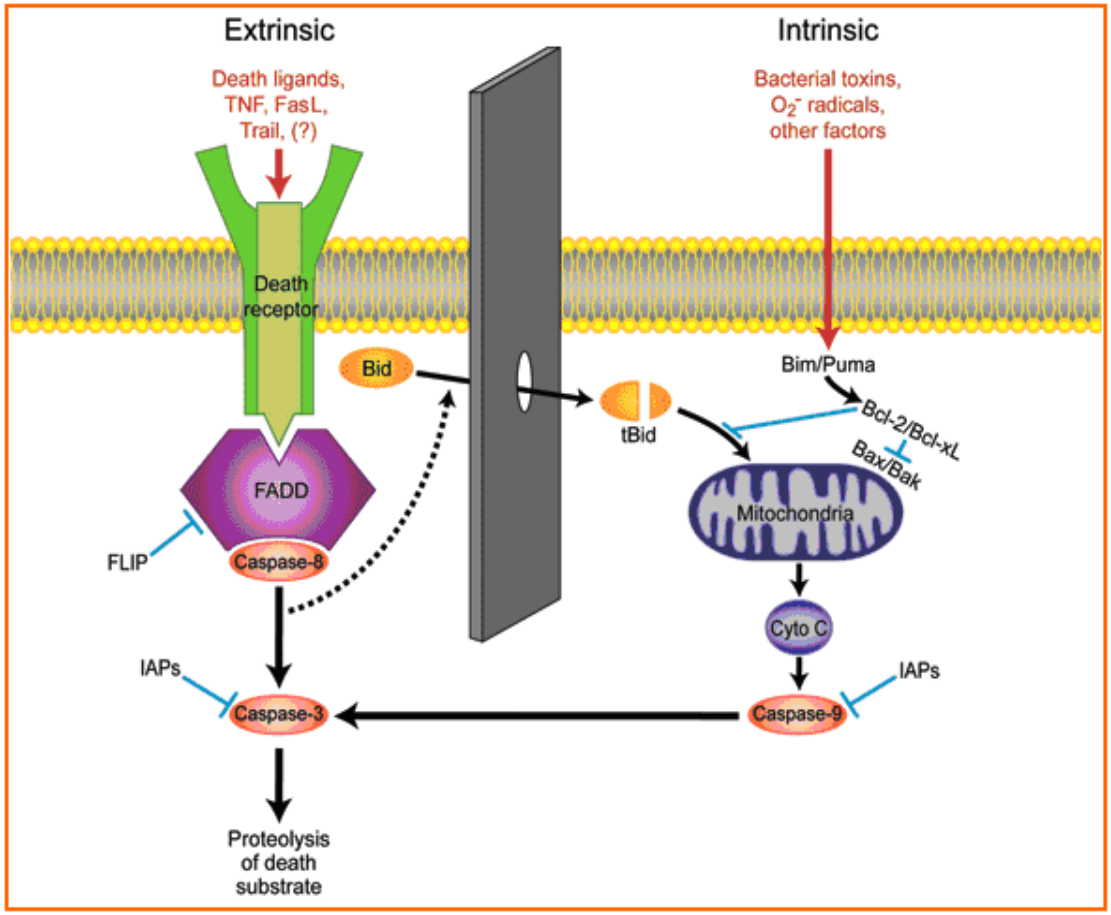


Figure 1.8: extrinsic (death receptor-mediated) and intrinsic (mitochondria mediated) pathways. Intrinsic pathway is mediated by mitochondrial and the ER pathways. Distinct initiator caspases are activated in each pathway of apoptosis. **(figure from web site: www. medscape.com)**.

molecule called TNFR associated death domain (TRADD) by interaction with the death domains on TNFR. TRADD in turn associates with another adapter molecule the Fas associated death domain (FADD). This complex then bind to procaspase-8 to form a death-inducing signal complex (DISC) **(Philchenkov, 2004; Thorburn, 2004)**, and becomes caspase-8. Active caspase-8 is rapidly released from DISC to the cytoplasm and is a downstream effector for caspase-3, -6, and -7. Activation of effector caspases especially caspase-3 results in of apoptosis **(Fuentes-Prior and Salvesen, 2004; Zimmerman et al., 2002)**.

1.8.3.1.2- Signalling by CD95

CD95/ Fas is expressed in various tissues, but its ligand (CD95-L) is expressed mainly in T lymphocytes and natural killer cells. It plays an essential role in the immune system, including the killing of pathogen-infected cells **(Suda et al., 1993)**. Several studies have shown that cytotoxic T cells, which express CD95L in a membrane-bound form (mCD95-L) on their surface, can kill CD95+ target cells **(Krammer, 2000; Nagata, 1997)**. CD95-L recruits an adapter protein called FADD which interacts with the death domain of CD95 **(Debatin and Krammer, 2004)**. FADD also contains DED and through the homologous interaction, it recruits procaspase-8 and/or procaspase-10 to form DISC, Which serves as a platform to initiate enzymatic activation of the apoptotic pathway by the autoproteolytic cleavage of procaspases-8 to active caspase-8 **(Gupta, 2003; Thorburn, 2004; Wolf and Green, 1999; Gloire et al., 2008)**. Further, Caspase-8 activation within the DISC occurs in two steps. First, recruitment of FADD to the intracellular region of CD95 promotes via homotypic interaction of DED dimerization and a conformational change in caspase-8 within the DISC that allows caspase-8 to gain full enzymatic activity. Second, active caspase-8 undergoes autoproteolytic processing, which allows the enzyme to leave the DISC and gain access to substrates in other cellular compartments **(Boatright et al., 2003)**. The active caspase-8 follows the apoptotic cascade to activate executioner caspase-3, which results in apoptosis **(Chowdhury et al., 2006) (fig. 1.8)**.

1.8.3.1.3- Signalling by TNF related apoptosis inducing ligand

TNF related apoptosis inducing ligand (TRAIL) is a member of the TNF superfamily that induces apoptosis upon binding to its receptors. TRAIL is also known as Apo-2L **(Pitti et al., 1996)** and is regulated by the expression of two death receptors TRAIL receptor 1 (TRAIL-R1 and TRAIL-R2) and two decoy receptors (TRAIL-R3 and TRAIL-R4) **(MacFarlane, 2003)**. Furthermore, there are also four decoy receptors (DcR) known TRAILR3 (also known as DcR1), TRAILR4 (also known as DcR2), DcR3 and osteoprotegrin (OPG). DcRs possess functional extracellular ligand binding domains, but do not contain the intracellular DD and therefore, cannot recruit adaptor proteins required for apoptosis. The principle function of DcR is to modulate the sensitivity of death-receptor-mediated apoptosis in-vivo **(Ashkenazi et al., 1999; Curtin and Cotter, 2003; Lavrik et al., 2005)**. TRAIL also binds DR4 and DR5. The signaling pathways of these are similar to CD95 and are mediated by FADD **(Almasan and Ashkenazi, 2003)**.

1.8.3.2- Mitochondrial (intrinsic) pathway of apoptosis

Mitochondria play a central role in the initiation of apoptosis. Several stimuli, such as cytotoxic stress, oxidative stress, heat shock and DNA damage, lead to changes in mitochondrial membrane integrity and the release of cytochrome c from mitochondria to the cytosol **(Armstrong, 2006; Finkel, 2001)**. The release of cytochrome c is one of the major factors in apoptosis related with permeabilization of outer mitochondrial membrane **(Fuentes-Prior and Salvesen, 2004; Aleo et al., 2006)**. Cytochrome c release therefore is followed by the formation of a high molecular mass cytoplasmic complex called the apoptosome **(Acehan et al., 2002)**. The apoptosome is a very large protein (700-1400 kDa) complex in the presence of cytochrome c, dATP and apoptotic protease activating factor-1 (Apaf-1). Procaspase-9 is then recruited to the complex by CARD interactions, which results in its activation **(Hu et al., 1998; Costantini et al., 2002)**. Active caspase-9 cleaves executioner caspases such as caspase-3 to induce apoptosis **(Aleo et al., 2006; Gupta, 2003; Chowdhury et al., 2006)**.

1.8.4- Regulation of caspase activation

Activation of caspases is vital for the induction of apoptosis

1.8.4.1- Bcl-2 family proteins

The mitochondrial pathway of apoptosis occurs when factors such as cytochrome c is released into the cytosol by permeabilization of outer mitochondrial membrane. This process is controlled by the Bcl-2 family proteins (named from B-cell lymphoma 2), which is composed of both pro- and anti-apoptotic proteins **(Gross et al., 1999; Chipuk and Green, 2008)**. The Bcl-2 family of proteins is divided into three groups based on number of Bcl-2 homology domains (BH1-4 domains) **(see fig. 1.9A)**. First group is Bcl-2 subfamily (named as anti-apoptotic) such as Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1, which contain BH1-BH4 domains and are generally integrated in the outer mitochondrial membrane and also found in association with ER and nuclear envelope **(Akao et al., 1994)**. These proteins function within the apoptotic pathway to directly bind and inhibit the pro-apoptotic Bcl-2 proteins. Second group is Bax subfamily (known as pro-apoptotic), which include Bax, Bak and Bok. They contain BH1-BH3 domains and permeabilize the outer mitochondrial membrane by creating the proteolipid pore responsible for cytochrome c release **(Leber et al., 2007)**. Third group is BH3 subfamily (known as pro-apoptotic) contain Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL; which function in distinct cellular stress pathways and, by protein-protein interactions with other Bcl-2 family members signal that a cellular stress has occurred **(Chipuk and Green, 2008)**.

Two pathways of caspases activation can be distinguished by whether they require Bcl-2 family proteins. The mitochondrial pathway (Bcl-2-regulated or intrinsic pathway) is strictly controlled by the Bcl-2 family of proteins. Furthermore, the pro-apoptotic family members Bax and Bak are essential for inducing permeabilization of the OMM and the subsequent release of cytochrome c, which leads to the activation of caspase-9**_(Hakem et al., 1998)**, but the role of Bax and Bak in release of cytochrome c is still unclear. **(Youle and Strasser, 2008)**. The anti-apoptotic family members, such as Bcl-2 and Bcl-xL, inhibit Bax and Bak. Recent evidence indicates that BH3-only proteins derepress Bax and Bak by direct binding and inhibition of Bcl-2 and other anti-apoptotic family members **(Youle, 2007)**. By contrast, an

opposing model postulates direct activation of Bax and Bak by some BH3-only proteins (specifically Bim, tBid and PUMA) **(Willis et al., 2007)**. One mechanism suggests that tBid induced the activation of Bax and Bak, resulting in dysfunction of mitochondria then release of cytochrome c **(Wei et al., 2001)**. However, the extrinsic pathway can activate caspase-8 which leads to caspase-3 activation and cell death. The Bcl-2 family regulates the intrinsic pathway and can modulate the extrinsic pathway when the cleavage of Bid cross-talks between the two pathways **(fig. 1.9B)** **(Youle and Strasser, 2008)**.

1.8.5- Apoptosis induced by beta-amyloid

It was found that DNA damage and increasing nuclear factor-kB (NF-kB) activation are associated with degenerating neurons in the brains of AD as the consequence of oxidative stress by Aβ deposition in brain **(Culmsee et al., 2003)**. Neurofibrillary tangles (NFTs) are involved in AD. These are formed from tau proteins which are a highly soluble microtubule-associated protein (MAP) and are abundant in neurons in the CNS (**Hyman, 1997; Goedert et al., 1991)**. Proteolytically cleaved and hyperphosphorylated tau proteins form paired helical filaments (PHFs) which aggregate to form NFTs **(Trojanowski and Lee, 1995)**. It is believed that caspase-3 cleaved tau proteins or its associated PHFs induces mitochondrial dysfunction causing mitochondrial Ca^{2+} release, reactive oxygen species (ROS) production and mitochondrial depolarisation **(Parihar and Hemnani, 2004)**. All these consequences can then contribute to mitochondrial-dependent apoptosis **(fig. 1.10)**.

Toxic peptide Aβ has been shown both to kill neurons and also to form Aβ-plaques **(Cantara et al., 2007)**. One current view is that Aβ forms oligomeric pores within the plasma membrane of neurons and this causes excessive Ca^{2+} influx and the generation of ROS. This then leads to lipid peroxidation, and caspase activation, mitochondrial Ca^{2+} overload, oxidative stress and ultimately apoptotic cell death **(Ferreiro et al., 2008)**. **(fig. 1.10)**.

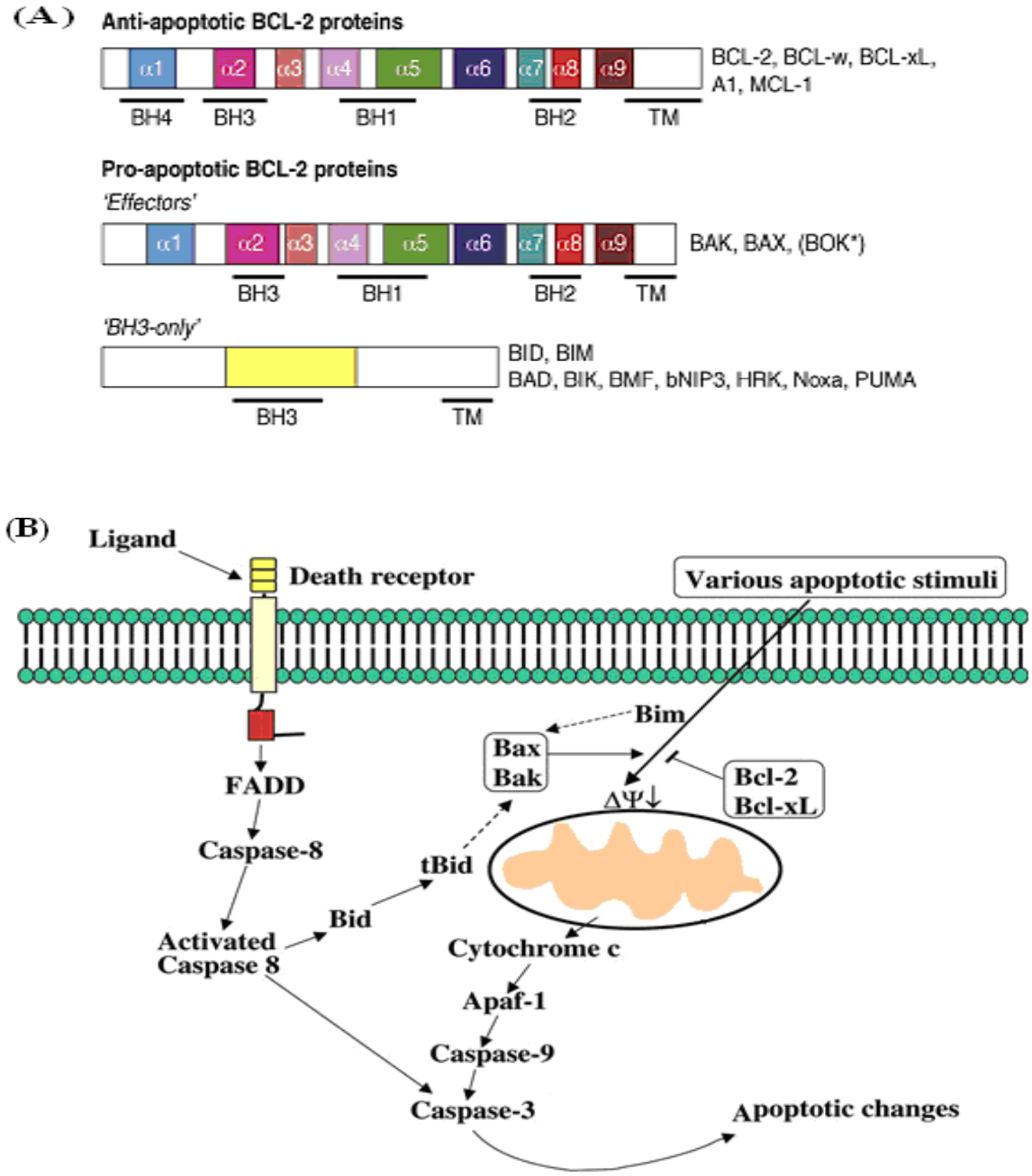


Figure 1.9: (A) Bcl-2 family of proteins, which is divided into three functional groups, based on their composition of Bcl-2 homology domains. The anti apoptotic members include Bcl-2, Bcl-xL, Bcl-w, A1 and Mcl-1 and contain four Bcl-2 homology domains (designated BH1–4). The pro-apoptotic multi-domains (Bax, Bak and Bok) contain BH1-3 domains. The BH3-only proteins are subdivided into direct activators (e.g. Bid and Bim) and de-repressors/ sensitizers (e.g. Bad, Bik, Bmf, bNIP3, HRK, Noxa and PUMA). **(Cory et al., 2003)**. **(B)** role of Bcl-2 family member in release of cytochrome c from the mitochondria. **(adapted from Mak and Yeh, 2002)**.

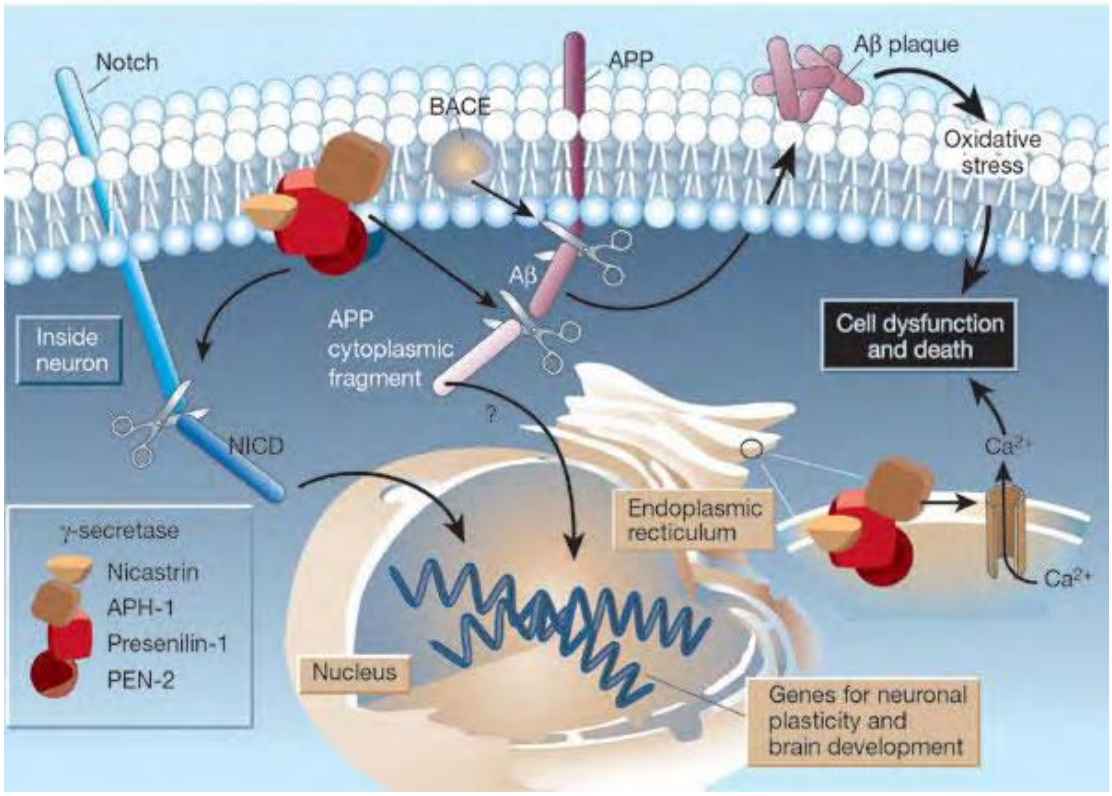


Figure 1.10: Aβ-mediated cell death in Alzheimer's disease.

The β-Secretase (also called BACE) cleavage of the amyloid precursor protein (APP), followed by γ-secretase complex (containing Presenilin-1, nicastrin, Aph-1 and PEN-2) forms Aβ. The Aβ then forms oligomers within the plasma membrane of neurons and this causes excessive Ca^{2+} influx then leads to lipid peroxidation, and caspase activation, mitochondrial Ca^{2+}overload, oxidative stress and ultimately apoptotic cell death **(figure from Mattson , 2003)**.

1.8.6- Role of reactive oxygen species in caspases activation

ROS such as hydrogen peroxides (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and superoxide anions (O_2^-), are produced as a natural product of cellular metabolism **(Fridovich, 1999; Inoue et al., 2003)**, excessive levels in cells can cause apoptosis by the activation of the mitochondrial pathway of apoptosis via the activation of c-Jun N-terminal kinase (c-JNK) and subsequently inducing cytochrome c release from mitochondria and activation of caspases **(Javvadi et al., 2008; Singh et al., 2005; Wang et al., 2008; Kim et al., 2009)**. Furthermore, activated c-JNK leads to phosphorylation of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, and inactivating their functions **(Wullaert et al., 2006)**.

1.9- Brominated flame retardants (BFRs)

Fires kill on average over 37,000 persons pear year **(CTIF, 2003)**, with more than 4,000 people killed and more than 80,000 injured in Europe Union (EU) alone **(Dawson, 2002)**. Flame retardants added to combustible materials prevent fires and thus save costs and human lives. A recent report has showed that the incidence of serious fires has dropped by 50% in the United States (US) over the past 30 years, because of the increasing use of flame retardant chemicals in many products (i.e. curtains, carpets, decorations, plastics in electrical and electronic equipment, upholstered furniture and textiles…etc) **(Karter, 2008)**.

Flame retardants (FRs) are chemicals that can be added to materials by blending or chemical incorporation, to reduce or delay the spread of a fire starting by providing increased resistance to ignition. There are more than 175 different commercial FRs in use on the market **(Alaee et al., 2003)**, and those can be divided into inorganic, organic halogenated and organophosphate compounds. Inorganic FRs includes metal hydroxides such as magnesium hydroxide, aluminum hydroxide and ammonium salts **(WHO, 1997)**. Inorganic FRs represent the largest portion of total amount of FRs produced **(WHO, 1997)**. Organic halogenated FRs are primarily based on chlorine and bromine, and also often contains other heteroelements, such as phosphorus or nitrogen. One mechanism for the action of FR uses its ability to form a protective coating of either liquid or solid above the surface of the combusting material, which

prevents oxygen and volatile combustion products reaching the flame **(BSEF, 2004)**. Another mechanism of halogenated FRs is to act as radical scavenger removing free radicals in material and reducing burning. Brominated flame retardants (BFRs) are the most efficient and possess the most constructive thermal stability of the halogenated products **(Kirk-Othmer, 1993; Orn and Bergman, 2004)**. In 2005, the total global use of BFRs was 311,000 metric tonnes which represent approximately 21 percent (%) of the total consumption of FRs **(Fink et al., 2005)**. Tetrabromobisphenol A (TBBPA) and hexabromocyclododecane (HBCD) are currently the most widely produced BFRs (since legislation to limit the use of polybrominated diphenyl ethers (PBDEs) was introduced a few year age).

BFRs are the most commonly employed group of FRs, used to date. These are the largest market group of FRs because of their low cost and high performance efficiency. Currently, more than 75 different chemicals have been employed as BFRs commercially **(WHO, 1994; BSEF, 2000)**. The major BFRs used are TBBPA, HBCD, PBDEs; which are include decabromodiphenyl ether (DBDE), octabromodiphenyl ether (OBDE), and pentabromodiphenyl ether (PBDE). These last three chemicals are referred to as commercial mixtures of PBDEs or biphenyl oxides. Each of the BFRs has very different properties and the structures **(fig. 1.11)**. They are generally produced synthetically as mixtures. Furthermore, these chemicals can be incorporated into the material to be protected in two different ways, either covalently linked (reactive) or only added to the material (additive). For examples, PBDEs and HBCD are additive BFRs and TBBPA are a reactive BFRs **(EHC-192, 1997)**.

BFRs are a highly diverse group of chemicals, some of them are aliphatic and others cycloaliphatic or aromatic compounds. BFRs are used in a variety of consumer products, including computers, electronics, electrical equipment, televisions, textiles, foam furniture, insulating foam and building materials and BFR production has increased dramatically during the last few years **(Alaee et al., 2003)**. TBBPA is the major BFR produced, followed by DBDE and HBCD. The production of BFRs differ by region, TBBPA is mostly produced in Asia. The EU produces most of the HBCD, while DBDE and PBDE are produced to a large extent in both the US and Asia. Interestingly, PBDE is produced nearly exclusively in the US **(Orn and Bergman, 2004)**.

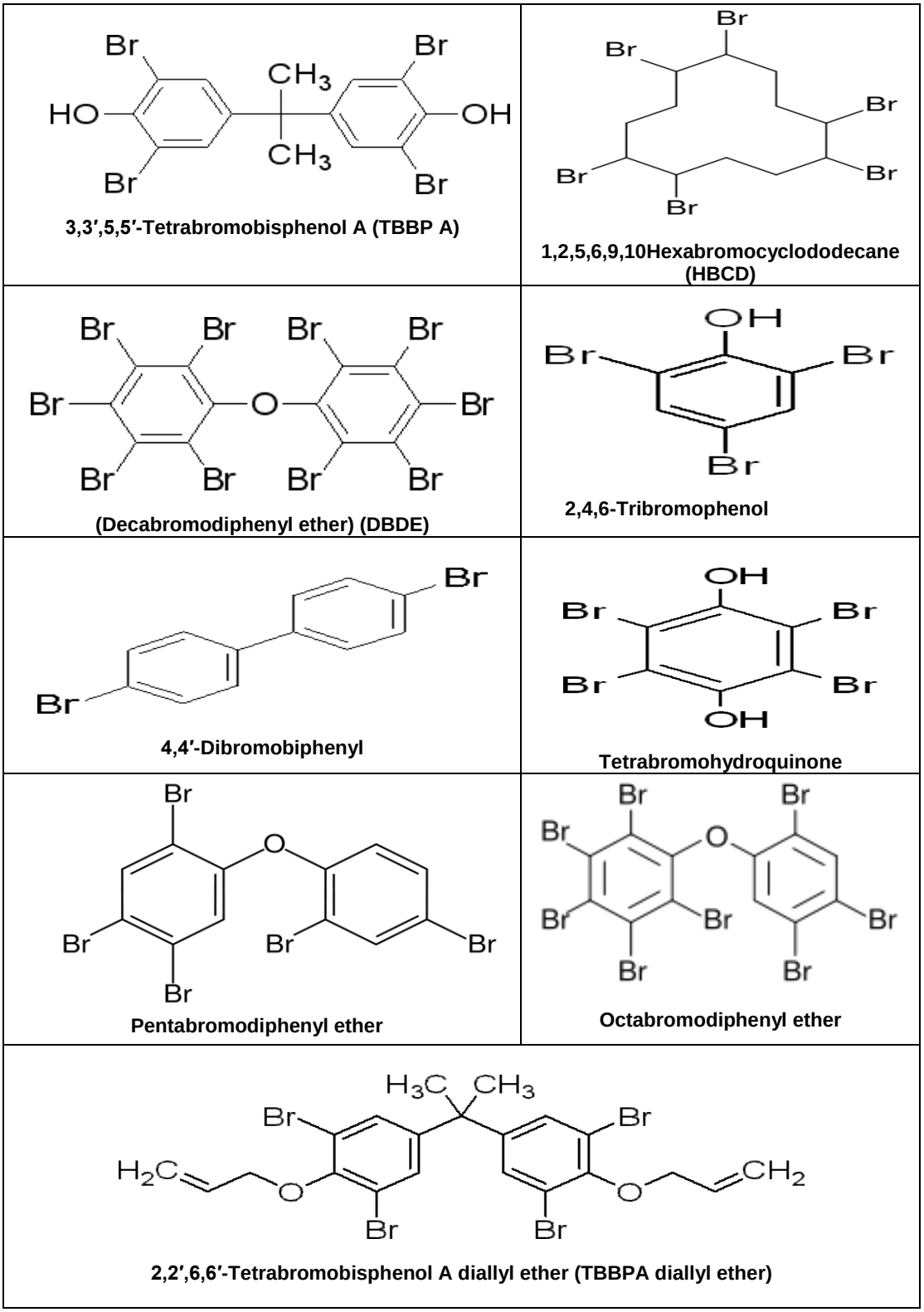


Figure 1.11: different structures of BFRs congeners.

1.9.1- Tetrabromobisphenol A

Tetrabromobisphenol A (TBBPA) is a crystalline solid and melting point of 180 - 184°C. TBBPA has the chemical formula: $C_{15}H_{12}Br_4O_2$. TBBPA consists of two brominated and hydroxylated aromatic phenyl rings, each bound to a propyl group as shown in fig. 1.11. TBBPA has proposed log partition coefficient (Kow) of 5.7, making it very hydrophobic/ lipophilic **(Ogunbayo et al., 2007)**. The TBBPA is one of the most widely used brominated flame retardants, employed to reduce the combustibility of a large variety of manufactured products. TBPPA is formed by bromination of bisphenol A (BPA) in an organic solvent. Around 90% of TBBPA is used as a reactive intermediate in the production of epoxy and polycarbonate resins used in printed circuit boards and electrical equipment. The remaining 10% of TBBPA is transformed into derivatives such as dimethyl TBBPA and used as additive BFR in acrylonitrile-butadiene-styrene (ABS) resin and high-impact polystyrene. Bis (2-hydoxyethyl ether) TBBPA, another TBBPA derivative, is used as a flame retardant for paper and textile, adhesives and coatings.

1.9.2- Hexabromocyclododecane

1,2,5,6,9,10-hexabromocyclododecane (HBCD) is a white crystalline powder with 74.7% bromine with the chemical formula: $C_{12}H_{18}Br_6$, and has a lipophilicity value of log Kow of 5.6 **(Birnbaum and Staskal, 2004)**. The configuration of the HBCD is presented in fig. 1.11. HBCD is a cyclic aliphatic and usually *melts at* a temperature range of 183-207 ºC. HBCD is one of the major BFR produced and mostly produced in the EU-region **(Orn and Bergman, 2004)**. The global product of HBCD was estimated to be more 20,000 metric tons in 2006 **(Heeb et al., 2007; BSEF, 2008)**, and is produced by bromination of cis, trans, trans-cyclododeca-1,5,9-triene **(Becher, 2005)**. HBCD has 16 possible stereo-isomers and the consequential industrial product consists of a mixture of three diastereomers of HBCD (α-, 6%; β-, 8% and γ-HBCD, 80%) **(ACC., 2001)**. The main application of HBCD is in polystyrene foam that is used in thermal insulation of building materials, electronics, textiles and upholstered furniture **(BSEF, 2008)**.

1.9.3- Polybrominated diphenyl ethers

PBDEs where the second highest production group of BFRs until recently. They are used in consumer items such as polyurethane foams, TV sets, computers, radios, textiles, paints, and plastics. These brominated compounds act in the gas phase of the fire by reacting with free radicals generated during combustion, thus terminating the reaction **(Rahman et al., 2001)**. PBDEs are additive flame retardants, which are mixed with the polymer instead of mixing with the plastic before polymerization. Therefore, PBDEs are more prone to leak out of products during their lifetime because they not covalently attached to the polymer **(Sjodin, 2003)**. PBDEs are produced by bromination of diphenyl ether. Diphenyl ether molecules contain 10 hydrogen atoms, any of which can be exchanged with bromine, resulting in 209 possible congeners, **(fig. 1.11)**. PBDE congeners are commercially produced in three different degrees of brominated forms such as pentabromodiphenyl ether (PBDE), octabromodiphenyl ether (OBDE) and decabromodiphenyl ether (DBDE). The PBDE and OBDE were banned in the EU in 2004, and in the US from 2006, while DBDE still to be widely used **(Giordano et al., 2008; fast facts: PBDEs, 2010)**.

1.9.4- Polybrominated biphenyls

Polybrominated biphenyls (PBBs) are a group of manufactured chemicals, of the polyhalogenated compounds. PBBs were produced as flame retardants in the early 1970s. The commercial production of PBBs in the form of Firemaster® in the United States continued until 1976 and approximately 6071 tonnes of PBBs were produced during those 6 years **(Di Carlo et al., 1978; Pomerantz et al., 1978)**. In 1973, Firemaster BP-6® and FF-1® were unintentionally mixed into cattle feed at a production site and distributed throughout Michigan. The prevalent contamination of Michigan farm products that resulted from this accident led to the ban of PBB in the USA in 1974 **(EHC-152, 1994)**. However, only three commercial PBB products were manufactured, hexabromobiphenyl (HxBB), octabromobiphenyl (OBB) and decabromobiphenyl (DBB), and these three products were based on a limited number of congeners **(Norris et al., 1975; Pomerantz et al., 1978)**. In Europe, particularly in Germany, a mixture of highly brominated PBBs was in manufacture until 1985, whereas in France, decabromobiphenyl **(EHC-152, 1994)** was in production until

2000 **(Hardy, 2002)**, then the EU banned the use of PBBs in electrical and electronic devices. Moreover, The PBBs are now limited in use; no more than 0.1 percent by weight of polybrominated biphenyl per homogenous material in electrical and electronic devices **(ERA, 2006)**.

1.9.5- Environmental and human exposure to BFRs

As BFRs are extensively used due to their chemical and physical properties, BFRs have become a ubiquitous group of environmental contaminants. Several detailed studies have shown that BFRs are found as contaminants in all environment media (indoor and outdoor) and concentrations of some of BFRs in both environment and humans are rapidly rising **(Alaee and Wenning, 2002)**.

Models have shown that a large part of the BFRs released to the environment will end up in soil or sediment **(Palm et al. 2002; Gouin and Harner, 2003)**. Furthermore, PBDEs persist in most environmental compartments such as soil, air, sea sediment and some of the PBDEs were also found to bio-magnify in the food chain **(de Wit, 2002)**. However, some of BFRs, such as the PBBs were removed from the market after a contamination incident in Michigan 1974, when PBBs was accidentally mixed into animal feed. The exposed animals showed symptoms such as a reduction in feed consumption, abnormal growth, lameness and deaths **(WHO, 1994)**.

Previous study have been shown that PBDEs levels in soil analyzed from a city centre can be as much as 50 times higher than those found in soil from rural sites **(Harrad and Hunter, 2006)**. Furthermore, this study also showed that the outdoor air levels of PBDEs were found to be about 8 times higher in the city compared to rural environments (i.e. 23.3 and 2.8 pg/m^3, respectively), and alarmingly air samples collected near HBCD-processing plants were excessively high at roughly 28500 ng/m^3 **(National Chemicals Inspectorate (KEMI), 2005)**.

Several BFR are bio-accumulating in both animals and humans **(Damerud, 2003)**. In fact some of the PBDEs congeners (BDE209) have been found in human tissues and fluids, including blood, breast milk, and adipose tissue at high concentrations i.e. 160ng/g serum lipid weight (lw) (equivalent to 0.3 µM) **(Sjodin et al., 2004)**. Several

Swedish studies showed that the monitored blood levels of some PBDEs congeners were significantly higher in electronic recycling workers and computer technicians compared to those than in control group of workers **(Sjodin et al., 1999; Jakobsson et al., 2002)**. Another recent study investigated the blood levels of PBDEs in workers from Chinese electronics dismantling plant, which showed that some individuals had highest ever recorded levels BDE-209 in their blood samples of 3400ng/g serum lw **(Qiu et al., 2007)**. In one study, TBBPA was found in 80 % of the serum samples from computer technicians, at levels ranging up to 3.4 pmol/g lw **(Jakobsson et al., 2002)** and in appreciably higher levels in workers continually exposed to high levels of this chemical, in electronics dismantling facilities **(Tomsen et al., 2001)**. In humans, the major intake of HBCD is from food, indoor air and dust **(Remberger et al., 2004)**. The concentrations of HBCD were measured in a variety of samples (air, animals, and human). Furthermore, high concentrations of up to 19200 ng/g lw were found in birds of prey (equivalent to 12µM) and more than 9600 ng/g Iw in marine mammals (equivalent to 6µM) while lower concentrations of 20 ng/g lw (equivalent to 30nM) were reported in a limited human study **(Covaci et al., 2006)**.

1.9.6- Toxicity of BFRs

In recent years, BFRs have been of considerable concern regarding their potential toxicity to humans **(Sjodin et al., 2003)**. Based on toxicity information from in-vitro and rodent studies, there are differences among the BFR groups, Some BFRs are capable of accumulating and levels of these chemicals have increased over time in some populations. For example, the PBDE congeners like PentaBDE may cause biochemical and toxicological effects such as altered growth, morphology and cardiac and neural functions in experimental animals **(Lema et al., 2007)**. Furthermore, environmentally relevant concentrations of TBBPA in rodents reduced their reproductive success **(Kuiper et al., 2007)**. However, several studies on animals and in cells using a variety of BFRs has shown them to have multiple disruptive effects on many physiological processes, such as neurological function and neuro development, endocrine function and immune function. Behavioural studies have demonstrated adverse neurodevelopmental effects on learning and memory after BFRs exposure, using some of the PBDEs congeners **(Reistad et al., 2002; Wiegand et al., 2001; Alm et al. 2006)**. Furthermore, **Viberg et al. (2006)** demonstrated that exposure of rat

neonates to higher brominated PBDEs, such as the PBDE-203 and PBDE-206, showed that they can cause developmental neurotoxic effects, manifested as defective spontaneous behavior, reduced or lack of habituation, and impaired learning and memory functions. Theses neurobehavioral effects were not only observed upon exposure of these chemicals in neonates, but rather exposure to low dose of these PBDEs in pregnant rats caused similar effects in their offspring **(Kuriyama et al., 2005)**. In-vitro studies suggest that a variety of other BFRs such as TBBPA and HBCD affect rat brain synaptosomes which influence memory and learning mechanisms in brain by inhibiting their ability to take up neurotransmitters like dopamine, and GABA **(Mariussen et al., 2002; Reistad et al., 2002; Fonnum et al., 1995)**. Excess glutamate in the synaptic cleft leads to increases in the neuron of excitotoxicity **(Fonnum, 1998)**. Further, BFRs have been shown to affect cholinergic **(Vilberg et al., 2003)** and catecholaminergeric systems within the developing brain **(Alm et al., 2006)**.

1.9.7- Effects BFRs on endocrine function

An endocrine disrupter has been defined as an exogenous agent which interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development or behaviour **(Kavlock et al., 1996)**. The extensive description includes all substances that can affect endocrine function via interference with hormone pathways (i.e. estrogen, androgen, or thyroid hormone). There are also several studies that have shown that BFRs have the potential to cause endocrine disruption **(Darnerud, 2008; Legler, 2008)**. A variety of BFRs at low concentrations have been shown to cause endocrine disruption by acting as agonists or antagonists of steroid hormones such as estrogen **(Meerts et al., 2001),** androgens **(Larsson et al., 2006)** and also thyroid hormones **(Kitamura et al., 2002)**.

1.9.7.1- Thyroid signalling

Several rodent studies have shown that exposure to BFRs such as PBDEs, (like some other organohalogenated compounds) leads to reduction of serum total and free forms of 3,3’,5-tetraiodo-L thyronine (T3) and 3,3’,5,5’-tetraiodo-L thyronine (T4) levels

(Fowles et al., 1994; Zhou et al., 2001, 2002; Hallgren et al., 2001; Hallgren and Darnerud, 2002). The mechanism of action of interference of organohalogens with the thyroid hormone (TH) system may include three different mechanisms: (1) thyroid gland function and regulation; (2) TH metabolism; and (3) TH plasma transport **(Brouwer et al., 1998)**. Some PBDEs congeners appear to interfere with the TH signalling at several levels by increasing the catabolism of T4 and T3 through induction of hepatic UDP-glucuronosyl transferases (UDPGT), thus lowering the circulating amounts of the hormones in the blood **(Zhou et al., 2001, 2002; Birnbaum and Staskal, 2004)**, and their hydroxylated metabolites can compete with T4 for binding to the plasma transport protein transthyretin (TTR) **(Meerts et al., 2000)**. Further, hydroxylated PBDEs (BDE 47, BDE 99 and BDE100) have a high affinity for thyroid receptors **(Marsh et al., 1998)**. Low micromolar concentration of TBBPA has also been shown to inhibit thyroid hormone sulfation **(Schuur et al., 1998)**.

1.97.2- Steroid signalling

Endocrine disruption happens also, when steroid synthesis, metabolism or function are unregulated. One of the major pathways involved in steroid metabolism is that of steroid sulfonation. Furthermore, Estrogen sulfates are pharmacologically inactive and are the main form of the steroids in plasma; free estrogens are then released at the target tissues by the action of cell-surface sulfatases. Formation of estrogen sulfates is carried out by sulfotransferase enzymes (SULT isoforms) which are cytosolic and use 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a co-factor in transferring a sulfonate group. The majority of endocrine disruptor research has focused on interference of compounds with the sex hormone homeostasis, resulting in effects on sexual differentiation and reproduction. FRs have also been described as endocrine disrupters **(Fonnum et al., 2006)**. Studies on the estrogenic effects of BFRs in-vivo are limited. In-vitro estrogenic activity of TBBPA has revealed no estrogenic effects in embryo development **(Berg et al., 2001)**. Furthermore, TBBPA was unable to alter circulating testosterone levels, testis weight and sexual behavior **(Halldin et al., 2001; 2005)**. However, other studies have been shown that TBBPA can inhibit estrogen sulfotransferase in-vitro **(Kester et al., 2002; Hamers et al., 2006)** and enhanced proliferation in an estrogen-dependent cell line **(Kitamura et al., 2002)**. TBBPA is

also able to detrimentally affect testicular Sertoli cell function (this cell is involved in sperm development and dictates the number and quality of mature sperm) **(Ogunbayo et al., 2008)**. Additional in-vitro studies indicated that several PBDEs congeners (BDE-71 BDE-47, BDE-99, BDE-100, and BDE-154) inhibited binding of the synthetic androgen (R1881) to cytosolic androgen receptor (AR) from the rat ventral prostate **(Stoker et al., 2005)**. Also, DE-71 has anti- androgenic activity causing a delay of puberty in male rats by inhibition of androgens binding to the AR **(Stoker et al., 2005)**, but the significance of these observations for exposure in vivo is not known.

1.9.7.3- Liver function (Hepatotoxicity)

Liver cytochrome P450 (CYP) plays a vital role in the oxidation of xenobiotics, including drugs and environmental pollutants, and therefore represents a primary focus of toxicological and drug metabolism research **(Anzenbacher and Anzenbacherova, 2001)**. Numerous in-vitro studies have shown that exposure of rodents and various cells lines to PBDE congeners induced both phase I and phase II detoxification enzymes in the liver by the increased activity of liver microsomal ethoxyresorufin-O-diethylase (EROD) after exposure **(von Meyerinck et al., 1990; Hanberg et al., 1991)**. EROD activity, a catalytic function of the CYP1A (microsomal oxygenase subfamily), is a popular biomarker for exposure to xenobiotics **(Zhou et al., 2002)**.

Hepatotoxicity of other BFRs such as TBBPA can inhibit hepatic enzymes such as CYP2C9 alanine aminotransferase (ALT), L-γ-glutamyl-transferase (γ-GT), glutathione (GSH) and malondialdehyde (MDA) by destabilizing membrane mitochondria **(Boecker et al., 2001; Szymanska et al., 2000)**.

1.9.7.4- Immune function

Many animal studies on the immune system have showed that exposure to some of the PBDEs congener's leads to decrease in the weight and size of the lymph glands, number of neutrophils, lymphocytes and certain subsets of T-cells **(Martin et al., 2007)**. Also, observed is a reduction in circulating both IgG and IgM and an increase in some interleukins **(Thuvander and Darnerud, 1999; Martin et al., 2007)**. In-

vitro immunotoxicology study has shown that exposure to low concentrations of TBBPA has immunotoxic potency in cell culture by its ability to specifically inhibit the expression of CD25 **(Pullen et al., 2003)**, which is required for the proliferation of activated T cells. Similar studies have also showed that the TBBPA and some of the PBDEs congeners (DE-71) can activate neutrophils leading to an activation of the NADPH oxidase primarily by an extracellular signal regulated kinase (ERK) pathway. This was followed by increase in intracellular and extracellular ROS formation. TBBPA also induced activation of mitogen-activated protein (MAP) kinases followed by a stimulation of immune functions in mussel hemocytes **(Reistad et al., 2005)**. Furthermore, in a preliminary study in Dr Michelangeli's lab has shown that TBBPA can induce degranulation of mast cells and release inflammatory factors (personal communication).

1.9.8- The cytotoxicity mechanisms of BFRs

Numerous in-vitro cellular studies using either primary cells or cell lines from different tissues organs has shown that most BFRs are toxic, and cause cell death at low concentrations. The toxic mechanism of BFRs, which causes cell death, is still not clear. Therefore, the mechanism of cytotoxicity for BFRs has been the subject of extensive study. Several studies with BFRs suggest that calcium unregulation is a possible mechanism of toxicity. Furthermore, in an in-vitro study on cultured cells have demonstrated that TBBPA and HBCD cause cell death by mitochondrial-regulated events involving cellular stress by either inducing the generation of ROS and abnormal increases in cytosolic Ca^{2+} concentration level which then induces mitochondrial Ca^{2+} overload and activation the PTP **(Ogunbayo et al., 2008; Zhang et al., 2008)**. Other BFRs either appear to cause cell death by non-typical apoptotic pathways which do not involved mitochondrial processes **(Reistad et al., 2005)**. Furthermore, some BFRs have been reported to cause cell death by a DNA fragmentation process, similar to apoptosis, yet this process is caspase-independent in some cell types and caspases-dependent in others **(Reistad et al., 2005, 2007; Ogunbayo et al., 2008)**. Such results may indicate the involvement of the apoptosis-inducing factor (AIF) which can trigger apoptosis without the initial involvement of caspases **(Lorenzo and Susin, 2007)**. All these observations suggest that different routes of BFR-induced cell death are activated, dependent upon the species of BFR

used and the cell type studied. Additional studies by others have also shown that some of the PBDEs congeners can cause translocation of protein kinase C (PKC) to the plasma membrane. As there appears to be a correlation between the extent of PKC translocation and the degree PBDE congeners accumulation within the cells, this is being considered as a potential indicator or "biomarker" for the assessment toxic levels of exposure **(Reistad et al., 2005)**. Similar effects were observed for mussel hemocytes exposed to TBBPA with a rapid increase in phosphorylation of the mitogen activated protein kinase (MAPK) family member, c-JNK, and PKC followed by a delayed increase in phosphorylated ERK2 **(Canesi et al., 2005)**.

Dr Michelangeli's group is the first to suggest a specific molecular target for BFR interactions inside the cell. Recent work by this group has shown that TBBPA, which causes abnormal elevation of intracellular Ca^{2+} levels within cells, causes its effects by directly binding to and inhibiting intracellular Ca^{2+} pumps (SERCA Ca^{2+}- ATPase) in sub-micro molar range and activating RyR, type Ca^{2+} channels **(Ogunbayo et al., 2008)**. This effect is not through indirect effects caused by oxidative damage of these proteins by ROS as was previously consideration **(Reistad et al., 2007)**. From enzymological, spectroscopic and modeling studies have also identified the potential binding site (s) for TBBPA on the Ca^{2+} pumping ATPase **(Ogunbayo and Michelangeli, 2007)**.

1.9.9- Metabolism and pharmacokinetics of BFRs

BFR metabolism is an important factor in determining the bioaccumulation as well as the fate, pharmacokinetics (toxicokinetics), and potential toxicity of BFRs in exposed organisms. Bioaccumulation is the net uptake and retention of a compound in living tissue from all routes of exposure **(van den Berg et al., 1998)** resulting in higher concentration within the organism compared with ambient concentration. Living organisms can be exposed to contaminants by water, air, food, and soil and contaminants can occur through the respiratory system, the skin, and the digestive system. This bioaccumulation process can lead to exposure of organisms to high concentrations of potentially toxic compounds. Although xenobiotic metabolism is the set of metabolic pathways that modify the chemical structure of xenobiotics, they are

compounds foreign to an organism's normal biochemistry, such as drugs and poisons. These pathways are a form of biotransformation present in all major groups of organisms, mainly in the liver, kidney, lung, intestine and placenta. These reactions often act to detoxify poisonous compounds; however, in some cases, the intermediates in xenobiotic metabolism can themselves be the cause of toxic effects.

Xenobiotic metabolism is divided into two phases. In phase I reactions (oxidation, reduction and hydrolysis), enzymes such as cytochrome P450 oxidases introduce reactive or polar groups into xenobiotics. These modified compounds are then conjugated to polar compounds in phase II reactions. These reactions are catalyzed by transferase enzymes such as glutathione S-transferases. Finally, the conjugated xenobiotics may be further modified and excreted.

Studies on the metabolism of a variety of BFRs have shown it to be susceptible to several metabolic processes including oxidative debromination, reductive debromination, oxidative CYP enzyme-mediated biotransformation, and/or phase II conjugation (glucuronidation and sulphation). Moreover, BFRs exposure can affect in the induction of phase I CYP monooxygenase enzymes and phase II conjugation enzymes (glucuronosyltransferases, sulfotransferases, and glutathione-S-transferases) **(Nelson et al., 1996; Lewis et al., 1998)**. For instance, the most common inducers of CYP1A are planar aromatics like polynuclear aromatic hydrocarbons (PAHs) and coplanar polychlorinated biphenyls (PCBs), although CYP2B and CYP3A are induced by globular molecules such as ortho-chlorine substituted (PCBs). Phase I hydroxylated metabolites (OH–PCBs) are subsequently metabolized via phase II conjugation, although competition with protective mechanisms such as protein binding may result in tissue retention. Reductive and oxidative dehalogenations are also both prospective enzyme-mediated processes that can lead to dehalogenated metabolites.

Metabolism studies on PBDE in rats have shown that both debromination and CYP enzyme-mediated OH-metabolite formations are the main biotransformation pathways **(Hakk and Letcher, 2003)**. The distribution of some of PBDE congeners (BDE-47, BDE-85 and BDE-99) have also been studied in animals. The animals were subjected to whole-body autoradiography using ^{14}C-labelled BDE-47, BDE-85 and BDE-99

(Klasson-Wehler et al., 1996; Orn and Klasson-Wehler, 1998; Hakk et al., 2002; Darnerud and Risberg, 2006). Labelled BDE-85 and -99 were also used in quantitative studies on milk transfer and tissue concentrations during the neonatal period (14 days post partum), **(Darnerud and Risberg, 2006)**. High radioactivity concentrations results show that PBDE congeners (BDE-47) were effectively absorbed in the fat *deposits* and other organs (i.e. liver, adrenal and ovary, lung and brain). At longer post-injection time, the concentration in most tissues was considerably lower, and radioactivity was mainly found in fat deposits and the liver. The data indicate that no considerable difference in distribution between the three studied PBDE congeners was observed. Following maternal exposure, the foetal uptake was limited. Alternatively, during lactation a considerable fraction of the dose (about 20% of the studied BDEs) given to the dam was transferred to the offspring **(Darnerud and Risberg, 2006)**. Furthermore, uptake, distribution, metabolism and excretion of PBDEs congeners (BDE-47) have been studied in animals (rats and mice) dosed orally with ^{14}C-labelled BDE-47 **(Klasson-Wehler et al., 1996; Orn 1997; Orn and Klasson-Wehler, 1998)**, results showed, 14% from labelled BDE-47 was excreted in rats faeces and less than 0.5% in urine while 20% of the same dose was excreted in faeces and 33% in urine of mice during 5-days. These studies also showed that all tissues analyzed contained labelled BDE-47 and the highest concentrations were found in adipose tissue; such as liver, lung, kidney, and brain. These results suggest that mice have a limited ability to metabolize BDE-47**(Orn and Klasson-Wehler, 1998)**.The liver also a contained low concentration of PBDE congener's hydroxylated metabolites **(Orn and Klasson-Wehler, 1998; Darnerud and Risberg, 2006)**.

Another study by **Meerts et al., (1999)** has shown that in exposure of pregnant rats to ^{14}C-labelled TBBPA, a major portion of radioactivity was excreted in faeces (79.8%) over a period of 13-days. In-vivo studies on TBBPA metabolism have shown that oral exposure of both humans and rodents to TBBPA lead to low blood levels of TBBPA and its metabolites were excreted in urine due to the high molecular weight of TBBPA metabolites **(Hakk and Letcher, 2003)**, while at the high doses used in rats, TBBPA or its metabolites were detected in bile, and excreted predominantly in faeces **(Hakk et al., 2000; Szymanska et al., 2001)**. In addition, TBBPA metabolites attributed to

conjugation reactions of TBBPA with glucuronic acid and with sulfate were also observed **(Schauer et al., 2006)**.

Yu and Atallah, (1980) have been shown that HBCD was rapidly absorbed, distributed and metabolized in the entire body with highest concentrations found in adipose tissue, followed by liver, kidney, lung and gonads when the animal was fed a single oral dose of ^{14}C-labelled HBCD. Another study found that 72% of fed HBCD was eliminated mainly in faeces and 16% by urine within 72h **(de Wit, 2002)**. Four metabolites were found but no information on their structures was given. HBCD absorption followed a two-compartment open model system, with the central compartment consisting of blood, muscle, liver, kidney and other non-adipose tissues, and the peripheral compartment consisting of fat tissue. Elimination from fat was slower than for the central compartment. Furthermore, another study by **Ryuich et al. (1983)** has shown that when Rats are fed HBCD daily for 5 days no urinary excretion of HBCD was detected **(de Wit, 2002)**, and excretion accounted for 29-37% of the administered amount. HBCD was found to accumulate mainly in the adipose tissue and absorbed from the intestine **(de Wit, 2002)**.

1.10- Environmental plasticisers and related chemicals

Environmental plasticisers (EPs) and related chemicals are global pollutants that are found ubiquitously in the environment, and which are used in food and drink packaging (e.g., water and infant bottles); the resins are used as lacquers to coat metal products such as food cans, bottle tops, and water supply pipes contaminating water, air, food and sediments. These pollutants are produced in enormous volumes (hundred of thousands of tonnes annually) **(Soto et al., 1991; Ben-Jonathan and Steinmetz, 1998; Balaguer et al., 1999)**.

Exposure to EPs may be from numerous sources and most probably from mixtures of different chemicals. It has been suggested that most EPs have been shown to have endocrine disrupting activities **(Sumpter et al., 1998; Kirk et al., 2003)**, and acts as 'xenoestrogens' that have the potential to interfere with hormonal regulations and the normal endocrine system and consequently cause health effects in animals and humans **(Sumpter et al., 1998; Guilette et al., 1999; Skakkebaek et al., 2001; Toppari et al., 1996)**. EDs such as bisphenol-A (BPA) and 4-n-nonylphenol (NP)

have oestrogenic effects **(Jobling et al., 1995; Routledge and Sumpter, 1997)**. BPA was shown to possess anti-androgenic activities and NP was also found to be a weak androgen agonist **(Sohoni and Sumpter, 1998)**. EPs are generally much less potent at activating hormone receptors when compared to endogenous hormones. For example, NP has much lower affinities for the oestrogen receptors (ERs) compared to endogenous hormone 17β-oestradiol **(Pillon et al., 2005)**. Some of these EDs have been shown to disrupt development, the immune system, and the neural system in animals such as fish, reptiles and possibly humans **(Bonefeld-Jorgensen 2004; Bonefeld- Jorgensen and Ayotte, 2003; Owens and Koeter, 2003; vom Saal and Hughes et al., 2000)**. Further, in-vitro studies have been shown that BPA and NP can hinder male sexual development and sperm production **(Han et al., 2004; de Jager et al., 1999)** and are highly toxic to spermatogenic and other testicular cells at low micromolar concentration **(Raychoudhury et al., 1999; Hughes et al., 2000)**.

Most of the EDs are synthetic compounds, some of which were designed to act as estrogens (e.g., oral contraceptives), while many were designed for other uses and accidentally found to be oestrogenic, because they contaminated in-vitro experiments studying natural oestrogens **(Krishnan et al., 1993; Soto et al., 1991)**. Naturally occurring xenoestrogens in the environment include phytoestrogens produced by plants which have numerous effects, including antioxidative and apoptotic activity, inhibitors of kinases, and suggested anticancer actions on prostate and breast carcinomas **(Basly and Lavier, 2005; Mueller, 2002; Sirtori et al., 2005)**.

1.10.1- Bisphenol-A (BPA)

BPA (2,2-bis(4-hydroxyphenyl) propane) was first synthesized in 1891. In the 1930's, BPA was investigated in the search for synthetic oestrogens. It was discounted as a therapeutic agent by the discovery of the much more potent synthetic hormone diethylstilbestrol (DES) **(Quesada et al., 2002)**. The structure, metabolism and action of BPA are very similar to DES **(see fig.1.12)**. BPA is a white solid, organic compound with two phenol functional groups **(fig.1.12)**. Global production of BPA in 2003 was estimated to be over 2 million metric tonnes **(NTP-CERHR, 2008)** and is used as a monomer in the manufacture of epoxy resins, polyester resins, polysulfone resins, polyacrylate resins, polycarbonate plastics, and flame retardants **(European-**

Union, 2003; Krishnan et al., 1993). Some polymers used in dental sealants and tooth coatings contain BPA **(European-Union, 2003)**. It is also used as an antioxidant and polymerization inhibitor in polyvinylchloride (PVC) production **(vom Saal et al., 1995)**.

BPA may be present in the environment as a result of direct releases from manufacturing or processing facilities via leaching from polycarbonate plastic products **(Krishnan et al., 1993)**. Exposure to the general population can occur through direct contact with BPA or by exposure to food or drink that has been in contact with a material containing BPA **(EFSA, 2006)**.

BPA is one of the most common EDs found ubiquitously in environment. It mimics 17β-oestradiol by binding ER (α) and ER (β) and can modulate target gene expression **(Kuiper et al., 1998, Matthews et al., 2001; Singleton et al., 2004)**, although it's oestrogenic activity was about four orders less potent than that of 17β-oestradiol **(Gaido et al., 1997)**. **Quesada et al., (2002)** also showed that low doses of BPA and 17β-oestradiol activated the transcription factor cAMP-responsive element binding protein (CREB) with similar potencies, which can modulate transcription of genes containing upstream cAMP/Ca^{2+} response elements. However, industry funded studies previously indicated that there were no human health risks from BPA at concentration found in the environment. Current risk assessments indicate that BPA is not thought to be a carcinogenic risk to humans. On the other hand, most of independent studies showed that there were potential human risks associated with BPA exposure **(vom Saal et al., 1995)**. BPA is demonstrated to induce male reproductive toxicities such as reductions in epididymis weight, seminal vesicle weight, sperm motility and cell function in in-vivo models **(Takahashi and Oishi, 2001; Takao et al., 1999)**. Furthermore, BPA induced cell death in many kinds of cells in in-vitro models **(Hughes et al., 2000)**. Results from epidemiological human studies have shown a relationship between high urine or blood concentrations of total or free BPA and a variety of health disorders including cardiovascular disease and diabetes **(Melzer et al., 2010)**.

Environmental exposure to BPA is calculated as less 1µg/kg body weight/day. It was suggested that BPA may be metabolized in-vitro to more toxic metabolites **(Bonefeld-**

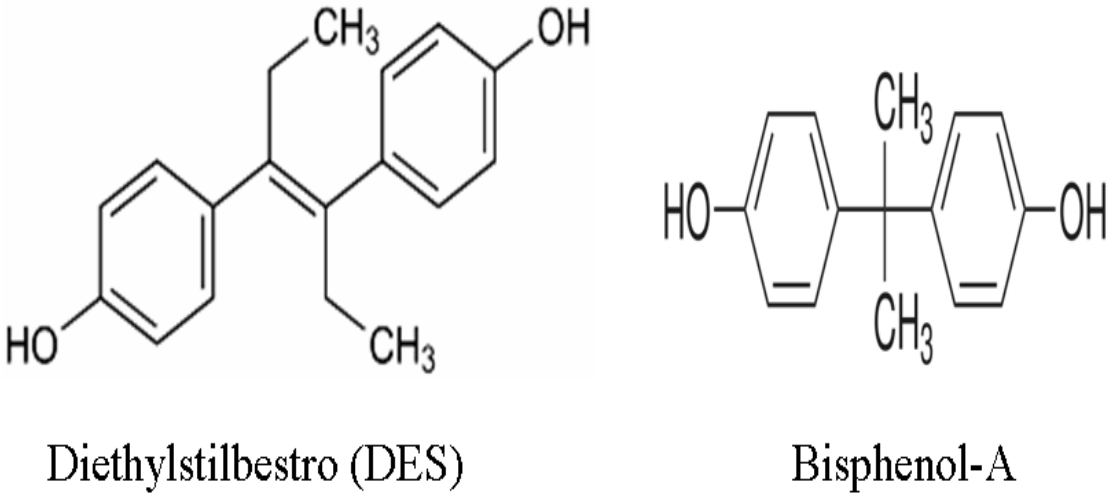
Jorgensen and Ayotte, 2003). Recent studies, however, propose that it is rapidly glucuronidated and excreted, without producing potentially reactive intermediates **(Haighton et al., 2002)**.

1.10.2- Alkylphenols

Alkylphenols (APS) are initiating materials for the synthesis of AP ethoxylates through the alkylation of phenols ring, which can differ in size, branching, and position. The most significant members are ethoxylates of nonylphenol and octylphenol (OP). AP ethoxylates are commonly used as non ionic detergents or dispersive agents in paper and leather manufacturing, emulsifiers for pesticide formulations and as auxiliary agents for drilling and flotation. The number of ethoxylate units can be more than 100. AP ethoxylates are produced in huge quantities; the annual worldwide usage is estimated to be approximately 600,000 tons **(Groshart et al., 2001)**. There is data indicating that both the position and branching of the alky group can influence endocrine disrupting effects of APs **(Routledge and Sumpter, 1997)**. There may be significant consequences of human exposure to APs, as these compounds are known to be persistent and bio-accumulate and be biomagnified through the food chain **(Guenther et al., 2002)**.

NP is a common name for several isomeric substances of the general chemical formula C_6H_4 (OH)C_9H_{19}, consists mainly of a phenol which is substituted in para-position with side chains of different degrees of branching. Moreover, 4-n-nonylphenol (NP) in which the n-name side chain is attached to the carbon directly opposite the hydroxyl group (OH), is the most common member of NP **(NRC, 1982) (fig. 1.13)**. They bio-accumulate within man, with levels in the µM concentration range reported in human tissues **(Michelangeli et al., 2008)** and have oestrogenic effects in-vitro and in-vivo **(Harris et al., 1997)**. **Kuiper et al., (1998)** showed that NP completes with 17β-oestradiol to bind to ER (α) and ER (β). Effects were observed with low concentration (0.1-1µM), although binding affinities to the ERs were much lower than those observed for 17β-oestradiol. NP is ubiquitous in food and data collected indicated that amounts in food varied between 0.1 and 19.4µg/kg, regardless of the fat content of the food **(Guenther et al., 2002)**. Recent studies have found levels of nonylphenol in samples of human milk to be as high as 56 ng/ml

(equivalent to 0.26 µM) **(Ademollo et al., 2008)** and, as high as 268 ng/g (equivalent to 1.2 µM) in some human blood plasma samples **(Chen et al., 2008)**. More recently however, other potential routes for endocrine disruption have been shown, which includes NP binding to ARs (where the IC_{50} value for androgen receptor binding was about 20µM for nonylphenol) **(Xu et al., 2005)**; cytochrome P450 expression (where effects were seen in the low µM concentration range) **(Hasselberg et al., 2004)**; aromatase activity (where the IC_{50} for nonylphenol was approximately 300µM) **(Benachour et al., 2007)**, sulfotransferase activity (where amylphenol was effective at less from 1µM) **(Harris et al., 2000)** and sulphate supply enzymes (where the effects for nonylphenol were also seen in the sub-µM concentration range) **(Turan et al., 2005)**.



Diethylstilbestro (DES) Bisphenol-A

Figure 1.12: chemical structures of DES and BPA.

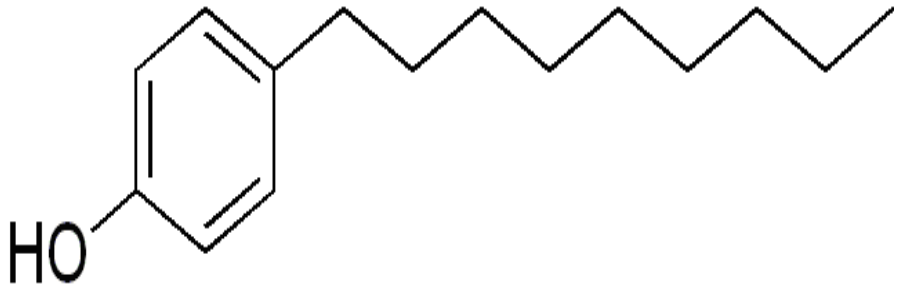


Figure 1.13: chemical structure of 4-n-nonylphenol.

1.11- Aims of this study:-

In order to understand the mechanism or mechanisms by which a number of environmentally prevalent brominated flame retardants (BFRs) and alkylphenols (AP) cause toxicity within neuronal cells (in-vitro) the following objectives will be addressed:-

1- To identify the types of BFRs and AP which cause neurotoxicity within SH-SY5Y human neuroblastoma cells (as model of brain cells) and to determine which are most toxic.

2- To what extent do commonly utilized BFRs and AP have the ability to dys-regulate Ca^{2+} signalling within SH-SY5Y cells by causing excessive changes in intracellular [Ca^{2+}] levels and to investigate if these abnormal changes in Ca^{2+} involve modulation of intracellular Ca^{2+} transport proteins.

3- To determine the mechanism by which BFRs and AP cause abnormal changes in intracellular Ca^{2+} levels leading cell death. Focus on the potential neurodegenerative effects of some commonly used BFRs and AP by investigating their effects on SHSY5Y human neuronal cells to elucidate the mechanisms of cell death, by monitoring there effects on caspases, mitochondria, cytochrome c release, ROS production and also their ability to secrete β-amyloid peptide, indicative of the potential to induce AD.

CHAPTER 2

Materials and Methods

2.1-Materials

2.1.1-Cell culture

All cell culture work was done using aseptic conditions and techniques. A Calls II Holten LaminAir Biological Safety Cabinet was used and all consumables and regents were purchased as sterile or were autoclaved prior to use. Ethanol, 70 % (v/v) was used to clean the cabinet and devices before and after use. Cells were cultured at 37°C and 5% carbon dioxide (CO_2) humidified sterile incubator.

2.1.1.1- Cell line investigated

The human neuroblastoma SH-SY5Y cell line is similar to common nerve cells in terms of shape, physiological and biochemical function. SH-SY5Y cells are therefore commonly used as a cellular model to investigating a number of neurological pathologies **(Gilany et al., 2008; Schaeffer et al., 2008)**. The SH-SY5Y cells are a thrice-cloned sub-line of SK-N-SH. Cells have dopamine-beta-hydroxylase activity and can convert glutamate to the neurotransmitter GABA. It is recommended not to use cells after passage 20 due to loss of neuronal characteristics **(ECACC)**. The SH-SY5Y and COS-7 cells were retrieved from the liquid nitrogen store of Dr. Frank Michelangeli, University of Birmingham, UK.

2.1.1.2- Tissues

Rabbit muscle was obtained from New Zealand white rabbits. Each rabbit was killed a by lethal dose of the anaesthetic, sodium phenobarbitone. Porcine cerebellar microsomes were obtained from a local abattoir and returned to the laboratory on dry ice. All tissues were frozen in liquid nitrogen and stored at -80 °C.

2.1.2- Chemicals

Nine different commercial available of BFRs were purchased from different sources; tetrabromobisphenol-A (TBBPA) (purity 100%) (CAS No. 79-94-7) was purchased from Acrôs Organics, dibromobiphenyl 98%, tetrabromohydroquinone 100%, hexabromocyclododecane (HBCD) 100%, decabromodiphenyl ether (DBPE) 100%, Tetrabromobisphenol A diallyl ether 99%, and tribromophenol 99% were purchased

from Sigma-Aldrich (UK), whereas, two commercial mixtures of polybrominated diphenyl ethers: pentabromodiphenyl ether (TBDE-71X) and octabromodiphenyl ether (TBDE-79) were purchased from Wellington Laboratories Inc, and 4-n-nonylphenol which were bought from Lancaster Synthesis Ltd and bisphenol-A (BPA) from Sigma-Aldrich (UK)

The caspases substrates: where specific peptides attached to 7-amino-4-methylcoumarin (AMC), caspase-3 fluorogenic substrate; Ac-DEVD-AMC, caspase-8 fluorogenic substrate; (Ac-IETD-AMC), and caspase-9 fluorogenic substrate; (Ac-LEHD-AMC) were purchased from Alexis Biochemicals (UK). Caspase-8 Inhibitor (Z-IETD-FMK) and caspase-9 Inhibitor (Z-LEHD-FMK) were purchased from Merck Chemicals Ltd (UK).

MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide thiazole blue), 2',7'-dichlorofluorescein diacetate (DCFH-DA), Rhodamine123 (Rh 123), Fluo-3, A23187 and fluorecein 5'-isothiocyanate (FITC) were purchased from Sigma-Aldrich (UK). [γ-^{32}P] ATP was purchased from Amersham Life Science Ltd. (Little Chalfont, Bucks, UK). Metafectene^{TM} transfection reagent was purchases from Biontex Laboratories, GmbH.

Dulbecco's Modified Eagle's medium (DMEM) were purchased from Lonza (UK). Phosphocreatine and creatine kinase were obtained from Boehringer Mannheim (Diagnostic and Biochemicals) Ltd. (Lewes Sussex, UK).

All other chemicals were of analytical grade and were obtained from either Bio-Rad Laboratories Ltd, (Hemel Hempstead, UK), Sigma-Aldrich Co. Ltd or BDH Laboratory Supplies (Poole, Dorset, UK).

2.1.3- Reagent preparation

All BFRs congeners that were dissolved in dimethylsulfoxide (DMSO, Sigma), which was no more than 2.0% (v/v) of the assay volume.

2.2- Methods

2.2.1- Culture medium and sub-culture routine

Human neuronal SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1% penicillin (20 units/ml), streptomycin (20 mg/ml), containing 10% (v/v) heat-inactivated foetal bovine serum (FBS), and 1% (v/v) non essential amino acids. SH-SY5Y cells were routinely sub-cultured by splitting sub-confluent cultures (70-90%) at a rate of 1:3 to 1:10 (seeding at density of 10^3- 10^5 cells/cm^2).

2.2.2- Storing Cells in liquid nitrogen

Stock aliquots of cells were frozen and stored under gaseous phase of liquid nitrogen at -80 °C. The aliquots contained a cryopreservant (10% v/v DMSO), which prevented cellular lysis (from intracellular ice crystals and alterations in osmotic balance) during the freezing process.

2.2.3- Preparation of SR Ca^{2+}-ATPase (SERCA1)

Sarcoplasmic reticulum (SR) was prepared from rabbit skeletal muscle as described by **Michelangeli and Munkonge (1991)** and typically had a purity of approximately 80% of SERCA Ca^{2+}-ATPase. Briefly, Minced rabbit muscle (around 300 g) was added to 300 ml of ice cold homogenization buffer (300 mM sucrose, 20 mM L-histidine, 1mM dithiothreitol (DTT), 5 µM phenylmethylsulponyl flouride (PMSF), pH 8.0) and homogenized in dual speed waring blender for about 1 min. The homogenized muscle was centrifuged for 20 min at 10000*g*. The supernatant was filtered through 2 layers of muslin and kept on ice. The pellet was re-homogenized and centrifuged as before, in further 300 ml of homogenization buffer. The resultant supernatant was filtered as previously done and then combined with the previous supernatant. The pooled supernatant was centrifuged at 30,000*g* for 90 min and the resultant pellet resuspended in 100 ml of cold buffer containing 300 mM sucrose, 10 mM L-histidine, 600 mM KCl, 1mM DTT, 5 µM PMSF, pH 8.0. The homogenate was then left on ice for 30 min to allow actin and myosin precipitation, before further

centrifugation for 20 min at 5,000*g*. The supernatant was carefully removed and centrifuged at 30,000*g* for 120 min. The pellet (SR) was resuspended in approximately 10 ml of buffer (250 mM sucrose, 1000 mM KCl, 50 mM K_2HPO_4, pH 8.0) and dialyzed overnight in 1000 ml of the same buffer. It was then aliquoted into 50µl fractions, snap-frozen in liquid N_2 and then stored at -80 °C.

2.2.4- Preparation of microsomal membranes

This method was described previously by **Michelangeli et al., (1991)**. Microsomal membranes were prepared from either SH-SY5Y cells or whole pig brain (cerebellum). SH-SY5Y cells ($1x10^6$ cells/cm^2) which were harvested by centrifugation at 1,000*g* for 10 min 4°C and washed with PBS, while pig brain was minced. Both were homogenized using a Teflon Potter-Elvejem homogenizer in 10 vol. of buffer containing 300 mM sucrose and 5 mM Hepes, pH 7.4, in the presence of 0.1 mM PMSF, 10 µM leupeptin, 10 µM pepstatin A and 50 µM benzamidine, and then centrifuged for 10 min at 500*g*. The pellet was resuspended in 5 vol. of the same buffer re-homogenized and centrifuged as above. The resulting supernatants were pooled and centrifuged for 20 min at 10,000*g*. The supernatant from this stage was centrifuged for 1 h at 100,000*g* and the resulting pellet (microsomal membrane) was resuspended in approximately 2 ml of the buffer, divided into 50-100 µl fractions, snap-frozen in liquid nitrogen and stored at -80 °C until use.

2.2.5- Determination of protein concentration

Protein estimations of the microsomal extracts were carried out using the Bio-Rad protein assay regent, following the manufacturer's instructions and using bovine serum albumin (BSA) as the standard. Protein estimations were measured by determining the absorbance at 590nm. Measurements were carried out in a spectrophotometer, (Amersham Pharmacia Biotech Ultraspec 1000 UV/Vis).

2.2.6- Cell viability assay

Cells were seeded in 24-well cell plates ($4x10^4$ cells/well) and allowed to cell grow at 37 °C until 80-90% confluence was reached. Pre-treatment with compound was

undertaken in the culture medium with DMEM (high glucose/without phenol or FBS). Stock solutions of compounds were prepared by dissolving in DMSO less than 2%. The cells were exposed to varying concentrations of compounds for 22-24h; cell viability was determined by MTT assay, using thiazolyl blue tetrazolium bromide (MTT reagent). Viable cells form an intracellular formazan product when incubated with MTT reagent for 2-3h, this product is solubilised with 1ml acidic isopropanol and then quantified spectroscopically by measuring the difference in absorbance ($\Delta A = A_{570} - A_{650}$) and compared to cells not exposed to compounds (but in DMSO alone up to 2%).

2.2.7- Caspase activity

Caspase-3, -8, and -9 activities in lysates were assayed using synthetic fluorogenic substrates (Ac-DEVD-AMC; substrate for caspase-3, Ac-IETD-AMC; substrate for caspase-8 and granzyme B, Ac-LEHD-AMC; substrate for caspase-9). SH-SY5Y cells ($5x10^5$ cells/35 mm dish) were incubated in the presence of varying concentration of compound for 12h and then lysed in 500 µl of lysis buffer (10 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1% Triton®-X- 100, 10 mM $Na_4P_2O_7.10H_2O$, and 10 mM Na_2HPO_4) on ice. After the addition of 1ml of protease buffer (20 mM HEPES, 10% glycerol and 2mM DTT) 20µM fluorogenic substrate (final concentration), was added to 100 µl protein of the cell lysates and the mixture was incubated for 1 h at 37 °C. The fluorescence (release of AMC) in the whole reaction mixture was measured with a spectrofluorimeter with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Fluorescence was converted to nmoles of AMC released, using the standard curve employing 7-amino-4- methylcoumarin.

2.2.8- Cytochrome c release assay

Detection of cytochrome c in mitochondrial and cytosolic protein extracts, using Immunoblotting. SH-SY5Y cells (5×10^5 cells/35 mm dish) were incubated in the presence of compound in DMEM culture medium for 12 h. The cells were suspended in lysis buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF), and disrupted using a Dounce

homogenizer. The cells lysates were centrifuged at 10,000*g* for 10min and the supernatants were further centrifuged at 100,000*g* for 30min. Both proteins in the final supernatant (cytosolic fraction) and the first centrifuged pellet (mitochondrial fraction) were separated by 15% SDS-PAGE, followed by electroblotting onto a nitrocellulose membrane (Protran Nitrocellulose**,** Whatman GmbH, Germany). Nonspecific binding sites were blocked by treatment with PBS containing 5% skimmed milk for 1 h. After a wash with PBS containing 0.1% Tween 20 (T-PBS), the membrane was incubated with primary antibody 'goat IgG' (cytochrome c (C-20), Santa Cruz Biotechnology, INC) at a dilution of 1:500 for 1 h. After washing, the membrane was then incubated with anti-goat IgG peroxidase (whole molecule) (1:3000) (Sigma, UK) in T-PBS for 1 h at room temperature. After further washing with T-PBS, the enhanced chemiluminescence assay was preformed, and the positive bands were detected with the chemiluminescent HRP substrate (Millipore Corporation, UK). Protein bands were visualized and analysed using a Fluor-S Multimager (Bio-Rad) and Quantity One 4.1.1 software.

2.2.9-Preparation of coverslips

Acid-etched sterile glass coverslips were placed into petri dish and coated with sterile 2% porcine skin gelatin (prepared in deionised water).

2.2.10- Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) in SH-SY5Y cells were monitored using the fluorescent dye Rhodamine123 (Rh123) as described in **(Hong and Liu, 2004)**. Depolarization of MMP results in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence **(Satoh et al., 1997)**. SH-SY5Y cells were plated on coverslips which were pre-coated with 2% gelatine; and incubated in the dark in Hanks Balanced Salt Solution (HBSS) (2 ml) containing 10 µM Rh123 (dissolved in ethanol) for 15 min at 37°C. Cells were washed twice with pre-warmed HBSS and exposed to varying concentrations of compounds and then analyzed by fluorescence microscopy, with the microscope fitted with a FITC filter cube. Up to 10 min video recordings were then made to observe the decrease in fluorescence with time upon exposure to the compounds.

2.2.11- Detection of reactive oxygen species

Reactive oxygen species (ROS) formation was measured by using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA is de-esterified by cells to 2',7'-dichlorodihydrofluorescein ($DCFH_2$), and then $DCFH_2$ is oxidized by ROS to form fluorescent 2',7'-dichlorofluorescein (DCF), as previously described **(Fotakis et al., 2005)**. Briefly, SH-SY5Y cells were cultured in 12-well plates, before exposure to compounds for 22-24 h, and then the cells were washed with PBS and then loaded with 40 µM DCFH-DA in hanks buffer (1 ml) (DCFH-DA was dissolved in DMSO ≤1%), at 37 °C with 5% CO_2 and constant humidity for 30 min. At the end of the incubation, the cells were washed with PBS. Then, 1000 µl NaOH (1 M) was added to extract the fluorescent product from the cells. These extract were transferred to cuvette and the fluorescent intensity was measured with a Perkin Elmer LS-50B spectrofluorimeter with an excitation wavelength of 485 nm and an emission wavelength range of 530 nm. ROS formation was expressed as the amount of DCF formed using a DCF standard curve (10-100 µM).

2.2.12- Fluorescence measurement of changes in intracellular [Ca^{2+}]

SH-SY5Y cells on the coverslips were allowed to grow to 40-50% confluence ($2x10^5$ cells/35 mm dish). The coverslips were incubated in sodium hydrogen carbonate-supplemented HBSS (pH 7.2), which contained 0.08 µM sulfinpyrazone, 1% bovine serum albumin, 0.025% pluronic acid and typically 10 µM Fluo-3-acetoxymethyl ester (Fluo-3 AM) for 50 min. This dye solution was then removed, replaced with fresh HBSS containing 0.08 µM sulfinpyrazone, and incubated for an additional 20 min. Both incubations were done with the coverslip kept in the dark (to minimise photo-bleaching) and at 35 °C (to keep cells viable and minimise dye leakage). Fluo-3 AM was dissolved in DMSO and cells were not exposed to more than 1% DMSO during the dye-loading process.

Each coverslip was then moved into a 35 mm plastic petri dish (Falcon) containing fresh HBSS (2 ml final volume), placed onto a heated microscope stage maintained at 35 °C and observed with a Nikon TS100F microscope in epi-fluorescence mode. The microscope was fitted with an FITC filter cube so that Fluo-3 fluorescence could be

monitored. Recordings of the cells, viewed at about 200x magnification, were taken using an Astrovid StellaCam-EX3 connected to a Hauppauge USB TV live video capture device for viewing on a PC. Win TV (Hauppauge; version 1.4) was used to record fluorescence videos of the cells at a frame rate of either 1frame/5s or 1frame/1s. Recordings were initiated about 50s before the compounds were added, which allowed the initial un-stimulated fluorescence intensity (F_o) to be determined. The compounds were dissolved in DMSO (some compounds such as HBCD need presence of 2-hydroxypropyl-β-cyclodextrin (450mg/ml) to improve the aqueous solubility) and cells were exposed to ≤ 1% DMSO (such as ≤ 30 µl in 3 ml of buffer) in experiments (this maximum concentration had no effect on the Fluo-3 fluorescence intensity of cells when added alone). Each series of images were analysed using Image J software (version 1.32j; National Institutes of Health USA). For each recording, the analysis involved the measurement of the mean intensity /cell area for a number of cells. After corrections for background fluorescence and photo-bleaching were made, these values were then converted into ratios of fluorescence intensities with respect to unstimulated fluorescence intensity (such as F/F_o) for each cell. These experiments were replicated at least three times.

2.2.13- Transfection

2.2.13.1- Making Luria-Bertani (LB) - Agar Plates

LB- Agar (1.2 % agar in LB media autoclaved) was heated in a microwave in a 80 ml bottle (for 1 minute on full power), followed by 5 minutes on medium power. The LB- Agar was allowed to cool on bench until warm enough to hold. Next, 1µl of 0.1g/ml Ampicillin for every 1ml of LB- Agar (e.g. 80µl of Ampicillin for 80ml of LB- Agar) was added and mixed gently to avoid bubbles in the LB- Agar). This was then poured into 10cm diameter petri dishes (80ml of LB- Agar should give 3 plates).

2.2.13.2- Plasmid preparation and transformation of DH5α cells

The expression vector pcDNA3.1 (+) (Invitrogen) containing an EGFP-tagged (at the C-terminal) of the SERCA1 sequence was constructed and provided by J.M. East (University of Southampton, UK) as described in **(Newton et al., 2003)**. Competent

DH5α cells were heat shocked to take up the plasmid DNA and transfered into LB-agar plates. Plates were incubated for 24h to allow colony growth. Selected colonies were transfered to LB liquid broth and an overnight culture was performed.

Plasmid DNA was then isolated from three overnight cultures, using PureLink™ HiPure Plasmid Filter Maxiprep Kits purchased from Sigma, (following the manufacturer's instructions).

2.2.13.3- Restriction digest and gel electrophoresis of maxiprep

A restriction digest enzyme such as NdeI was performed to ensure the maxiprep consists of only plasmids containing the SERCA1 gene. NdeI cuts at only one restriction site, which is not within any multiple cloning sites of pcDNA3.1 (+) or SERCA1 gene sequence. This allows the insertion of almost any specific fragment of DNA into plasmid vectors, which can be efficiently "cloned" by insertion into replicating bacterial cells. After restriction digest, DNA can then be analysed using 0.8 % agarose gel electrophoresis for 30 min at 80 volt then the gel was stained with Invitrogen SybrSafe DNA gel dye and visualized under UV light as shown in chapter 5 figure 5.2.1.1

2.2.13.4- Estimating plasmid DNA quality

The DNA concentration was estimated by measuring its absorbance at 260 nm (A_{260}). The DNA solution was diluted in 1in 10 mM Tris-HCl, pH 7.5 in a spectrophotometer where an absorbance of 1 (1 cm path length) is equivalent to 50 µg DNA / ml. The concentration of DNA was calculated using the formula in equation.1 (Eq.1):

$$\text{DNA (µg/ml)} = A_{260} \times 50 \text{ dilution factor for DNA (100µl of 10mM Tris-HCl)} \quad \text{Eq.1}$$

The plasmid DNA was relatively pure with an estimated concentration of 0.91µg/ml.

2.2.13.5- Transfection of SH-SY5Y cells

The SH-SY5Y cells were grown in 24-well tissue culture plates ($4x10^4$ cells/well) in 1ml of in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin, at 37°C under a 5% CO_2 atmosphere. The optimum transfection condition ratio of (1:8) was used the SERCA1 plasmid DNA: Metafectene^{TM} (Biontex). To over-express SERCA1, the SERCA1-EGFP pcDNA3.1 (+) plasmid was added to SH-SY5Y cells, according to the manufacturer's instructions. Briefly, when the cells reached 40-50% confluence they were washed once with PBS. The solutions of plasmid and metafectene transfection reagents were mixed with media (without FBS or antibiotics) and added to the cells and incubated for a period of 24 h. After that, the metafectene was discarded and fresh culture media, supplemented with FBS and antibiotics were added to the cells. Metafectene alone was used as a positive control for transfection, added in DMEM without phenol red, serum and antibiotics, following manufacturer procedures. Transfection required 48 h to complete.

2.2.13.6- MTT cell viability assay: after transfection

Fluorescent microscope images were taken 48 h after transfection to observe the proportion or efficiency of cells transfected with SERCA1-EGFP. In order to assess whether the transfected SH-SY5Y cells provided protection against compound-induced cell death, MTT cells viability assay were also carried out after transfection. The cells were exposed with the compounds for 22 h. MTT assays were then conducted using the same method as described previously and percentage cell viability was determined.

2.2.14- Flow cytometry

The cells were transfected and incubated at 37°C for 48 h before analysis for green fluorescent protein (GFP) expression. Cells were exposed to compound for 22-24h then washed with PBS and incubated with 0.05% trypsin-EDTA (Invitrogen, USA) until cells detached from the surface. After incubation, complete medium was added to inhibit trypsin, followed by centrifugation and washing with PBS. They were then

replenished with PBS containing 2% serum and 3µM of PI (Propidium iodide, Invitrogen, USA). Transfection efficiency was assessed by Flouresence-activated cell sorting analysis (FACS) analysis with the aid of a FACScalibur (BD Biosciences, CA), which used the 488 nm line of an argon laser to excite both PI and GFP. After cell observation with fluorescent microscopy, cell samples were transferred to FACS tubes and directly analyzed without any additional treatment. Data was then analyzed with Flowjo software (Treestar; USA), using appropriate controls and gates. Dead cells (PI positive) and cell debris were excluded from the calculation of the percentage of cells expressing GFP.

2.2.15- Ca^{2+}- ATPase activities

2.2.15.1- Measurement of SR Ca^{2+}-ATPase activity employing the coupled enzyme assay

The effects of compounds on SERCA activity in rabbit skeletal muscle SR were investigated at pH 7.2 and 25°C, using a coupled enzyme assay as previously described by **Michelangeli and Munkonge (1991)**. Typically, 10 µg/ml of SR Ca^{2+}-ATPase protein was added to a buffer containing 40 mM HEPES/KOH (pH 7.2), 1mM EGTA, 5 mM $MgSO_4$, 2mM ATP, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 8.0 units pyruvate kinase, and 20 units lactate dehydrogenase. SR Ca^{2+}-ATPase was incubated for 10 min at 25°C in 2.5 ml of assay buffer. ATPase activity was initiated by the addition of 90 µl of 25 mM $CaCl_2$, to give a free Ca^{2+} concentration of 6.5 µM (pCa 5.2). SR Ca^{2+}-ATPase activities were also investigated as a function of [Ca^{2+}], [ATP] and [Mg^{2+}] at pH 7.2, employing the coupled enzyme assay. In all experiments where the Ca^{2+} was varied, free Ca^{2+} concentrations were calculated by using the binding parameters as in **Gould et al., (1987)**.

The Ca^{2+}-ATPase activity was measured by monitoring the consequent oxidation of NADH to NAD^+, using an Amersham Pharmacia Biotech Ultrapspec 1000 UV/Vis spectrophotometer to monitor the change in absorbance at 340 nm. ATPase activity is expressed as international I.U/mg of protein employing the following (Eq.2):

I.U/mg = [OD/min X assay volume (2.5ml)/ [6.22 X mg protein] -**Eq.2**

The SR Ca^{2+}- ATPase activity data measured as a function of free [Ca^{2+}] gave a typical bell-shape profile. This profile was fitted using Eq.3:

$$V = \left(\frac{V_{max} \; x \; [Ca^{2+}]}{Ks \; + \; [Ca^{2+}]} + \frac{V_{max}}{(1 + [Ca^{2+}]/Ki)} \right) - V_{max} \quad \text{-Eq.3}$$

Where K_s is the stimulatory constant and K_i is the inhibitory constant.

While the activity data as a function of [ATP] was fitted into Eq.4:

$$V = \frac{[ATP] \; x \; V_{max}(cat)}{K_m(cat) + [ATP]} + \frac{[ATP] \; x \; V_{max}(reg)}{K_m(reg) + [ATP]} \quad \text{-Eq.4}$$

Where Vmax (cat) and Km (cat) are the enzymatic parameters for the catalytic ATP site and Vmax (reg) and Km (reg) are the enzymatic parameters for the regulatory ATP site as previously described in **(Coll and Murphy, 1991)**.

The Ca^{2+}-ATPase activity data was also measured as a function of [Mg^{2+}] was fitted to a simple enzyme inhibition equation; Eq.5:

$$V = \frac{V_{max}}{(1 + [Mg^{2+}]/K_i)} \quad \text{-Eq.5}$$

2.2.15.2-Determination of Ca^{2+}-ATPase activity in microsomal extracts employing the phosphate liberation assay

The Ca^{2+}-dependent ATPase activity of pig brain and SH-SY5Y microsomal membranes was performed using the phosphate liberation assay as described by **Longland et al., (1998)** with minor modifications as described in **Wootton and Michelangeli (2006)**. Briefly, microsomal extracts (5 µg) were re-suspended in 200 µl of buffer containing 45 mM HEPES/KOH (pH 7.0), 6 mM $MgCl_2$, 2 mM NaN_3,

250 mM sucrose, 12.5 µg/ml A23187 ionophore, and EGTA with $CaCl_2$ added to give a free [Ca^{2+}] of 1µM. Assays were pre-incubated at 37 °C for 10 minutes prior to addition of ATP (final concentration 6 mM) to initiate activity. The reaction was stopped by addition of 50µl 6.5% (w/v) trichloroacetic acid (TCA) after 30 min. The samples were put on ice for 10 min before centrifugation for 10 min at 14,000*g*. The supernatant (100 µl) was added to 150 µl buffer containing (11.25% (v/v) acetic acid, 0.25% (w/v) copper sulphate, and 0.2 M sodium acetate pH 4.0). Ammonium molybdate (25 µl of 5% (w/v)) was then added, followed by the addition on 25µl of ELAN solution (2% (w/v) p-methyl-aminophenol sulphate and 5% (w/v) sodium sulphate). The samples were mixed and the blue colouration was allowed to develop for 10 min prior to measuring the absorption at 870 nm using a Dynatech Laboratories ELISA plate reader. The amount of Pi liberated due to Ca^{2+} independent ATP breakdown could then be calculated and subtracted from the amount liberated in the presence of 1µM free [Ca^{2+}], to give the Ca^{2+} -dependent ATPase activity.

2.2.16- ATP-dependent Ca^{2+} uptake and release

ATP-dependent Ca^{2+} uptake from SH-SY5Y microsomal membrane and release from SR were by monitoring changes in fluorescence of the Ca^{2+} indicator dye Fluo-3 (free acid) as described by **Michelangeli (1991)**. Briefly, microsomal membranes (100-300µg) were added to a stirred cuvette containing 2 ml of 40 mM Tris/phosphate, 100 mM KCl (pH 7.2) in the presence of 1.25 µM Fluo-3 (free acid), 10 µg/ml creatine kinase, and 10 mM phospho-creatine. Range of concentration of compounds was then added prior to Ca^{2+} accumulation. Ca^{2+} uptake within the membranes was then initiated by the addition of 1.5 mM Mg-ATP. Total Ca^{2+} accumulation was measured by the addition of the calcium ionophore A23187 (12.5µg/ml). The initial rate of Ca^{2+} uptake was calculated by measuring the [Ca^{2+}] change during the first two min. Fluorescence changes were measured in a Perkin Elmer LS-50B spectrofluorimeter, using excitation and emission wavelengths of 506 nm and 526 nm, respectively. Ca^{2+} concentrations were determined using the following Eq.6

$$[Ca^{2+}]_i = Kd \; X \; ((F-F_{min})/ (F_{max}- F)) \quad \text{-Eq.6}$$

where K_d is the dissociation constant for Ca^{2+} binding to Fluo-3 (900 nM at 37 °C, pH 7.2, in 100 mM KCl), F is the fluorescence intensity of the sample, and F_{min} and F_{max} are the fluorescence intensities in the presence of 0.25 M EGTA and 1 M $CaCl_2$, respectively **(Mezna and Michelangeli, 1996)**. Percentage Ca^{2+} released (%) was determined comparing the amount of Ca^{2+} released by the compounds with that observed with the addition 12.5µg/ml A23187 (Ca^{2+} ionophore).

2.2.17- Phosphorylation studies

Phosphorylation of SERCA by [γ -^{32}P] ATP was carried out at 25 °C as described in **(Michelangeli et al., 1990; Longland et al., 1999; Bilmen et al., 2002; Ogunbayo and Michelangeli, 2007)**. The SERCA was diluted to 0.1 mg/ml in 40 mM Hepes/Tris (pH 7.2) containing 100 mM KCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$, BSA (final conc. 1 mg/ml) and 12.5µg/ml A23187 in a total volume of 1 ml of ATP stock (0.5 mM) with specific radioactivities of 10 Ci/ mol was made in the above buffer to cover the range between 0 and 20 µM. The reaction was initiated by the addition of [γ -^{32}P] ATP and stopped by the addition of 250µl ice-cold 40% (w/v) TCA after 15s. The samples were placed on ice for 30 min. SR Ca^{2+}-ATPase was separated from the solution by filtration through Whatman GF/C filters. The filters were washed with 30 ml of 12.5 % (w/v) TCA containing 0.2 M H_3PO_4 and left to dry. The filters were placed in scintillant and counted.

2.2.18- Effects of HBCD on FITC-labelled Ca^{2+}-ATPase

SR Ca^{2+}-ATPase was labelled with fluorescein 5'-isothiocyanate (FITC), according to the method described by **Michelangeli et al. (1990)**, to monitor the E2 → E1 transition. The Ca^{2+}-ATPase (1.1mg/ml) was added in equal volume to the starting buffer (1 mM KCl 250 mM sucrose and 50 mM potassium phosphate pH 8.0). FITC in dimethylformamide was then added at a molar ratio of FITC/ATPase, (0.5: 1 molar ratio). The reaction was incubated for 1h at 25 °C and stopped by the addition of 250µl of stopping buffer (0.2 M sucrose, 50 mM Tris/HCl pH 7.0), which was left to incubate for 30 min at 30 °C prior to being placed on ice until required. Fluorescence measurements of FITC-ATPase were made in a buffer containing 50 mM Tris, 50

mM maleate, 5 mM $MgSO_4$ and 100 mM KCl at pH 6.0. Fluorescence was measured in a PerkinElmer LS50B fluorescence spectrophotometer at 25 °C (excitation 495 nm, emission 525 nm). Ca^{2+} (400 µM) was then added to induce changes in fluorescence intensity.

2.2.19- The effects of different types of BFRs and APs on Amyloid Precursor Protein (APP) processing in SH-SY5Y cells

2.2.19.1- Measurements of Beta-Amyloid (Aβ) peptide level by ELISA

Aβ 1-42 level was determined by using the BetaMark x-42 ELISA kit (Covance). In brief, SH-SY5Y cells (seeded at $4x10^4$ cells/100 mm petri dish) were incubated in the presence of different concentrations of compounds for up to 12h. After the desired incubation times, cell culture supernatants were removed and centrifuged to precipitate any cellular debris. Proteinaceous material was precipitated with 10% TCA for 15 min at 4°C followed by centrifugation at 21,000g. The resultant pellets were resuspended in a buffer containing 150 mM Tris- HCl buffer (pH 7.5) containing 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, in presence of protease inhibitors (10µM leupeptin) for 24 h at 4 °C and protein concentrations determined with the protein assay of Bradford. Samples were diluted to 1mg/ml with working incubation buffer according to the manufacturer's instructions. Antigen is coated on the surface of a microtiter plate as capture antibody. A second antibody conjugate serves as the detection 1-42 Aβ antibody in the assay. The antibodies and the respective amyloid-peptide form antibody-amyloid-antibody (sandwich) complexes. Peroxidase enzyme catalyzes the conversion of a substrate (Chromogen) into a coloured product, which subsequently is measured by photometry and correlates directly to the 1-42 Aβ concentration present in the sample. Measured values are quantified in correlation to a synthetic peptide standard. The assay is run on standard plate reader and all Aβ-ELISAs were performed in triplicate. The standard curve was constructed by plotting the mean value of each duplicate absorbance of Aβ x-42 standard peptide and then this was used to determine the concentration of Aβ x-42 of unknown samples.

2.2.19.2- Immunoblotting of APP fragments

Cellular proteins from cultured SH-SY5Y cells (growing in $4x10^4$ cells/100 mm petri dish) were extracted after expose to different of compounds of 12 h by lysis buffer containing 150 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, and 2 mM EDTA, pH 7.5, in the presence protease inhibitors; 1 mM PMSF and 10 µg/ml leupeptin). Lysates were completed by sonication and protein levels determined. Samples were separated by SDS-PAGE on an 8% polyacrylamide Tris/glycine gels and transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., USA). Non-specific binding sites will block by treatment with PBS containing 10% skimmed milk for 1 hr, and then washed with PBS containing 0.1% Tween 20 (T-PBS). The membrane was incubated with a 1:100 dilution of the β-Amyloid antibody (20.1): sc-53822, Santa Cruz Biotechnology, Inc). After 1 h incubation at room temperature the membrane was washed and incubated with a second antibody (1:2000) alkaline phosphates conjugated anti-mouse IgG for an additional hour, washed again and finally the blot developed by incubated for 5-10 with the BCIP/NBT ((5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolume): Sigma, UK) and the APP positive bands were recorded on Kodak digital science camera (DC120).

CHAPTER 3

Cell Toxicity Mechanism

3.1- Introduction

Brominated flame retardants (BFRs) are incorporated into a wide range of consumer products that have been the ability of potentially being a fire hazard. These include every day items such as TV-sets, household appliances, computers, and textiles and their addition is used as an essential safety feature of modern design and production **(Karter, 2008)**. BFRs are a diverse group of chemicals, which are used to slow down or inhibit the development of fires but are also a group of global environmental contaminants **(de Boer et al, 1998)**. Recently, concern for this emerging class of chemicals has a risen because of the occurrence of several classes of BFRs in the environment and in human and animal biota. The widespread production and use of BFRs, the increasing contamination of the environment, wildlife, and people and the limited knowledge of their potential adverse health effects, increases the importance of identifying emerging issues associated with the use of BFRs **(Birnbaum and Staskal, 2004)**. Commonly occuring BFRs include tetrabromobisphenol-A (TBBPA), hexabromocyclododecane (HBCD), polybrominated diphenyl ethers (PBDEs) such as Deca-, Pent- and Octa-bromodiphenyl ethers, tribromophenol, dibromobiphenyl, tetrabromohydroquinone, and tetrabromobisphenol-A diallyl ether, which are all examined in this study. Cell viability, caspase activation, cytochrome c release, measurement of mitochondrial membrane depolarisation, detection of reactive oxygen species and intracellular [Ca^{2+}] levels measurement assays were used to elucidate the mechanism of cytotoxicity of these chemicals in the SH-SY5Y human neuronal cells line. Additionally, this study also examined environmental plasticisers and related chemicals of which some are known to be endocrine disruptors (EDs), such as bisphenol-A (BPA) and 4-n-nonylphenol (NP) **(Jobling et al., 1995, Routledge and Sumpter, 1997)**. EDs may have impacts at much lower levels than those of traditional concern to toxicologists **(Geck et al., 2000)**. BPA induces cell death in many kinds of cells in in-vitro models **(Hughes et al., 2000)**. Recently alkylphenols have also been shown to affect Ca^{2+} signalling pathways **(Michelangeli et al., 2008)**.

This study focuses on two alkylphenol EDs, these being NP and BPA and investigates their ability to modulate neuronal SH-SY5Y cell viability, mitochondrial depolarisation and Ca^{2+} homeostasis.

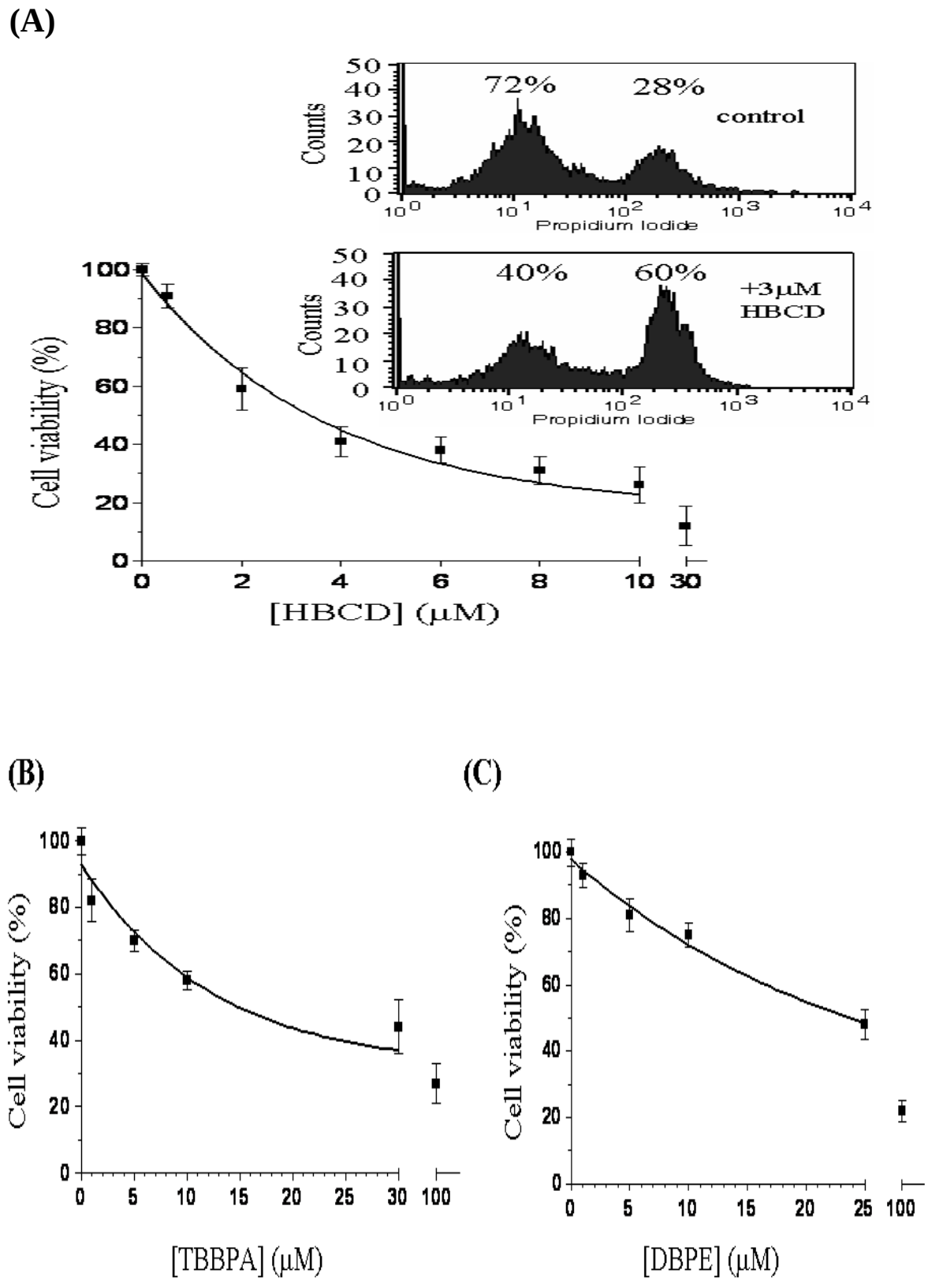
3.2- Results

3.2.1- The effects of various BFRs, BPA, and NP on the viability of SH-SY5Y cells as monitored using the MTT assay

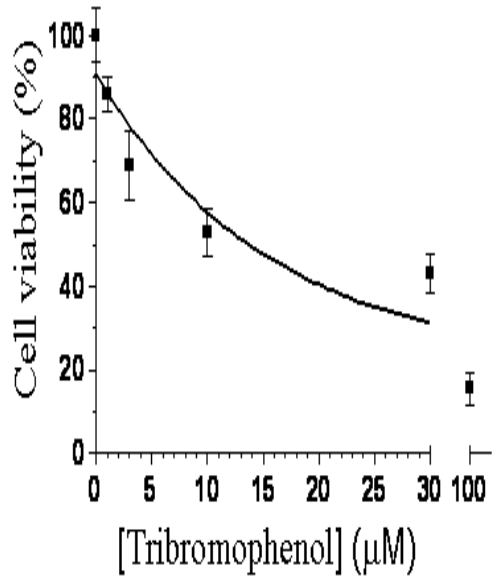
Initially a series of neuronal cell viability assays were performed with a range of concentration of BFRs and APs to determine their potential for neurotoxicity.

Figure 3.2.1A-K; shows the dose-dependent affects of different types and concentrations of BFRs and APs on SH-SY5Y cell viability. The cells were incubated for about 22-24h of exposure at 37 °C in CO_2 incubator and cell viability was measured using the MTT based assay and compared to cells not exposed to these compounds but to DMSO (2%) alone. All data represent the mean ± the SD of between 3-4 determinations. Figure 3.2.1A insert shows cell viability was measured by flow cytometry. SH-SYSY cells treated with 2% DMSO (control) and 3µM HBCD for 22h. 3µM HBCD also caused about 50% cell death as determined by propidium iodide stained cells and FACS analysis (fig.3.2.1A insert). Table 3.2.1; lists the lethal concentration 50 (LC_{50}) for cell viability of these compounds. It can be seen that HBCD is the most potent inhibitor (the LC_{50} was 2.7 ± 0.7µM) compared to other compounds. In contrast, TBBPA-diallyl ether and BPA were weakly toxic (with LC_{50} of > 300 µM and 243 ± 38 µM, respectively). All other compounds tested had LC_{50} values of between 12-150µM.

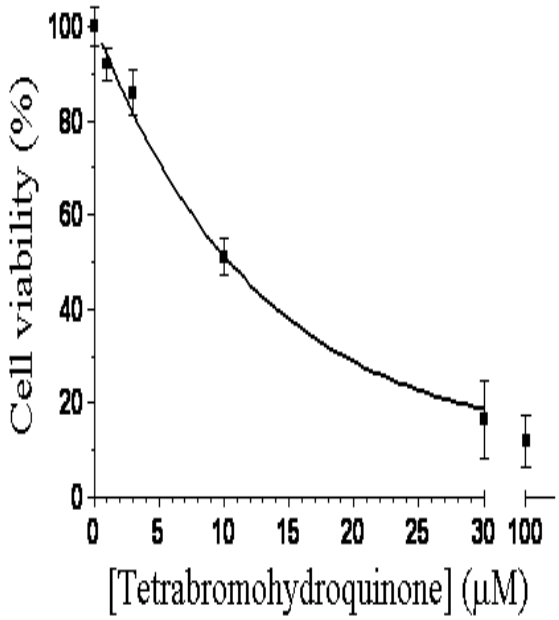
Figure 3.2.1


(A)
72%
28%
control
Counts
Propidium Iodide
40%
60%
+3μM
HBCD
Cell viability (%)
[HBCD] (μM)
(B)
[TBBPA] (μM)
(C)
[DBPE] (μM)

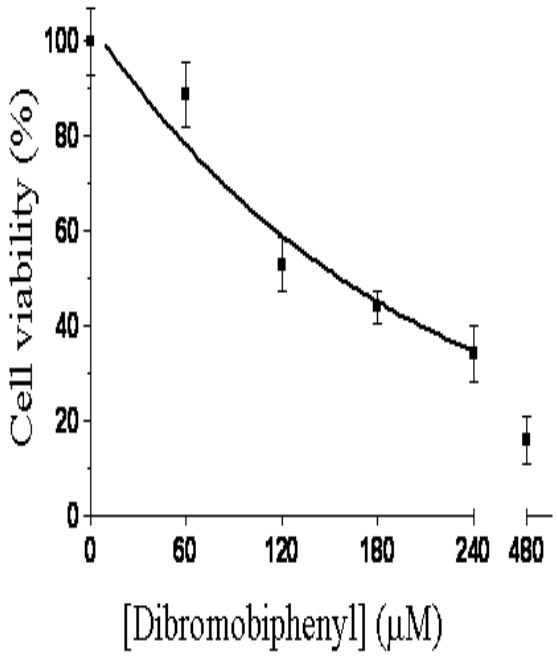
(D)


Cell viability (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 100
[Tribromophenol] (μM)

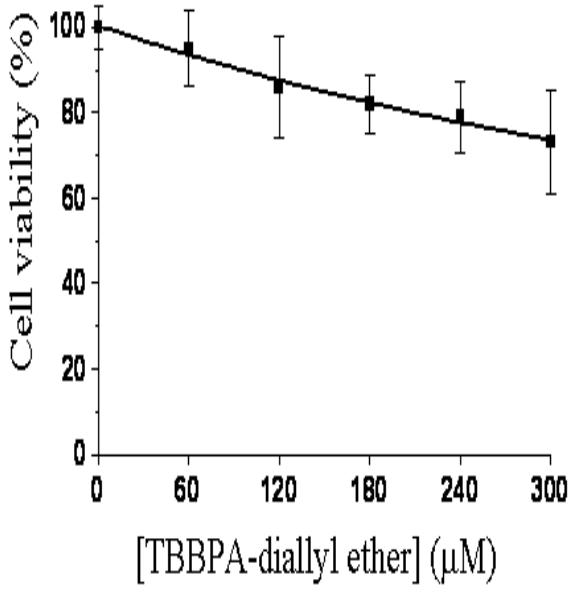
(E)


Cell viability (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 100
[Tetrabromohydroquinone] (μM)

(F)


Cell viability (%)
100
80
60
40
20
0
0 60 120 180 240 480
[Dibromobiphenyl] (μM)

(G)


Cell viability (%)
100
80
60
40
20
0
0 60 120 180 240 300
[TBBPA-diallyl ether] (μM)

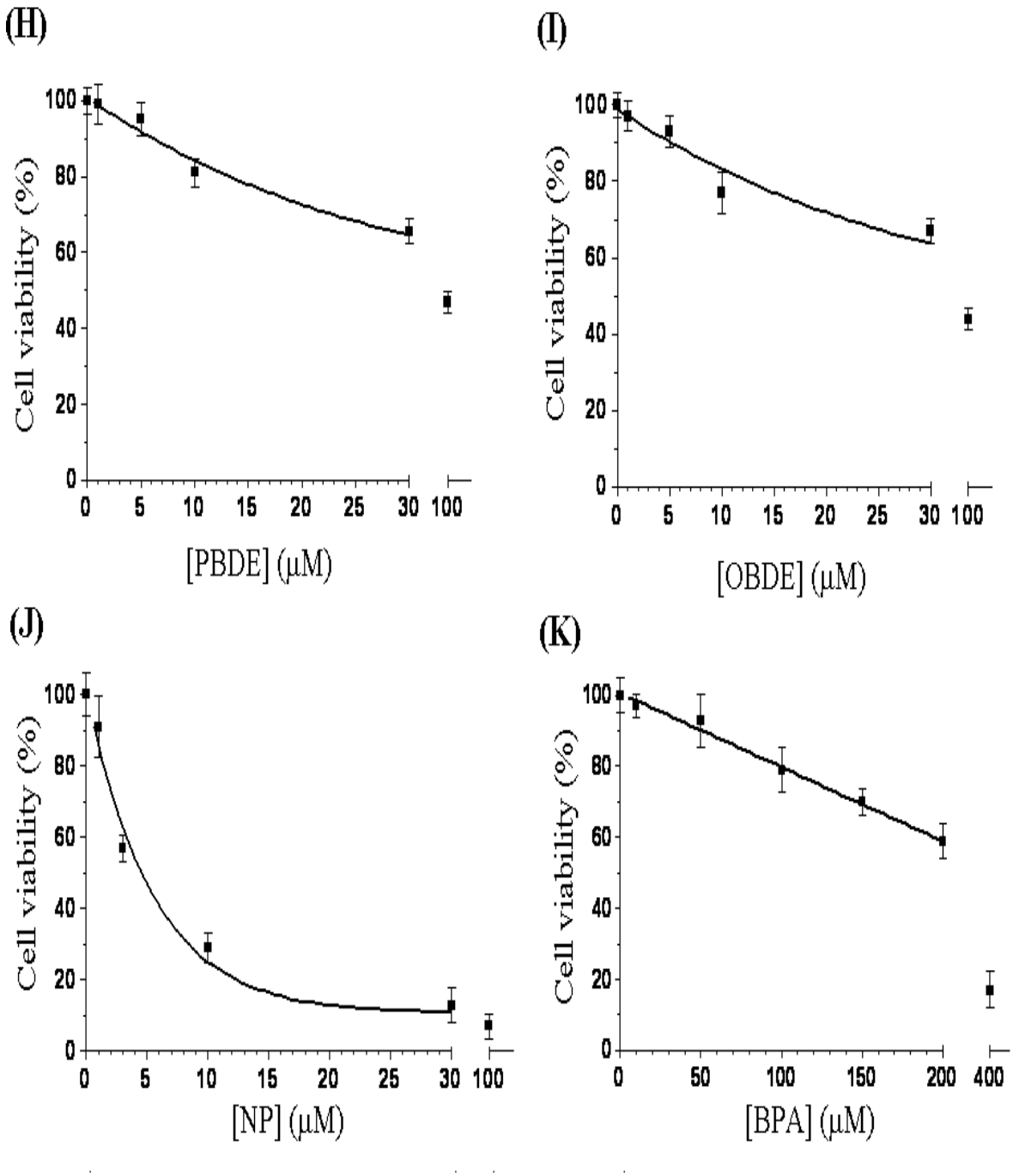


Figure 3.2.1A-K; shows the cytotoxic effects of different concentrations of BFRs, NP and BPA on the viability of SH-SY5Y cells. The cells were treated with different [compound] for ~ 22 h prior to measurement, using the MTT assay. Figure 3.2.1 (A) insert shows cell viability measured by FACS analysis, (histograms of both control cells treated with 2% DMSO and cells treated with 3µM HBCD for 22h). The data shows that viable cells were 72% (in control) compared to 40% of viable cells when exposed with 3µM HBCD. All data represent the mean ± the SD of between 3-4 determinations.

Table 3.2.1 comparison of lethal concentration 50 (LC_{50}) values of different compounds. All data represent the mean ± the SD of between 3-4 determinations.

Name of compound	50% SH-SY5Y cell death (LC_{50}) (μM)
3,3′,5,5′-Tetrabromobisphenol A (TBBPA)	15 ± 4.2
1,2,5,6,9,10-Hexabromocyclododecane (HBCD)	2.7 ± 0.7
2,4,6-Tribromophenol	14 ± 4.2
4,4′-Dibromobiphenyl	150 ± 17
Tetrabromohydroquinone	12 ± 6.4
2,2′,6,6′-Tetrabromobisphenol A diallyl ether (TBBPA-diallyl ether)	> 300
Decabromodiphenyl ether (DBDE)	24 ± 6.4
Pentabromodiphenyl ether (PBDE)	67 ± 10.5
Octabromodiphenyl ether (OBDE)	63 ± 7.5
4-n-nonylphenol (NP)	6 ±1.8
Bisphenol-A (BPA)	243 ± 38

3.2.2- Caspases activation induced by TBBPA, HBCD, DBPE, NP and BPA using a fluorogenic substrate (Ac-DEVD-AMC (casp-3), Ac-IETD-AMC (casp-8), and Ac-LEHD-AMC (casp-9))

In order to determine whether cell death by these compounds was, at least in part by apoptosis, caspase activities were measured.

As shown in figure 3.2.2.1; caspases-3, -8 and -9 activities were determined in SH-SY5Y cells where they were incubated in the presence of three different concentrations of HBCD, TBBPA, NP, DBPE and BPA for 12h. In figure 3.2.2.1A; all compounds tested showed activation of the executioner caspase (caspase-3) at all of the concentrations tested. TBBPA, HBCD and NP were particularly potent at increasing caspase-3 activity as they showed statistically significant levels of activity, above those of control, at 1µM. However, DBPE and BPA were least effective only showing significant activity above control at 10 µM and 50 µM, respectively.

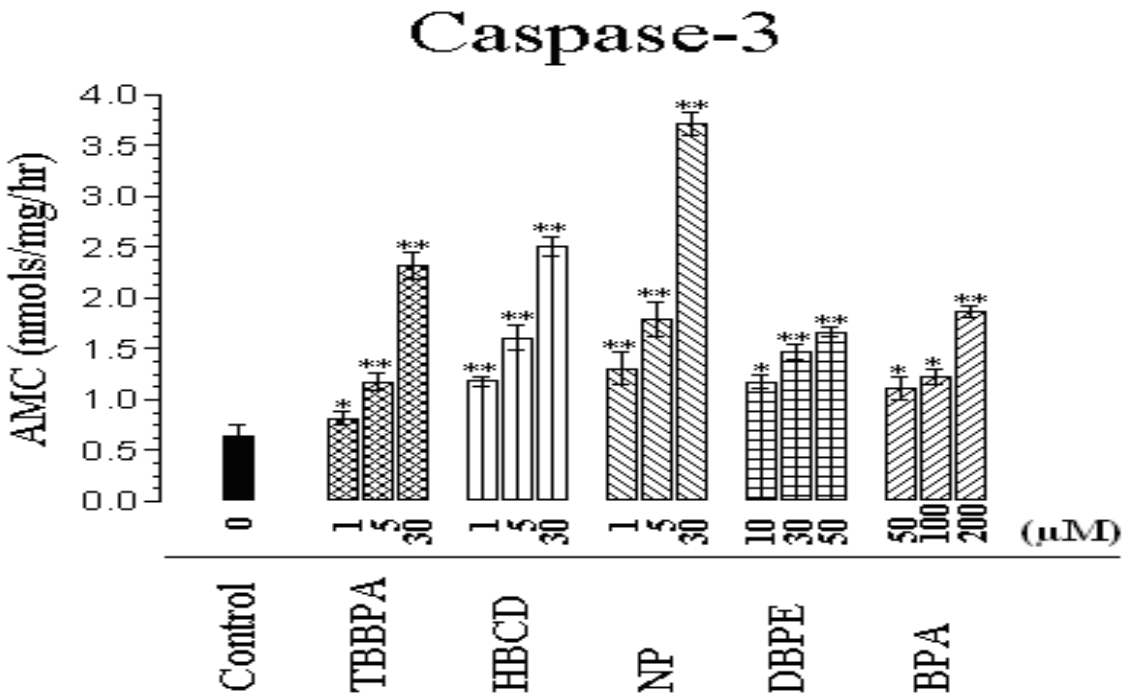
In order to determine whether caspase activation was by intrinsic (via caspase-9) or the extrinsic (via caspase-8) apoptotic pathways, the activities of these caspases were also measured (figure 3.2.2.1B&C). The results showed that most compounds tested were equally effective at increasing the activities of both caspase-8 and -9. However, a notable exception was NP which appeared to preferentially activate caspase-9 compared to -8, highlighting that it activated a specific apoptotic pathway.

In order to understand whether BFRs caused activation of the two caspases pathways independently or concertedly, a time course of caspase-8 and caspase-9 activation was undertaken with HBCD (5µM). Figure 3.2.2.2A shows that the time course for the induction of caspase-8 and -9 activities were similar.

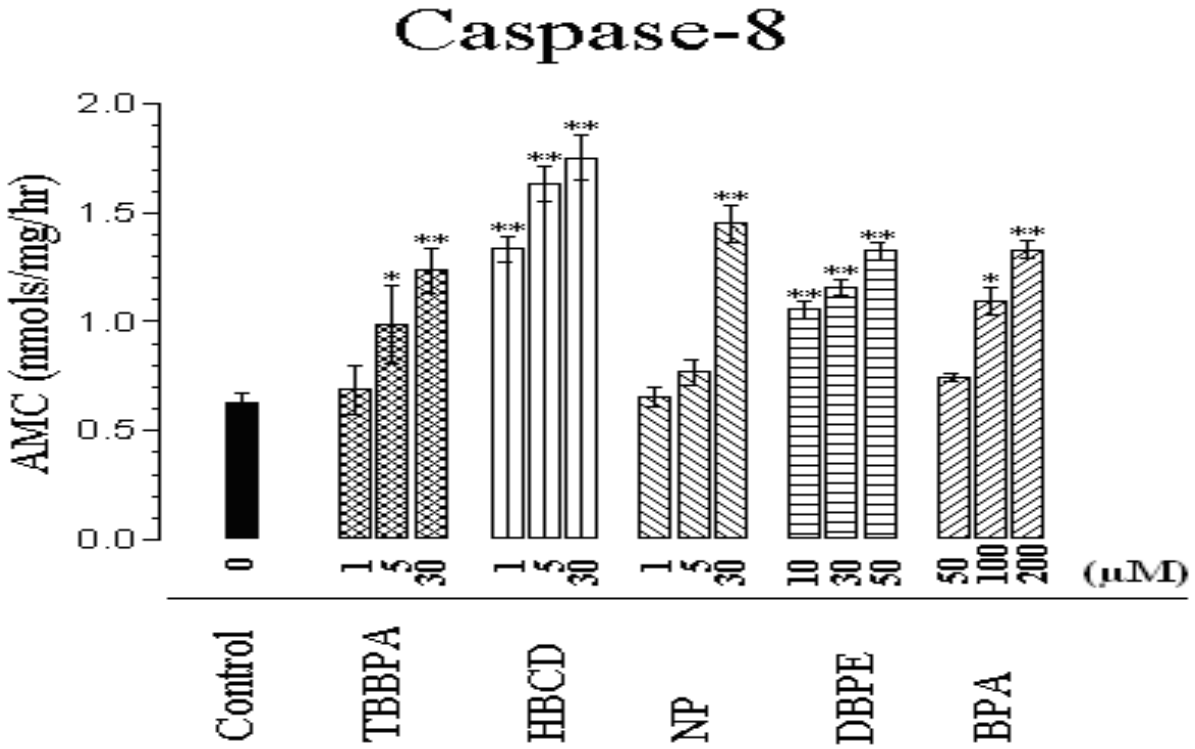
In order to assess whether there was sequential activation of either pathways by HBCD, experiments with caspase-8 and -9 inhibitors were undertaken. Figure 3.2.2.2B shows that cells pretreated with either caspase-8 or -9 inhibitors partially protected the cells from cell death. It should be noted, however, that the addition of either caspase inhibitors did not fully protect against 2µM HBCD-induced cell death.

Figure 3.2.2.1

(A)



(B)



(C)

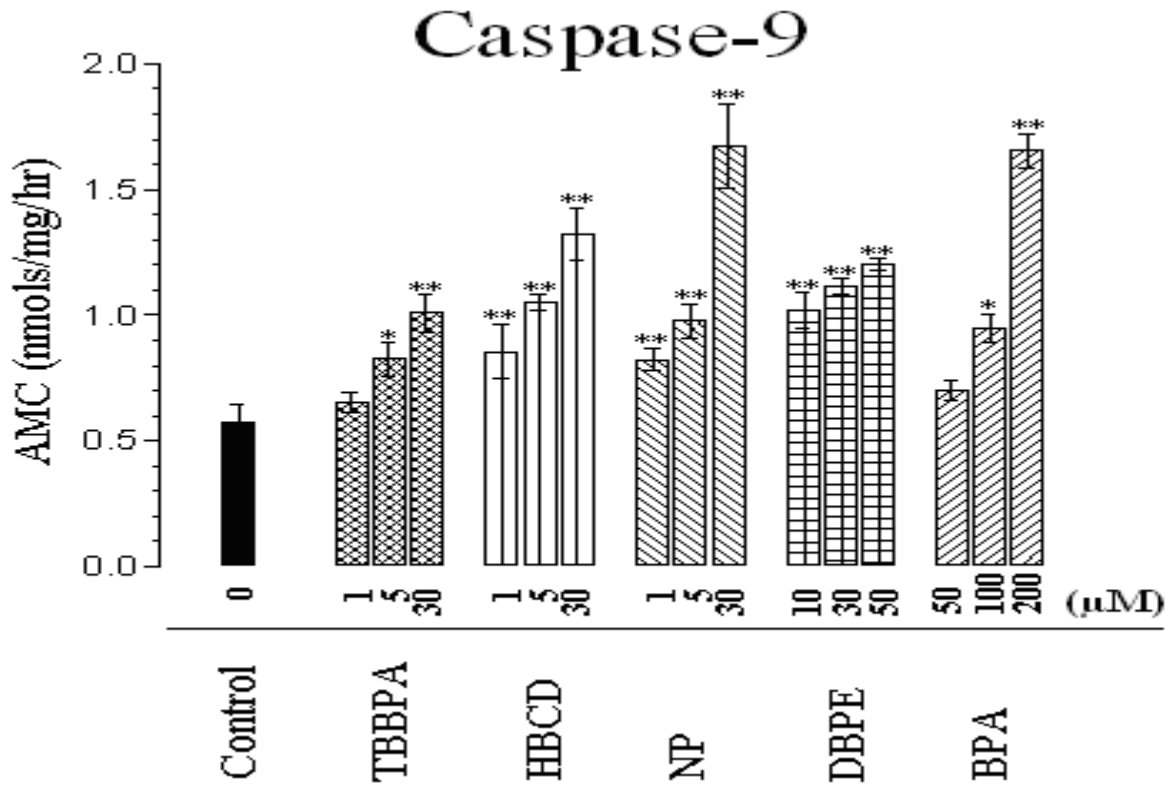


Figure 3.2.2.1; activation of caspases by HBCD, TBBPA, NP, DBPE and BPA using caspase-specific fluorogenic substrates. (A) Shows the effects of different concentration of HBCD, TBBPA, NP, DBPE and BPA on DEVD-ase activity (indicative of caspase-3 cleavage activity), (B) IETD-ase activity (indicative of caspase-8 cleavage activity) and (C) LEHD-ase activity (indicative of caspase-9 cleavage activity), after 12 h of exposure in SH-SY5Y cells. Values are presented as means ± SD of 3-4 determinations (* value points were significant different from the controls ($P \leq 0.05$, ** $P \leq 0.001$), using t-test.

Figure 3.2.2.2

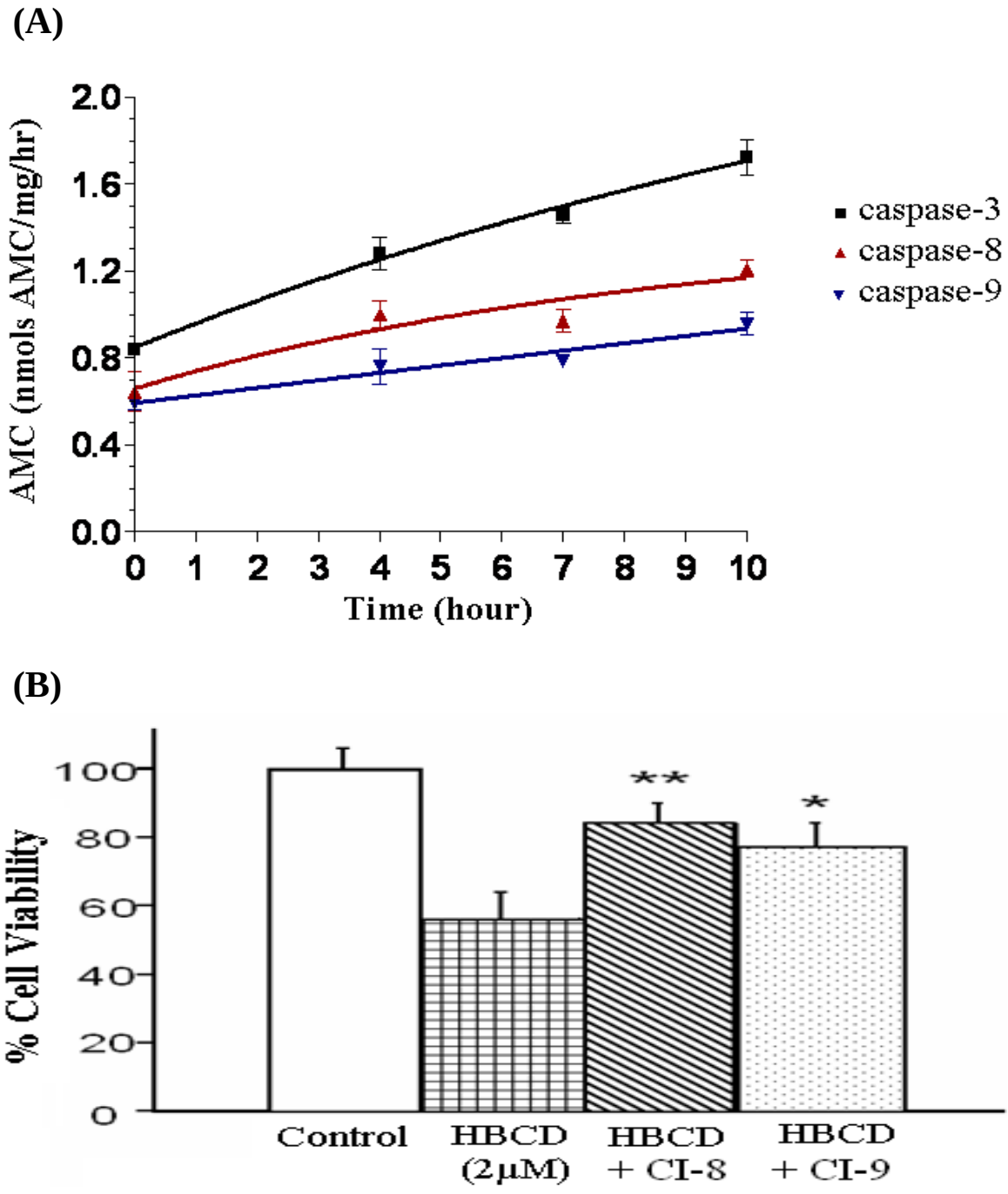


Figure 3.2.2.2 (A) Time course of the effects of 5μM HBCD on caspases-3, -8 and -9 activities in SH-SY5Y cells. (B) Showed that inhibition of either caspase-8 (CI-8) or caspase-9 (CI-9) enhanced the viability of cells after the SH-SY5Y cells were treated with 2μM HBCD. The data was analysed using the t-test (* indicates significant difference from the control ($P \leq 0.05$, ** $P \leq 0.001$).

3.2.3- Detection of cytochrome c in mitochondrial and cytosolic extracts from SH-SY5Y cells by using immunoblotting with a cytochrome c antibody

As the caspase-9 pathway was activated, which involves the mitochondria releasing pro-apoptotic factors such as cytochrome c, experiments were undertaken to assess whether some of the BFRs and APs caused cytochrome c to be released from the mitochondria in to the cytoplasm.

Figure 3.2.3 shows cytochrome c was detected by immunoblotting analysis of the mitochondrial and cytosolic fractions which were obtained from SH-SY5Y cells treated with HBCD, TBBPA, NP and DBPE for 12h. Cytochrome c appeared in cytosolic fraction of SH-SY5Y cells after treatment with HBCD, TBBPA, NP and DBPE. However, in control cells no cytochrome c in the cytoplasm was detected.

Figure 3.2.3

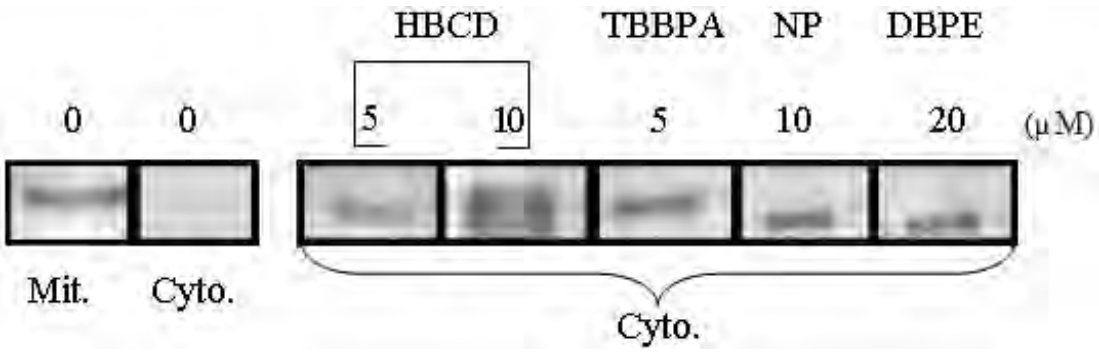


Figure 3.2.3; shows cytochrome c was detected by immunoblotting analysis. SH-SY5Y cells (5×10^5 cells/35 mm dish) were incubated in the presence of HBCD (5-10 μM), 5μM TBBPA, 10μM NP and 20μM DBPE for 12 h. Samples form the mitochondrial fraction (Mit.) and cytosolic fraction (Cyto.) were extracted from SH-SY5Y cells by homogenization and centrifugation at 100,000*g* and were subjected to 15% SDS-PAGE followed by western blotting with anti-cytochrome c IgG. The blots were representative of two repeats.

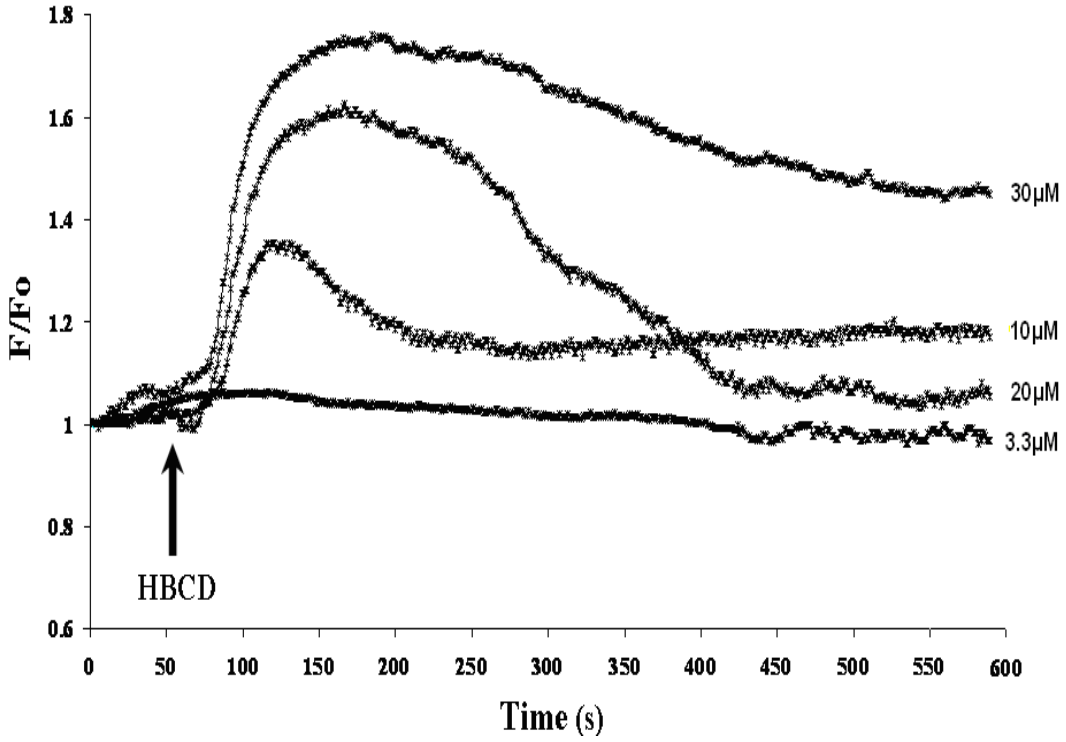
3.2.4- The effects of HBCD, TBBPA, DBPE, NP and BPA on the intracellular concentration ($[Ca^{2+}]_i$) levels within SH-SY5Y cells loaded with Fluo-3 AM

As cell-death can be induced by prolonged elevation of $[Ca^{2+}]_i$ levels, since previous studies have shown that related compounds can cause similar effects **(Ogunbayo et al., 2008; Michelangeli et al., 2008)**, experiments were undertaken to investigate whether these compounds also elevated $[Ca^{2+}]_i$ in SH-SY5Y cells.

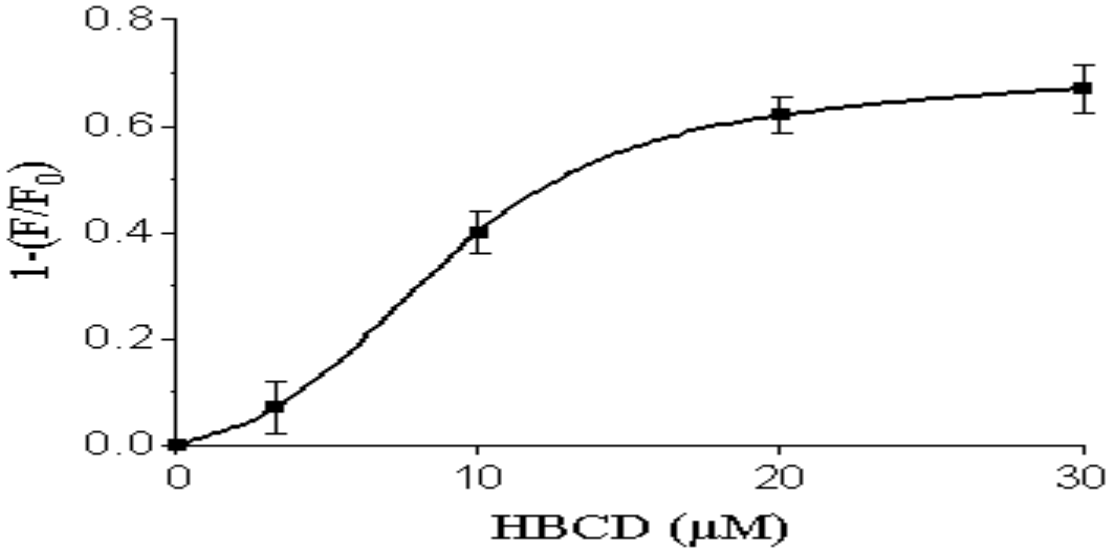
The figure 3.2.4.1A; shows traces of the relative fluorescence change averaged for 8 individual cells viewed, using the fluorescence microscope, the profiles of which were typical of the majority of cells observed. The arrow indicates the addition of HBCD after around 60s from the start of the recording. Under these experimental conditions, more than 70% of all cells viewed showed changes in their $[Ca^{2+}]$i levels upon exposure. The rise in $[Ca^{2+}]_i$ measured as an increase in relative fluorescence levels attained maximal levels within about 1-2 min of the addition of HBCD. The elevations in $[Ca^{2+}]_i$ levels were transient in nature and returned back towards un-stimulated levels after about a 5 min period. Figure 3.2.4.1B shows the dose-dependent effects of increasing [HBCD] on $[Ca^{2+}]_i$ levels within SH-SY5Y cells. (The EC_{50} was calculated to be 7 ± 0.6 µM). Figure 4.2.4.1C; shows fluorescence micrograph images of a number SH-SY5Y cells rendered in greyscale (i.e. black is low $[Ca^{2+}]$, white is higher $[Ca^{2+}]$), before 20 µM HBCD addition (60s), at a time point where the maximal increase $[Ca^{2+}]_i$ is occurring (240s) and towards the end of the experiment (500s). The magnification was equivalent to 500x. Figure 3.2.3.2 shows that TBBPA, NP, DBPE and BPA also all increase $[Ca^{2+}]_i$ levels of Fluo-3 AM loaded SH-SY5Y cells.

Figure 4.2.4.1

(A)



(B)



(C)

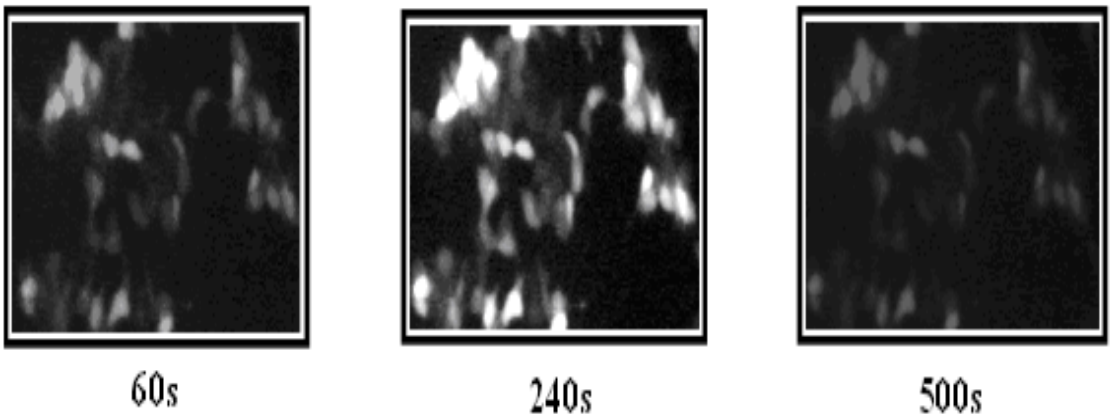


Figure 3.2.4.1; the dose-dependent effects of [HBCD] on changes of intracellular [Ca^{2+}] levels of SH-SY5Y cells. (A) Shows the changes in relative fluorescence intensity (F/Fo) versus time of 8 averaged SH-SY5Y cells loaded with Fluo-3, at pH 7.2, 37 °C, when treaded with 3.3, 10, 20 and 30 µM HBCD (at the point marked with the arrow). These traces were typical of those observed in three replicate experiments. (B) (■) Shows the summary of effects of increasing [HBCD] on intracellular [Ca^{2+}] levels within single SH-SY5Y cells. The results represent the mean ± SD of 3 determinations. (C) Shows fluorescence micrograph images of Fluo-3 AM loaded SH-SY5Y cells, just prior to exposure (at 60s from start of recording) and after exposure to 30 µM HBCD (i.e. at 240s and 500s from the start of recording). The fluorescence micrographs are presented in greyscale (where black indicates the lowest level of [Ca^{2+}] and white the highest).

Figure 3.2.4.2

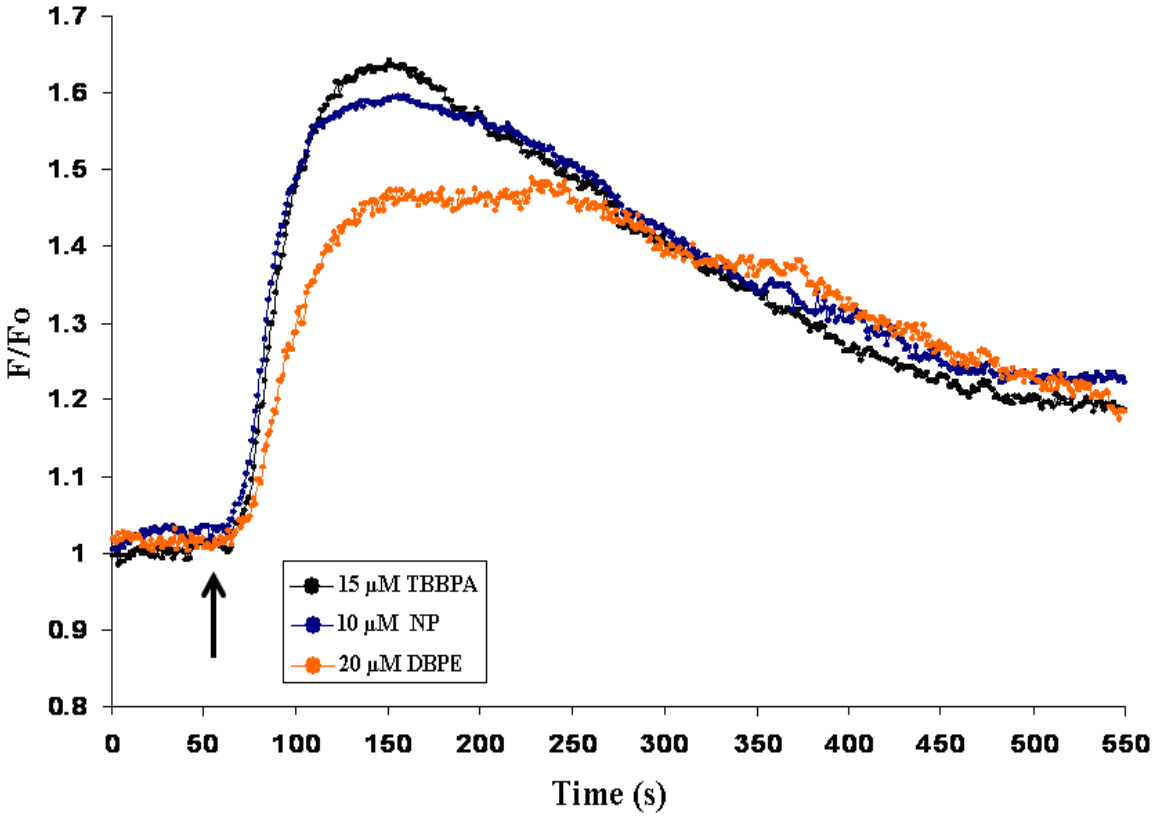


Figure 3.2.4.2; the effects of TBBPA, NP and DBPE on $[Ca^{2+}]_i$ levels within single SH-SY5Y cells. This figure shows the effect of TBBPA, NP and DBPE on the increase in $[Ca^{2+}]_i$ levels within single SH-SY5Y cells loaded with Fluo-3, at pH 7.2, 37 °C, when treaded with TBBPA, NP and DBPE, 15 µM, 10 µM and 20 µM respectively (at the point marked with the arrow). Each trace corresponds to the mean of 8 cells averaged together and all traces were typical of those observed in three replicate experiments.

3.2.5- Mitochondria membrane depolarization of SH-SY5Y cells after exposure to HBCD, TBBPA, NP, DBPE and BPA

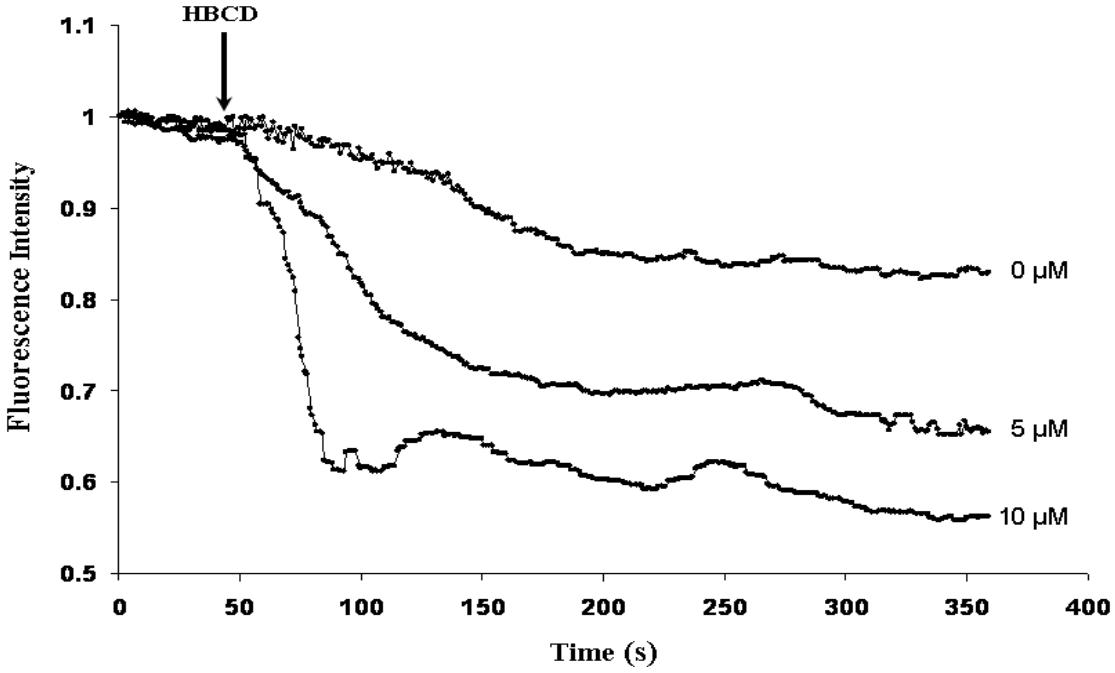
In order to further explore the relationship between elevation of $[Ca^{2+}]_i$ and cell death, it is known that changes in intracellular Ca^{2+} levels cause Ca^{2+}-dependent depolarization of the mitochondria membrane **(Gunter et al., 2004; Michelangeli et al., 2005; Hom et al., 2007)**. Rhodamine123 (Rh123) is fluorescent dye which preferentially goes into mitochondria. When the mitochondria depolarise the dye leaks out and decreases its fluorescence.

Figure 3.2.5.1A; shows how the fluorescence intensity of the Rh123-loaded SH-SY5Y cells decreases after exposure to HBCD. Each trace corresponds to the average fluorescence change of 8 cells combined together and the traces show that the decrease in fluorescence occurred at all concentrations of HBCD tested. The HBCD was added to the cells about 60s after the start of the recordings. When no HBCD was added a small decrease in fluorescence was observed probably due to some photo-bleaching. However, the rate of fluorescence decrease was significantly enhanced upon exposure to HBCD, suggesting rapid mitochondrial depolarisation within a few seconds of exposure. Figure 3.2.5.1B; shows the fluorescence images of Rh123-SH-SY5Y cells, prior to 10 µM HBCD exposure (50s) and at the around end of the experiment (320s).

Figure 3.2.5.2; also shows similar effects of TBBPA, NP, DBPE and BPA on the mitochondria membrane depolarisation. The compounds were added to SH-SY5Y cells about 50s after start of the recordings under fluorescence microscopy. The traces (each trace contains an average of 8 individual cells) show that the decrease in fluorescence occurred for all compounds.

Figure 3.2.5.1

(A)



(B)

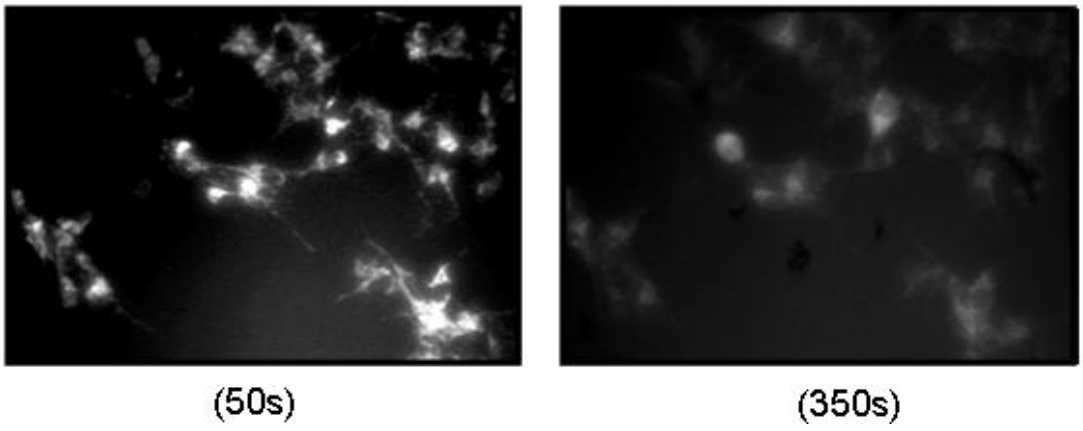


Figure 3.2.5.1 (A); shows the effects of different of [HBCD] on the mitochondrial membrane depolarisation. This was monitored in Rh-123-loaded SH-SY5Y cells. Traces are the combined average of 8 individual cells recorded from within the field of view from the fluorescence microscope. The arrow indicates the time point where the HBCD was added. (B); shows the fluorescence images of SH-SY5Y, prior to 10µM HBCD exposure (50s) and at the around end of the experiment (350s).

Figure 3.2.5.2

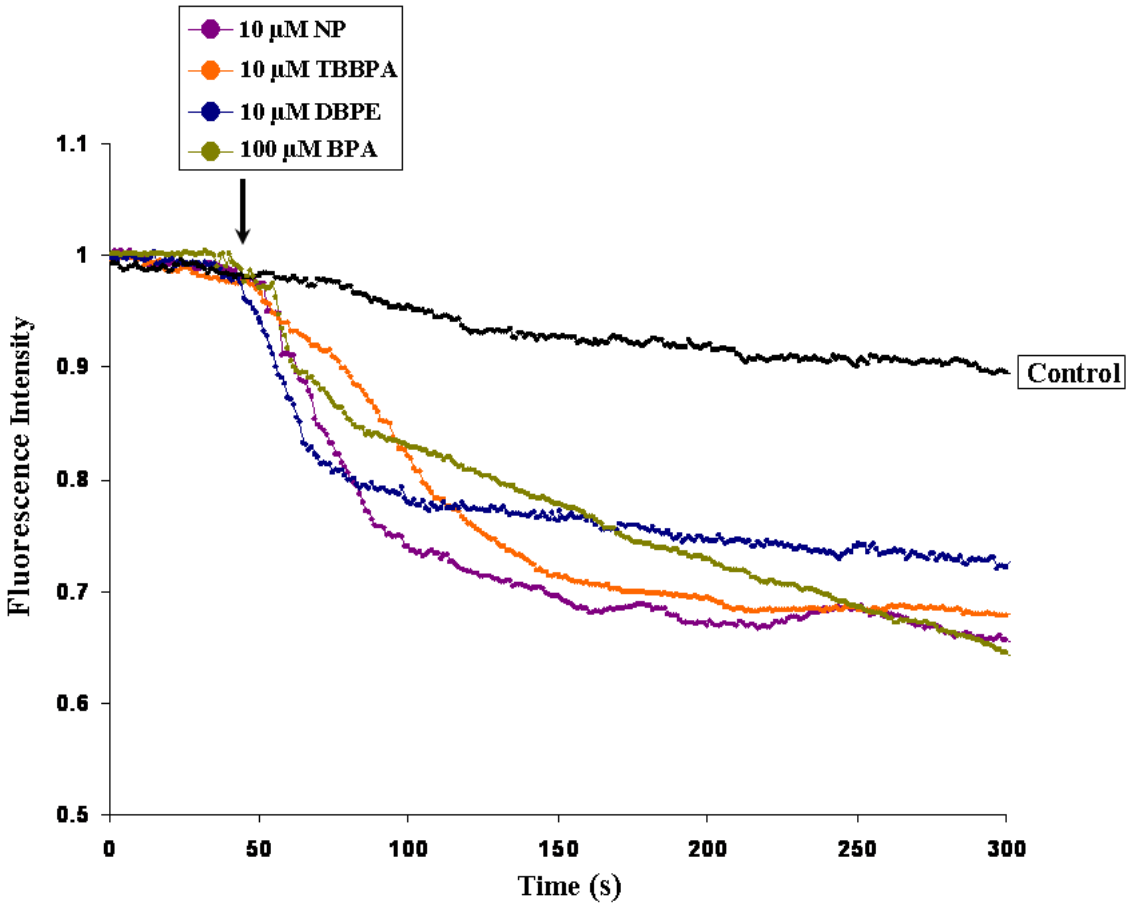


Figure 3.2.5.2 shows the effects of TBBPA, NP, DBPE and BPA on the mitochondrial membrane depolarisation of SH-SY5Y cells loaded with Rh123. Upon exposure to 10µM of TBBPA, NP, DBPE and 100µM BPA. The traces are the combined average of 8 individual cells, within the field of view, and are typical of 3 replicate experiments.

3.2.6- The induction of ROS by HBCD, NP, TBBPA, DBPE and BPA

As mitochondrial disfunction can lead to oxygen free radicals production and ultimately to cellular damage, the ability of compounds to cause oxidative stress in SH-SY5Y cells was determined by measuring reactive oxygen species formation via monitoring 2',7'-dichlorofluorescein (DCF) fluorescence.

Figure 3.2.6.1A shows the production of ROS in SH-SY5Y cells after exposure to different concentrations of HBCD for 22-24 hr. Fluorescence of DCF relative to control was measured after 30 min treatment with 2',7'-dichlorofluorescein diacetate (DCFH2-DA). Of the other compounds tested (i.e. TBBPA, DBPE, NP and BPA), all except DBPE and BPA, showed some formation of ROS compared to control. Data are presented as means ± SD. (* indicates significant difference from the control ($P \leq 0.05$, ** $P \leq 0.001$).

Figure 3.2.6.1B ROS formation was also shown to be concentration dependent with HBCD having an EC_{50} of 6 ± 0.4 µM.

Figure 3.2.6.1

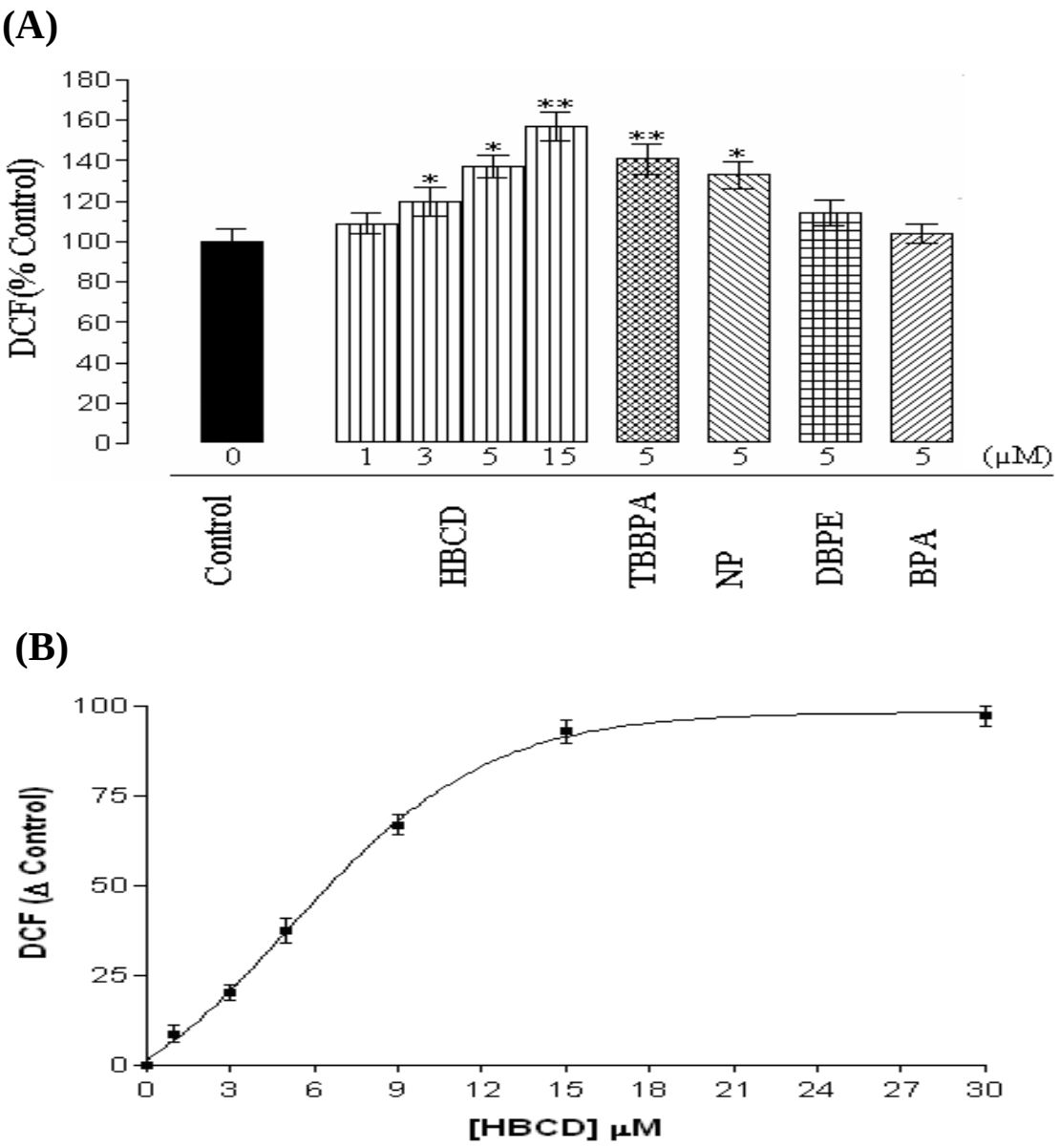


Figure 3.2.6.1 (A); shows the effects of HBCD, TBBPA, NP, DBPE and BPA on ROS production as measured by increase in DCF fluorescence. SH-SY5Y cells were exposed to the compounds for 24 h. The fluorescence intensity was monitored after the reaction mixture was incubated for 30 min (37°C). Data represents the mean ± S.D of 3-4 determinations. (*) and (**) indicates statistically significant difference from control (2% DMSO), ($p < 0.05$) and ($p < 0.001$) respectively. (B) the dose-dependent increase of DCF fluorescence intensity in SH-SY5Y cells after exposure to different concentrations of HBCD (EC_{50} value was 6 ± 0.4 µM).

3.3- Discussion

Brominated flame retardants (BFRs) and alkylphenols (APs) are chemicals pollutants commonly found within the environment and have human health concerns due to their endocrine disrupting and cytotoxic effects. BFRs are used to reduce the flammability of a variety of consumer products such as foam furnishings, whereas APs are found in plastic products used by the food industry. In-vitro studies have shown that a variety of the BFRs pollutants are neurotoxic and can affect a range neurological cell functions, such as cell signalling processes, neurotransmission and cell death **(Barclay et al., 2005; Dingemans et al., 2008)**. Little is, however, known as to the molecular mechanisms of action of these BFRs and also whether APs pollutants also act on neurons in a similar fashion. The human neuroblastoma SH-SY5Y cell line is similar to common nerve cells in terms of shape, physiological and biochemical function and is commonly used as model for neurons.

In this chapter, the cell viability, caspases activation, cytochrome c release, mitochondria membrane depolarisation, ROS formation and elevation of $[Ca^{2+}]_i$ levels assays were used to assess the mechanism of cytotoxicity of some commonly utilized of BFR and APs in SH-SY5Y human neuronal cells.

The data in fig.3.2.4.1A and fig.3.2.4.2, show that HBCD, TBBPA, DBPE and NP induce a transient elevation in $[Ca^{2+}]_i$ within SH-SY5Y cells, these Ca^{2+} transients were comparable similar to those observed when these cells were treated with the potent Ca^{2+} pump inhibitor, thapsigargin, which is also used to induce cell death by Ca^{2+}-dependent mechanism **(Hughes et al., 2000; Laskay et al., 2005; Hom et al., 2007)**. It is well known that mitochondria are important cellular organelles regulating metabolism and cell death pathways **(Acton et al., 2004)**. The MTT cell viability assay is an indicator of mitochondrial activity and specificity of mitochondrial dehydrogenase activity **(Fotakis and Timbrell, 2006)**. It can be deduced that of the 11 chemicals tested (over a 22 h exposure), the data present here shows that HBCD was the most potent at inducing cell death in the SH-SY5Y neuroblastoma cells (see table 3.2.1). The LC_{50} for HBCD and NP calculated to be 2.7 ± 0.7μM and 6 ± 1.8μM, respectively. This range of HBCD potency on inducing cell death is similar

that observed by **Reistad et al., (2006)** (the LC_{50} value was estimated to 3μM in cerebellar granule cells) and may indicate that a range of neuronal cell types are similarly affected by this chemical, while for NP the LC_{50} values were similar to that found in TM4 Sertoli cells (i.e. LC_{50} = 10 μM) **(Michelangeli et al., 2008)**.

Furthermore, these results show that HBCD, TBBPA, NP can depolarize mitochondria in these neuronal cells at low micromolar concentration (fig.3.2.5.1A and fig.3.2.5.2), with the degree and rate of depolarization also being dependent upon the concentration of HBCD (fig.3.2.5.1A). Specific toxic stress events can cause Ca^{2+} overload in the mitochondrial matrix which can trigger mitochondrial membrane depolarization, this then causes release of pro-apoptotic factors, by activating the PTP and loss of mitochondrial membrane integrity **(Michelangeli et al., 2005; Hajnoczky et al., 2006; Hom et al., 2007)**. However, oxidative stress also triggers apoptosis that is associated with decreased levels of MMP, increased generation of intracellular ROS and accumulation of intracellular Ca^{2+} level **(Satoh et al., 1997; Hoyt et al., 1997)**. Although it is established that mitochondrial respiration is an important source of ROS production, excessive ROS generated within the mitochondria can also cause damage to cellular components **(Koizumi et al., 1996)** such as proteins, membranes and DNA. The fact that micromolar concentrations of HBCD, TBBPA and NP are able to generate measurable levels of ROS in SH-SYSY cells, (an observation also seen that BFRs cause increased ROS formation in SH-SY5Y cells **(He et al., 2008)** and in Hep G2 cells **(Zhang et al., 2008)**). This would indicate that further experiments investigating the effects of these compounds on DNA damage should be undertaken.

There are two major pathways of caspase activation during apoptosis. The first of these is the intrinsic (mitochondrial) pathway and the second extrinsic (death receptor) pathway. The mitochondrial pathway releases cytochrome c into the cytosol, which binds and activates of apoptotic protease activating factor-1 (Apaf-1), promoting the activation of caspase-9, which then results in activation of caspase- 3 **(Riedl and Salvesen, 2007)**. Alternatively, Death receptors are activated by ligand binding to the extracellular domain of the receptor causing activation of caspase-8 by forming the death-inducing signalling complex (DISC) **(Thorburn, 2004)**. However, a recent study has shown that some BFRs cause an increase in $[Ca^{2+}]_i$ which induced

apoptosis in human neuronal SH-SY5Y cells by activation of two caspases pathways **(He et al., 2009)**. HBCD, TBBPA and DBPE are shown to induce the activation of both caspase-8 and -9, resulting in the activation of caspase-3 (see fig.3.2.2A, B& C), while NP appeared to specifically activate caspase-9. The apoptotic pathway through mitochondria was further confirmed by cytochrome c release (see fig.3.2.3.4).

Some BFRs have been shown to disrupt Ca^{2+} homeostasis by increasing $[Ca^{2+}]_i$ in SHSY5Y cells which can then activage the death receptor which induces caspase-8. This results in activation of the pro-apoptotic Bid which enters the mitochondria and blocks the functioning of Bcl-2 resulting in the release of cytochrome c. This stimulates caspase-9 which the actives caspase-3 and leads to cell death **(He et al., 2009)**. These results, at least for HBCD, indicate it unlikely that caspase-9 is activated via caspase-8 as shown in studies **(Sprick and Walczak, 2004; He et al., 2009)**, as the resuts with caspase inhibitors and the time-course of induction of caspase -8 and -9 activities, would likely preclude this.

In summary, this study has shown that these pollutants are neurotoxic at low concentrations. Some compounds such as HBCD, TBBPA, DBPE and NP induce cell death (apoptosis) by caspases activation (Caspse-8, -9 and -3) and cytochrome *c* release at low micromolar concentration. These compounds also cause mitochondria membrane depolarisation and increase in intracellular Ca^{2+} levels and some also cause ROS formation supporting the possible mechanisms as described in see fig.3.3

Figure 3.3

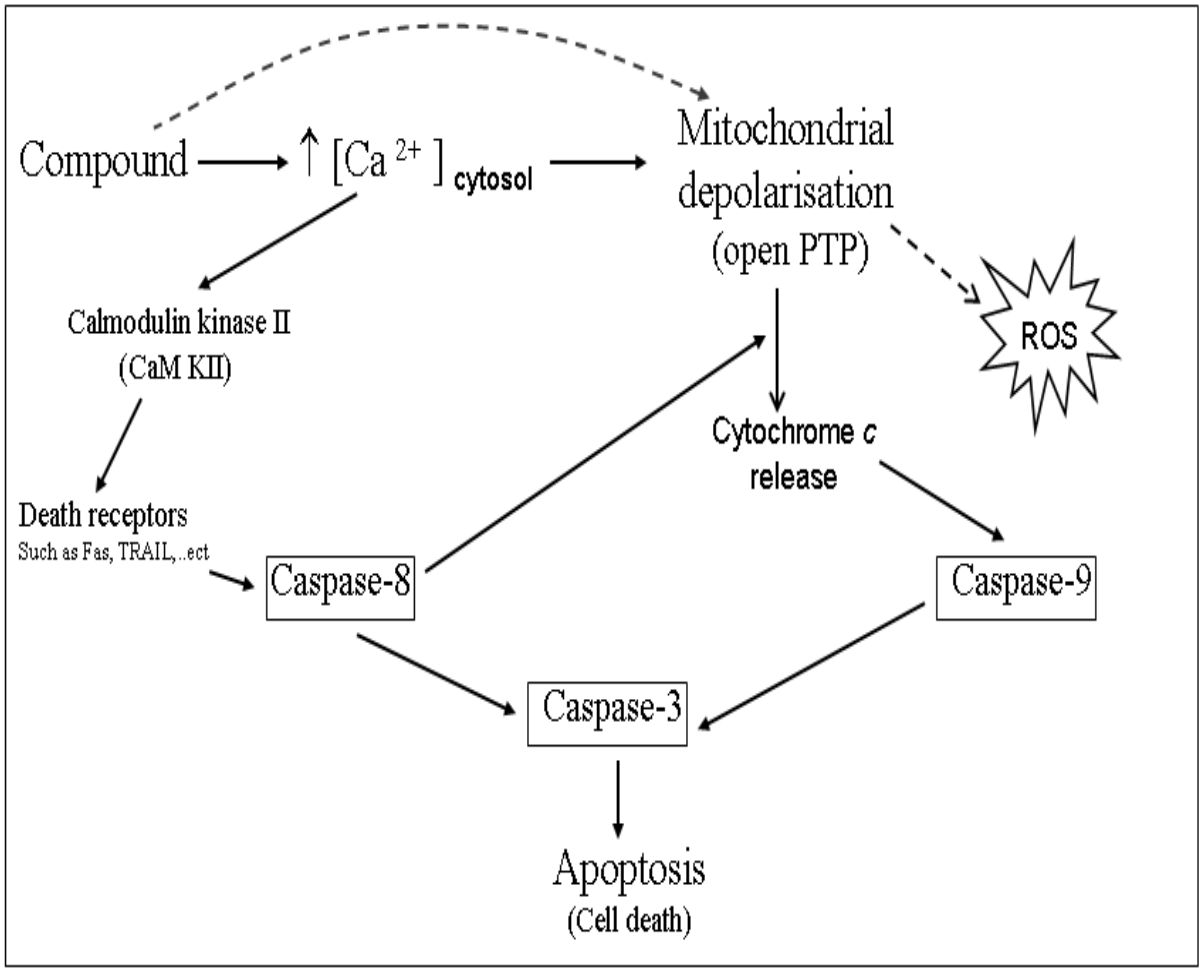


Figure 3.3: schematic representations of the effects of these compounds on apoptosis SH-SY5Y cells.

CHAPTER 4

Molecular Toxicity on Ca^{2+} Transporters

4.1- Introduction

Ca^{2+} is a commonly employed signal within cells that regulates many different cellular processes such as contraction, secretion, fertilisation and proliferation by increasing in $[Ca^{2+}]_i$ levels **(Khan et al., 2003; David et al., 2000)**, inadequate or prolonged elevation of $[Ca^{2+}]_i$ may be lead to deleterious effects **(Berridge et al., 1998)** and to avoid these effects, $[Ca^{2+}]_i$ levels must be strictly controlled by SERCA Ca^{2+} pumps **(Khan et al., 2003; David et al., 2000)**, which help to maintain low cytosolic $[Ca^{2+}]$ levels in resting cells **(Khan et al., 2003; Ogunbayo et al., 2007)**.

SERCA Ca^{2+} pumps belong to the P-type ion transporters **(Toyoshima and Inesi, 2004)** that exist in three isoforms and exhibit a tissue-specific distribution **(Khan et al., 2003; Ogunbayo et al., 2007)**. SERCA1 is expressed exclusively in skeletal muscle **(MacLennan et al., 1985)**, SERCA2a isoform is expressed predominantly in cardiac tissues and SERCA2b is dominating isoform found in neuronal tissues including SH-SY5Y cells **(Baba-Aissa et al., 1998, Mbaya et al., 2010)**. SERCA3 is expressed in non- muscle tissues **(Wu et al., 1995)**.

A number of brominated flame retardants (BFRs) have recently been recognized as widespread environmental contaminants **(Reistad et al., 2006)**. It is clear that some of BFRs are non-isoform specific inhibitors of the SERCA Ca^{2+} pumps at low micromolar (μM) concentrations within cells **(Ogunbayo and Michelangeli, 2007)**. Mobilisation of intracellular Ca^{2+} stores by inhibiting SERCA activity is one of the mechanisms by which BFRs and APs interfere with the Ca^{2+} signalling pathway **(Hughes et al ., 2000; Ogunbayo and Michelangeli, 2007; Ogunbayo et al., 2008)**.

It is now evident that a number of BFRs and APs are cytotoxic at low μM concentrations within cultured cells **(Ogunbayo et al., 2007; Michelangeli et al., 2008)**. This has led to investigation the effects of nine BFRs and two APs on SERCA Ca^{2+} pumps in skeletal muscle vesicles (SERCA1a), SH-SY5Y microsomal and cerebellar microsomes (both SERCA2b) **(Plessers et al., 1991; Mbaya et al., 2010)**.

4.2- Results

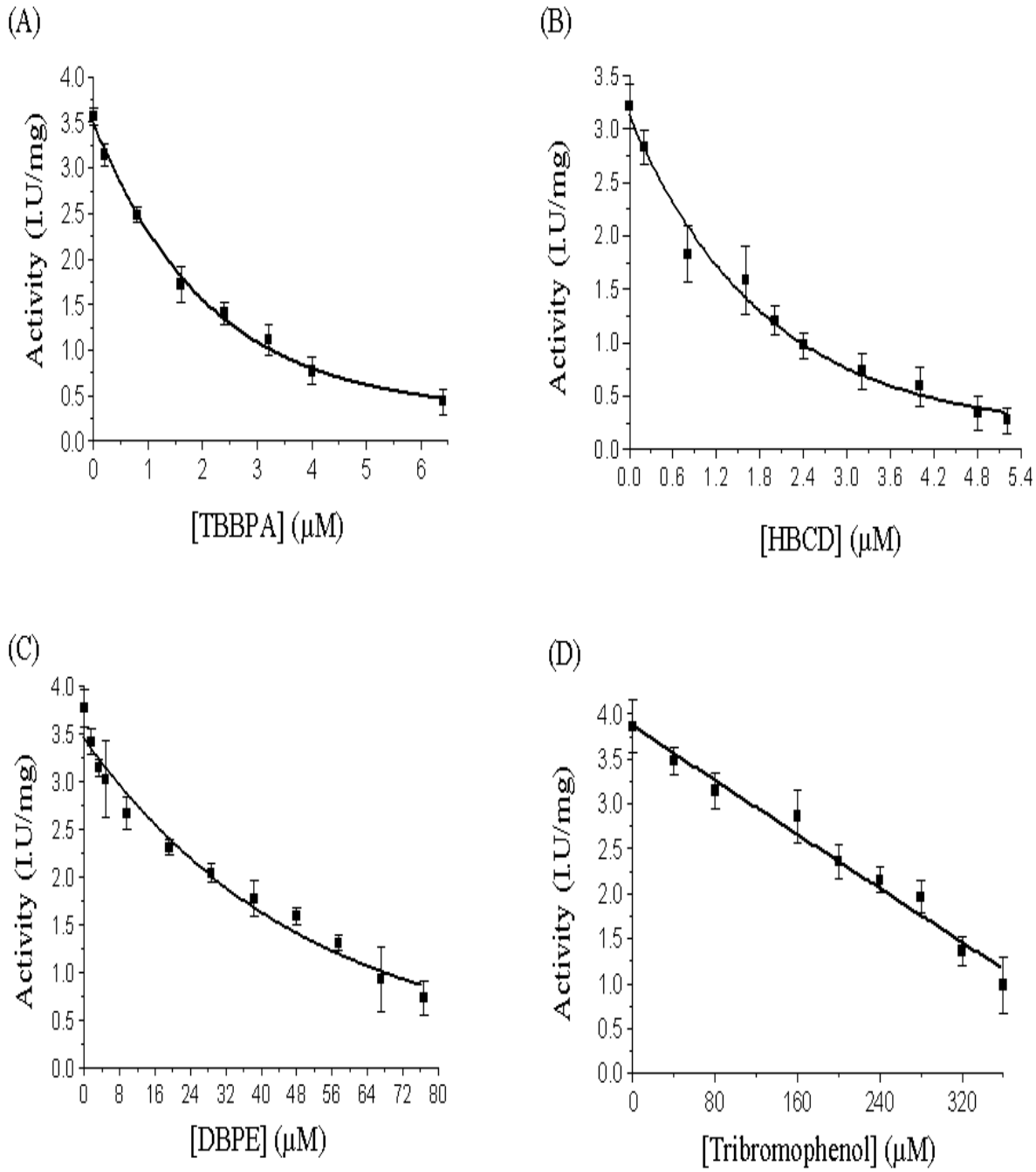
4.2.1- The effect of BFRs, Thapsigargin (Tg), NP and BPA on skeletal muscle (SR) Ca^{2+}-ATPase activity

Initially a number of BFRs and APs were tested on the SR Ca^{2+}-ATPase using the coupled enzyme method, which contains the SERCA 1a isoform.

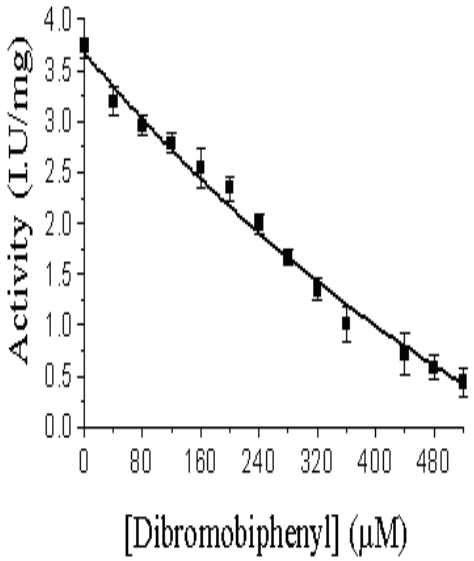
Figure 4.2.1.1 (A-I) shows effect of nine different types of BFRs on the skeletal muscle SERCA 1A isoform of Ca^{2+} -ATPase activity, measured at pH 7.2 at 25°C. Of the BFRs tested HBCD was the most potent inhibitors of the SERCA 1a Ca^{2+}-ATPase, with IC_{50} values calculated to be 1.2 ± 0.4 µM, then TBBPA and apart from DBPE (IC_{50}, 1.8 ± 0.6 µM and 36 ± 4.4µM, respectively), the other BFR's tested proved to be weak inhibitors of SERCA 1a Ca^{2+}-ATPase, since the IC_{50} were calculated to be around 100-300µM (see table 4.2.1).

Figure 4.2.1.2 (A-C) shows the effect of different concentrations of Tg, NP and BPA on skeletal muscle SERCA 1A isoform of Ca^{2+}-ATPase activity, measured at pH 7.2 at 25°C. Tg has been shown to be a specific inhibitor of all SERCA isoforms **(Lytton et al., 1991; Wootton and Michelangeli, 2006)**. In this study, the IC_{50} for Tg, the most potent SERCA1A inhibitor, was 100 ± 5.6 nM, while that for NP was 9 ± 1.3µM and for BPA, 180 ± 27µM.

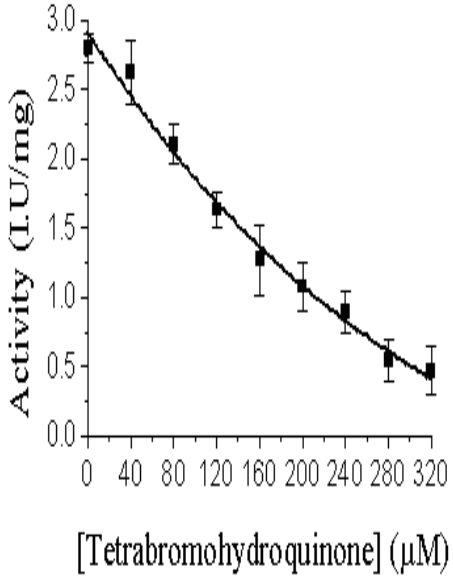
Figure 4.2.1.1


(A)
Activity (I.U/mg)
[TBBPA] (μM)
(B)
Activity (I.U/mg)
[HBCD] (μM)
(C)
Activity (I.U/mg)
[DBPE] (μM)
(D)
Activity (I.U/mg)
[Tribromophenol] (μM)

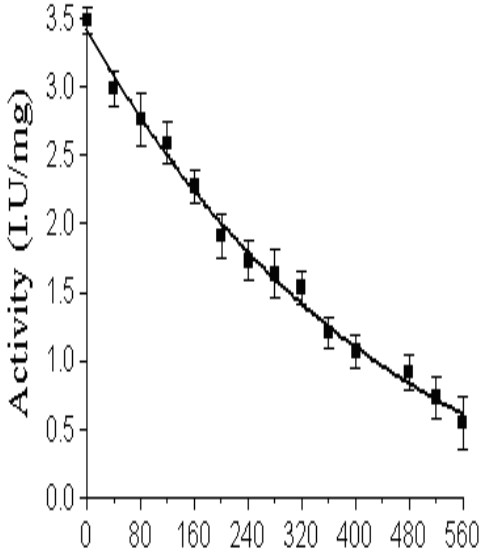
(E)


Activity (I.U/mg)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
80
160
240
320
400
480
[Dibromobiphenyl] (μM)

(F)


Activity (I.U/mg)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
40
80
120
160
200
240
280
320
[Tetrabromohydroquinone] (μM)

(G)


Activity (I.U/mg)
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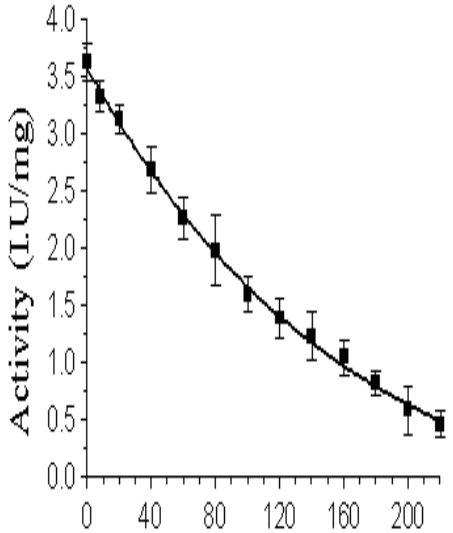
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
80
160
240
320
400
480
560
[TBBPA-diallyl ether] (μM)

(H)


Activity (I.U/mg)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
40
80
120
160
200
[PBDE] (μM)

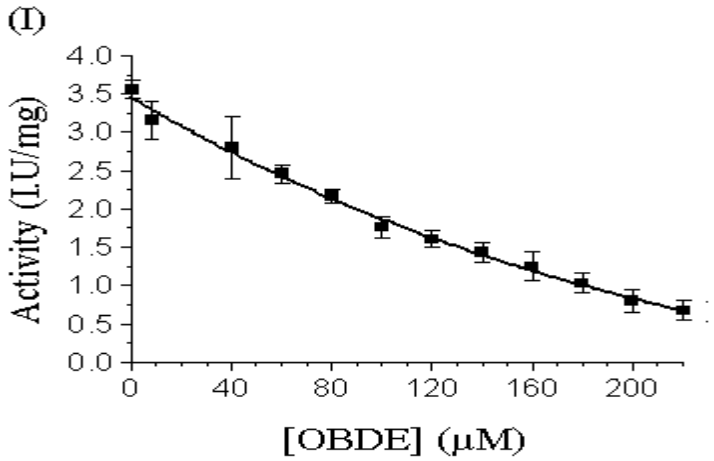


Figure 4.2.1.1 (A-I); shows the inhibition of nine BFRs on the skeletal muscle the Ca^{2+}-ATPase (SERCA1a) activity measured in rabbit skeletal muscle SR membranes at 25 °C in buffer at pH 7.2. The data points represent the mean ± SD of between 3-4 determinations.

Figure 4.2.1.2

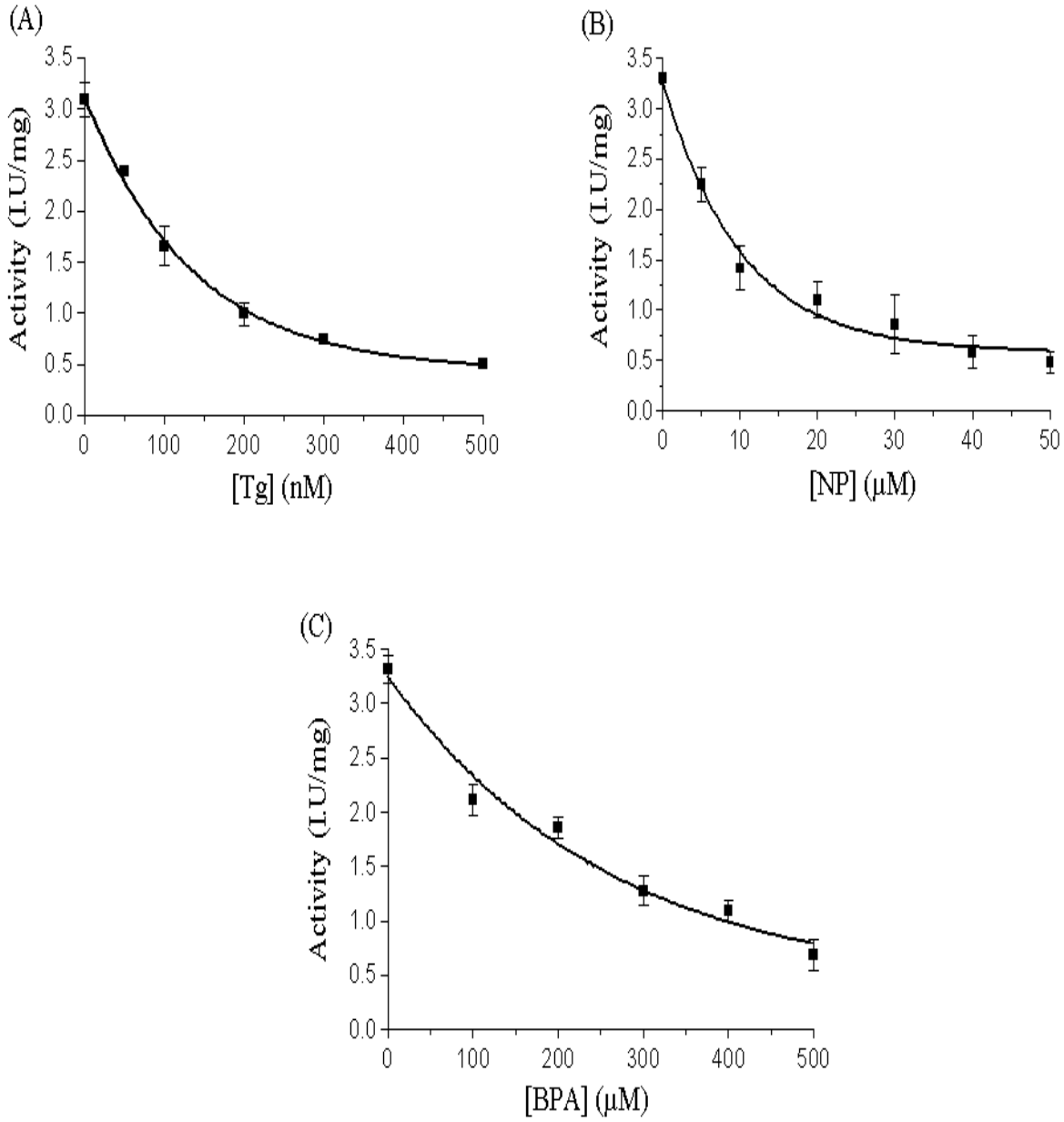


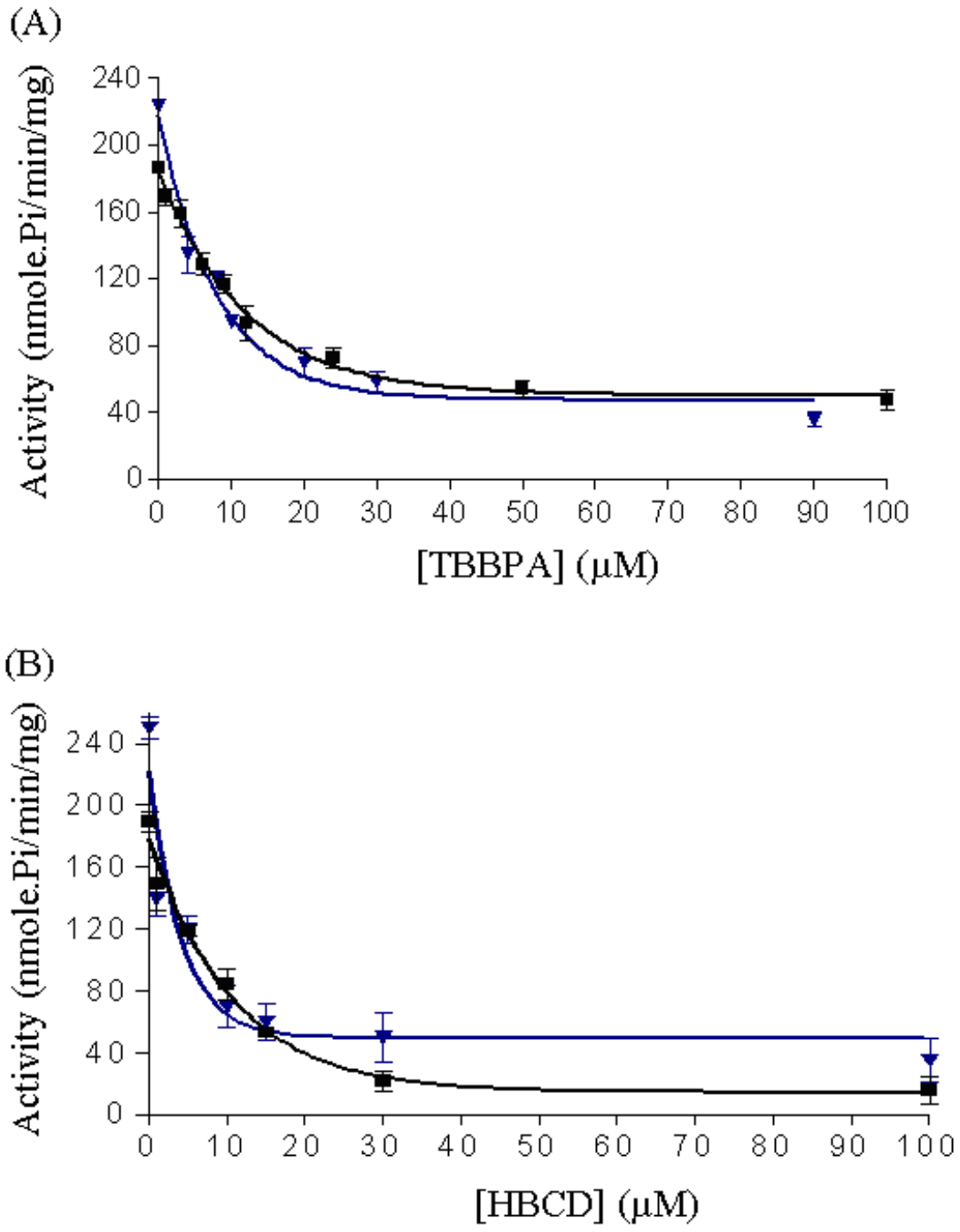
Figure 4.2.1.2; the effect of Tg (A), NP (B) and BPA (C) on the skeletal muscle SR of Ca^{2+}-ATPase (SERCA1a) activity, measured at pH 7.2 and at 25°C. The data points represent the mean ± SD of between 3-4 determinations

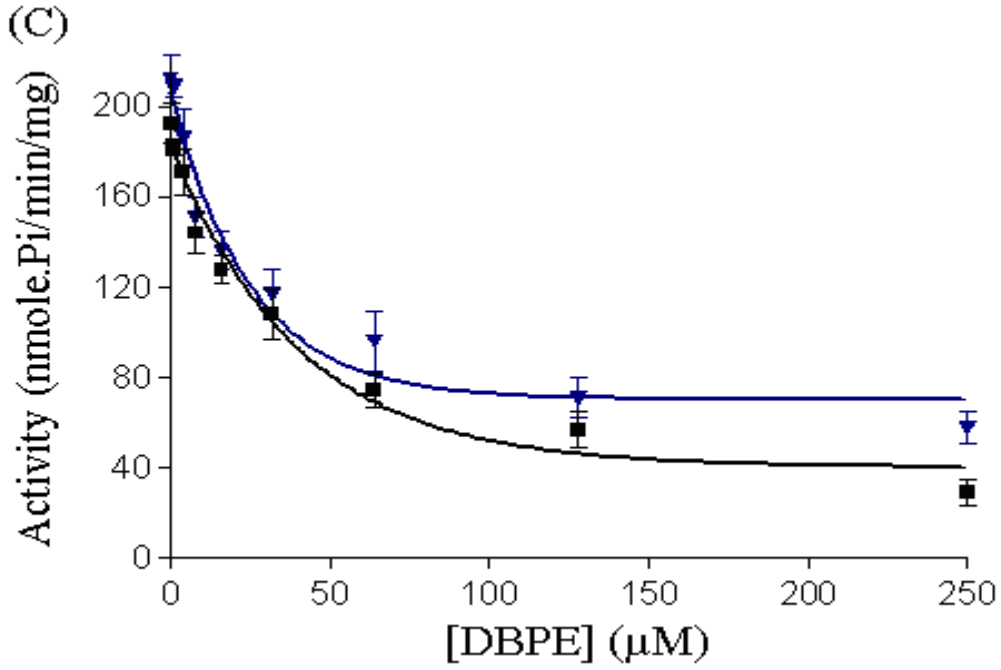
4.2.2- The dose-dependent effects of BFRs on the SH-SY5Y microsomal membranes and cerebellar microsomes measured by the phosphate liberation assay

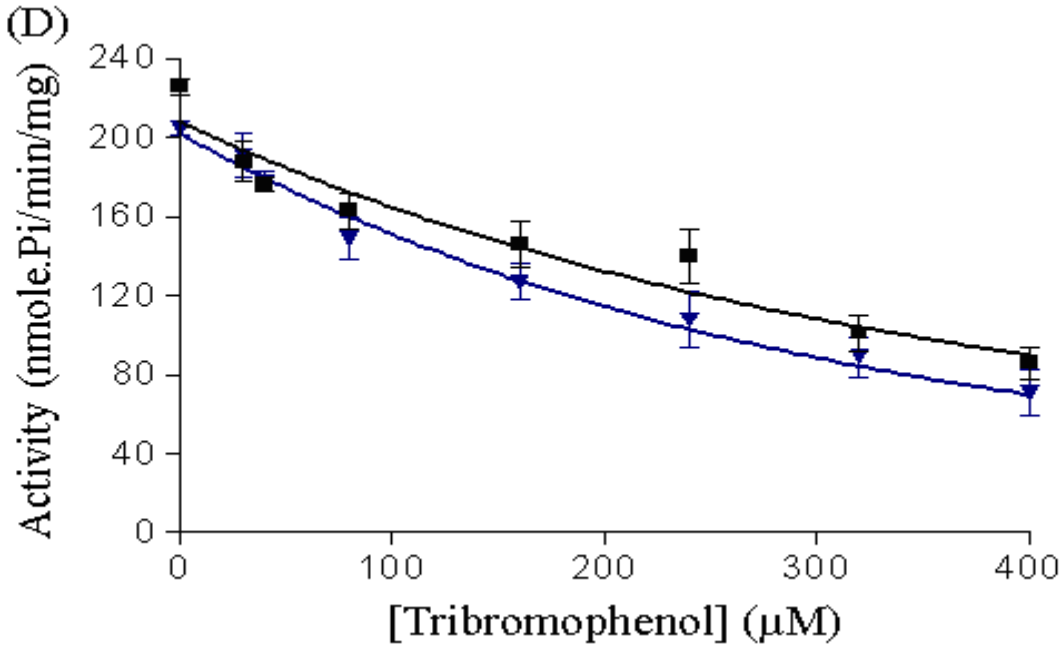
Microsomal extracts from brain and cells are a good source of Ca^{2+} stores which contain Ca^{2+} pumps, and Ca^{2+} release channels such as IP_3 receptor and RyR **(Berridge et al., 2000)**. Cerebellum is known to express mostly SERCA2b **(Plessers et al., 1991; Baba-Aissa et al., 1998)** and SH-SY5Y cells also contains mainly SERCA2b **(Cecchi et al., 2000; Mbaya et al., 2010)**.

Figure 4.2.2 (A-J) shows the effects of a number of BFRs on Ca^{2+}- ATPase activity in SH-SY5Y microsomal membranes (▼) and cerebellar microsomal (■), using the phosphate liberation method. Again for the BFRs, HBCD and TBBPA proved to be the most potent at inhibiting the SERCA2b Ca^{2+} pumps of cerebellar microsomal and SH-SY5Y microsomal, membranes, with IC_{50} values for the inhibitors in 4.3-13µM concentration range. In general the IC_{50} values determined for pig cerebellar microsomes and SH-SY5Y membranes were comparable. (see table 4.2.1). Of the other BFR's tested it was clear that the polybrominated diphenyl ethers were reasonably effective inhibitors of SERCA2b, since DBPE, OBPE and PBPE had IC_{50} values of between 38 to 93µM. All other Brominated compounds tested were poor inhibitors and had IC_{50} values which ranged from approximately 200-300µM. NP was also determined to be an effective inhibitor (IC_{50} = 13-21 µM).

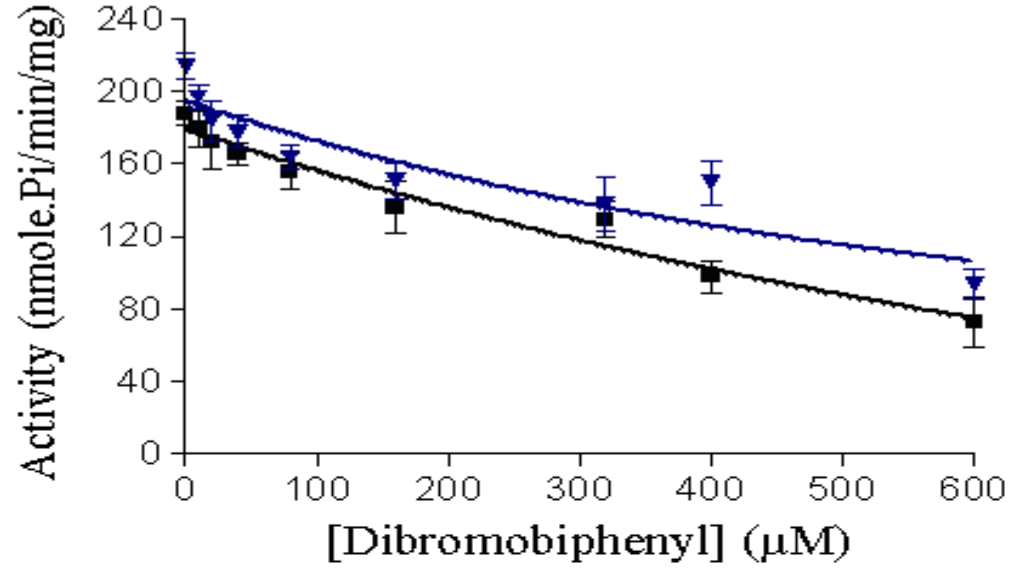
Figure 4.2.2


(A)
Activity (nmole.Pi/min/mg)
240
200
160
120
80
40
0
0 10 20 30 40 50 60 70 80 90 100
[TBBPA] (μM)
(B)
Activity (nmole.Pi/min/mg)
240
200
160
120
80
40
0
0 10 20 30 40 50 60 70 80 90 100
[HBCD] (μM)


(C)
Activity (nmole.Pi/min/mg)
200
160
120
80
40
0
0
50
100
150
200
250
[DBPE] (µM)


(D)
Activity (nmole.Pi/min/mg)
240
200
160
120
80
40
0
0
100
200
300
400
[Tribromophenol] (µM)

(E)


Activity (nmole.Pi/min/mg)
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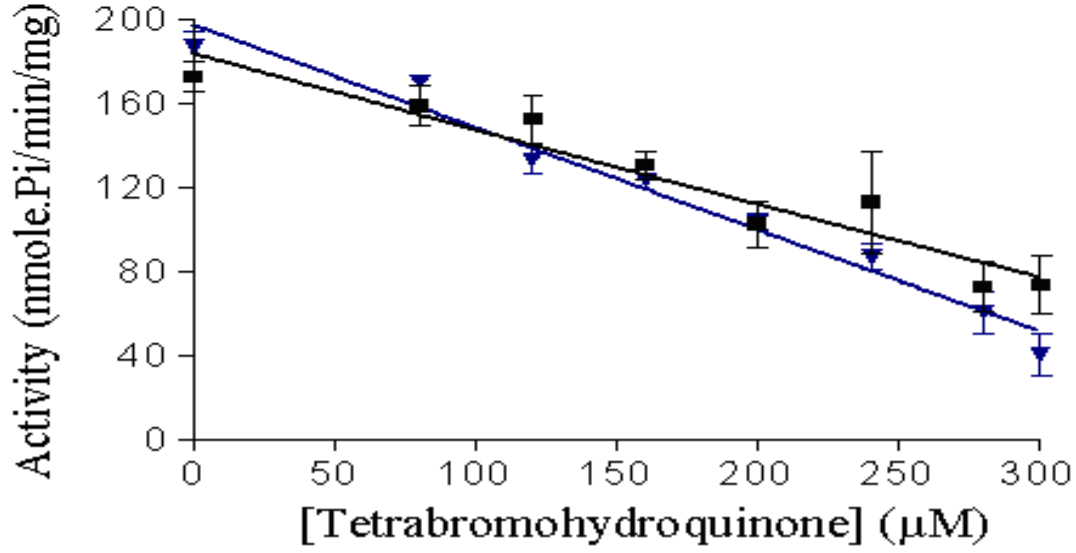
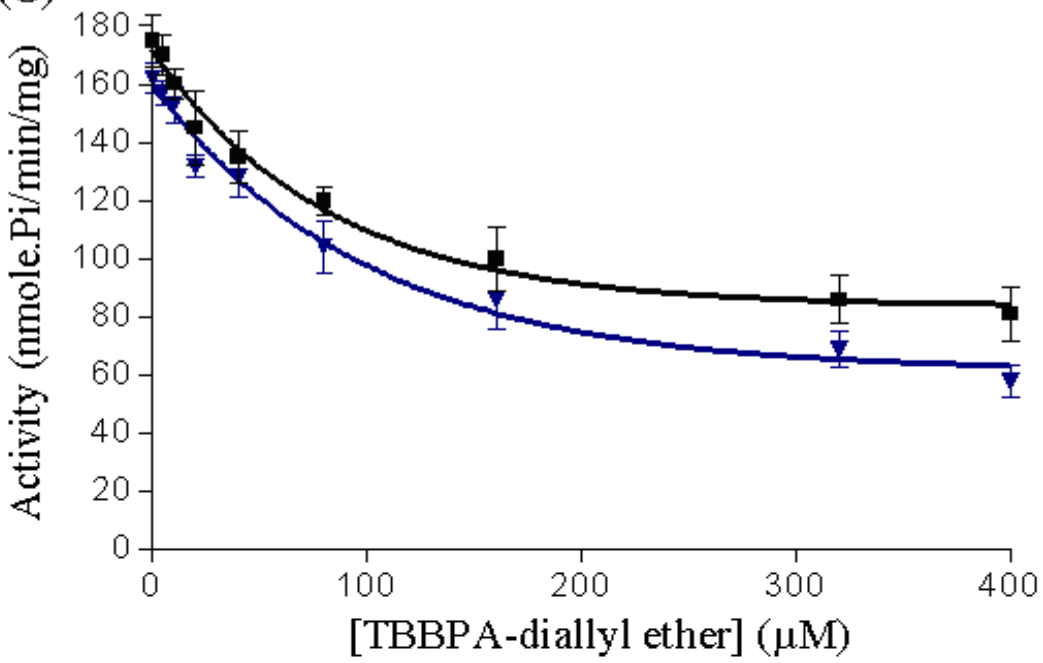
200
160
120
80
40
0
0
100
200
300
400
500
600
[Dibromobiphenyl] (μM)

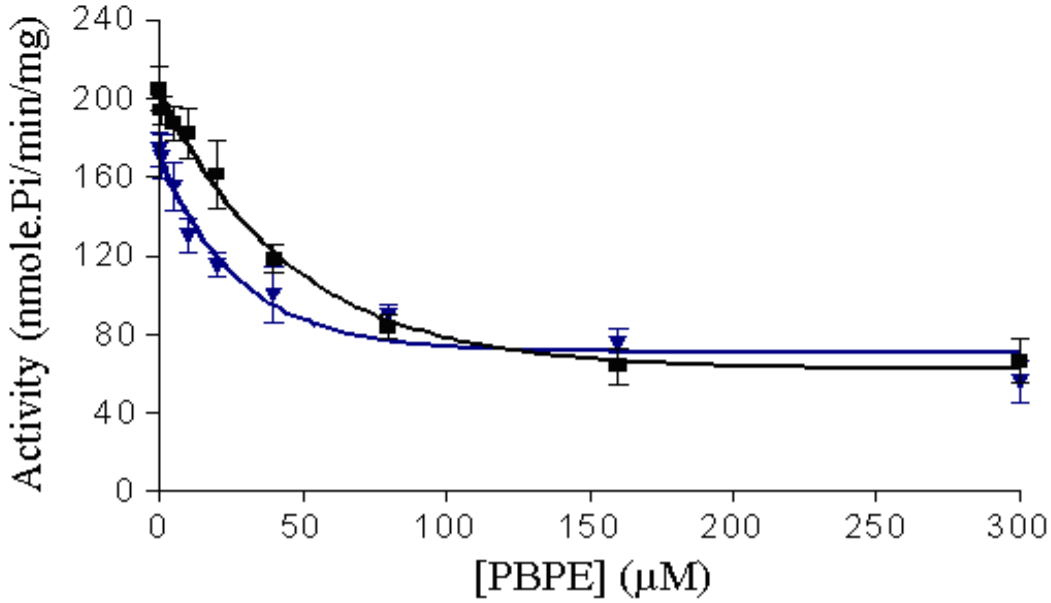
(F)


Activity (nmole.Pi/min/mg)
200
160
120
80
40
0
0
50
100
150
200
250
300
[Tetrabromohydroquinone] (μM)

(G)


Activity (nmole.Pi/min/mg)
180
160
140
120
100
80
60
40
20
0
0
100
200
300
400
[TBBPA-diallyl ether] (μM)

(H)


Activity (nmole.Pi/min/mg)
240
200
160
120
80
40
0
0
50
100
150
200
250
300
[PBPE] (μM)

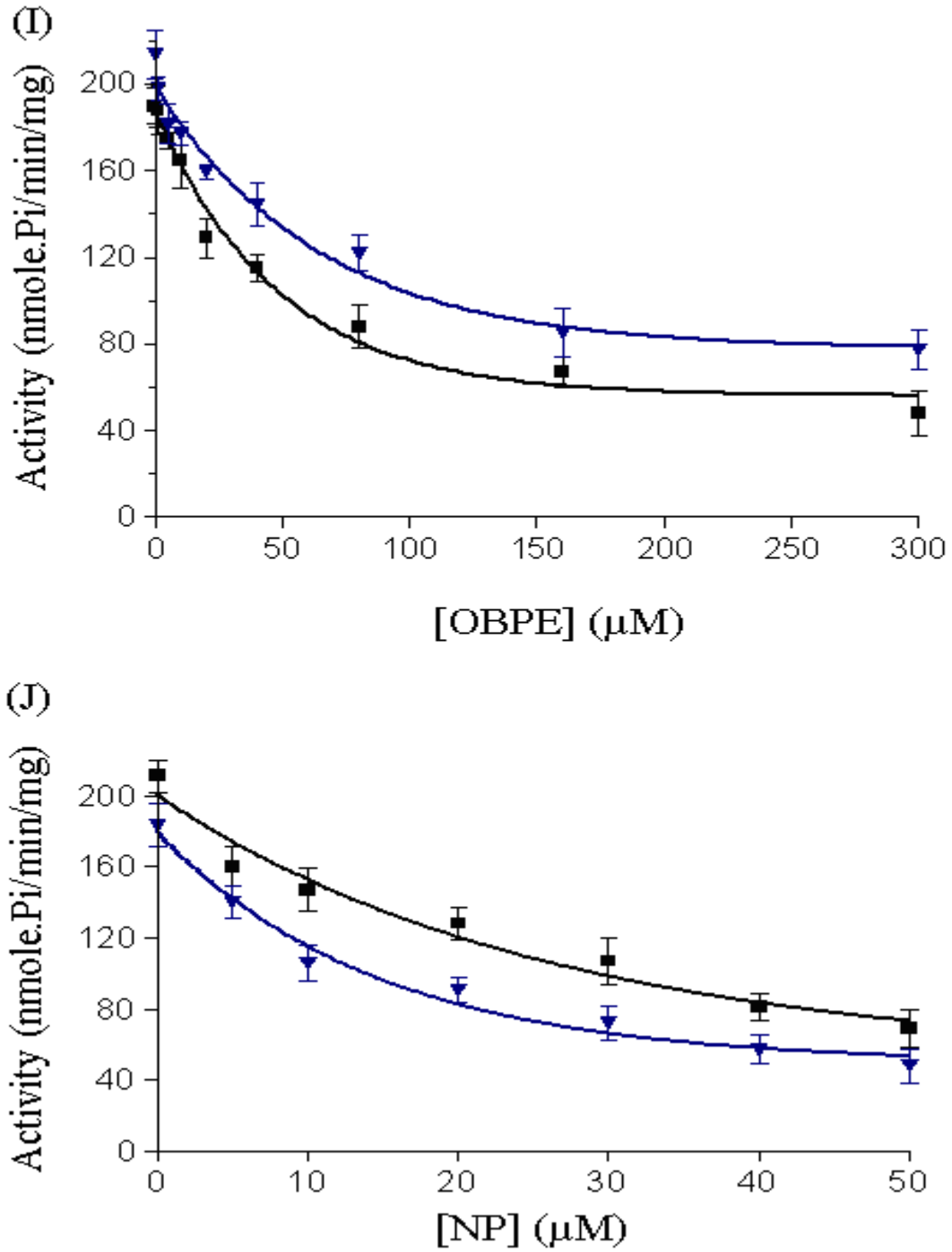


Figure 4.2.1(A-J); shows the inhibition of SH-SY5Y membranes and cerebellar microsomal the Ca^{2+}-ATPase activity by nine types of BFRs and NP. The effects of nine types of BFR and NP on Ca^{2+}-ATPase (SERCA2b) activity in SH-SY5Y microsomal membranes (▼) and cerebellar microsomal (■) measured at pH 7.2, 37 °C by phosphate liberation method The data points represent the mean ± SD of 3-5 determinations.

Table 4.2.1: comparison of IC_{50} values of the effects of different compounds on Ca^{2+}-dependent ATPase activity from various tissues. All experiments were performed in the presence of ATP 6mM and 1µM free [Ca^{2+}], at pH 7.2, 37 °C. Results are the mean ± SD of 3-5 determinations.

Name of compound	IC_{50} of Ca^{2+}ATPase inhibition (µM)		
	Skeletal muscle (SR)	Pig brain membrane	SH-SY5Y membrane
3,3′,5,5′-Tetrabromobisphenol A (TBBPA)	1.8 ± 0.6	13.0 ± 1.4	8 ± 1.9
1,2,5,6,9,10-Hexabromocyclododecane (HBCD)	1.2 ± 0.4	7.5 ± 2.2	4.3 ± 1.1
2,4,6-Tribromophenol	280 ± 11	300 ± 32	230 ± 18
4,4′-Dibromobiphenyl	270 ± 9	420 ± 12	481 ± 26
Tetrabromohydroquinone	170 ± 14	265 ± 19	220 ± 24
2,2′,6,6′-Tetrabromobisphenol A diallyl ether	247 ± 10	213 ± 33	172 ± 28
Decabromodiphenyl ether (DBPE)	30.0 ± 5.5	38 ± 4	41.0 ± 8.2
Pentabromodiphenyl ether (PBPE)	93 ± 16	72.0 ± 5.8	78.0 ± 7.8
Octabromodiphenyl ether (OBPE)	110 ± 12	61.0 ± 3.8	93 ± 15
4-n-Nonylphenol (NP)	9.0 ± 1.6	21 ± 4	13.0 ± 2.2

4.2.3- Effects of HBCD, TBBPA, DBPE and NP on intracellular Ca^{2+} transport proteins

As these compounds increased intracellular [Ca^{2+}] levels within cells other types of Ca^{2+} transporters could be involved. Therefore, the different compounds were tested on SH-SY5Y microsomes and SR vesicles to assess whether they affected both pumps and channels. Figures 4.2.3.1A shows the effects of varying concentrations of HBCD on the initial rate of ATP-dependent Ca^{2+} uptake in SH-SY5Y microsomal membranes. Ca^{2+} uptake rate in SH-SY5Y microsome is clearly inhibited by HBCD at low concentrations. An IC_{50} value for Ca^{2+} uptake rates was 1.9 ± 0.61 µM, similar to that seen in Ca^{2+}-ATPase pump inhibition. Figure 4.2.3.1B shows that HBCD is also able to induce substantial Ca^{2+} release by itself when skeletal muscle SR vesicles are first preloaded with Ca^{2+}. When SR is treated with HBCD in presence of 1mM tetracaine (a blocker of the release RyR Ca^{2+} release channel) **(Tovey et al., 1998)**, the extent of Ca^{2+} release is considerably reduced compared to the absence of tetracaine. Also if the SR was also pre-treated with 20 mM caffeine (a known as RyR channel activator **(Tovey et al., 1998)** which depletes the RyR containing Ca^{2+} stores of Ca^{2+}), again HBCD, under these condition released little Ca^{2+}. These results suggest that HBCD may also activate RyR Ca^{2+} channel.

Figure 4.2.3.2 B-C shows effects of TBBPA, DBPE and NP on intracellular Ca^{2+} transport proteins. Ca^{2+} uptake from SH-SY5Y microsomal membrane (■) and release from SR vesicles (▼). Also Ca^{2+} uptake rate in SH-SY5Y microsome is inhibited by TBBPA and NP at low concentrations, the IC_{50} values determined for TBBPA was 7.2 ± 0.52 µM, and for NP, 9.6 ± 1.3µM. Again, the Ca^{2+} uptake results and Ca^{2+}-ATPase activity are also similar. All other compounds tested also released Ca^{2+} from SR vesicles preloaded with [Ca^{2+}], suggesting that they might also activate the RyR is a similar fashion to HBCD, but this requires further investigation.

Figure 4.2.3.1

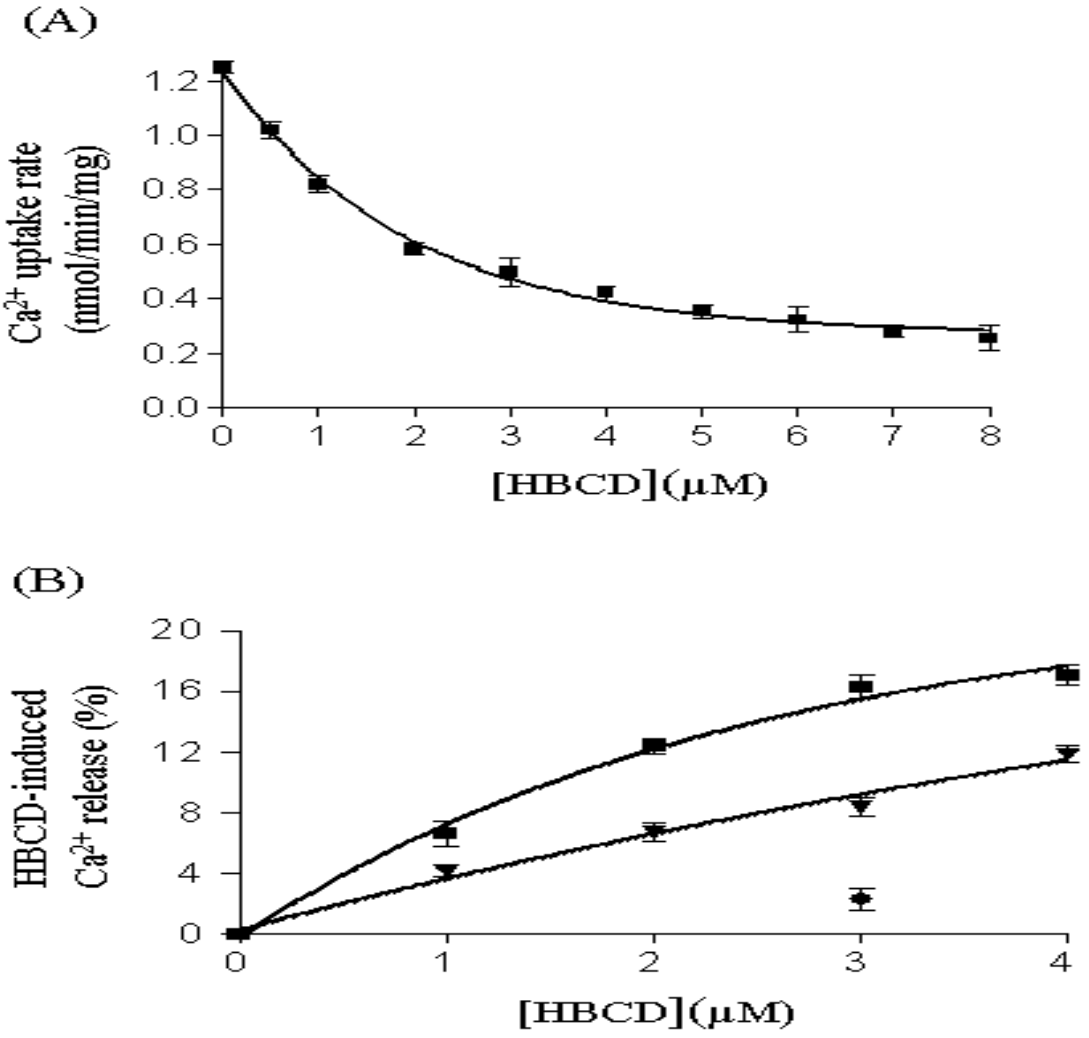
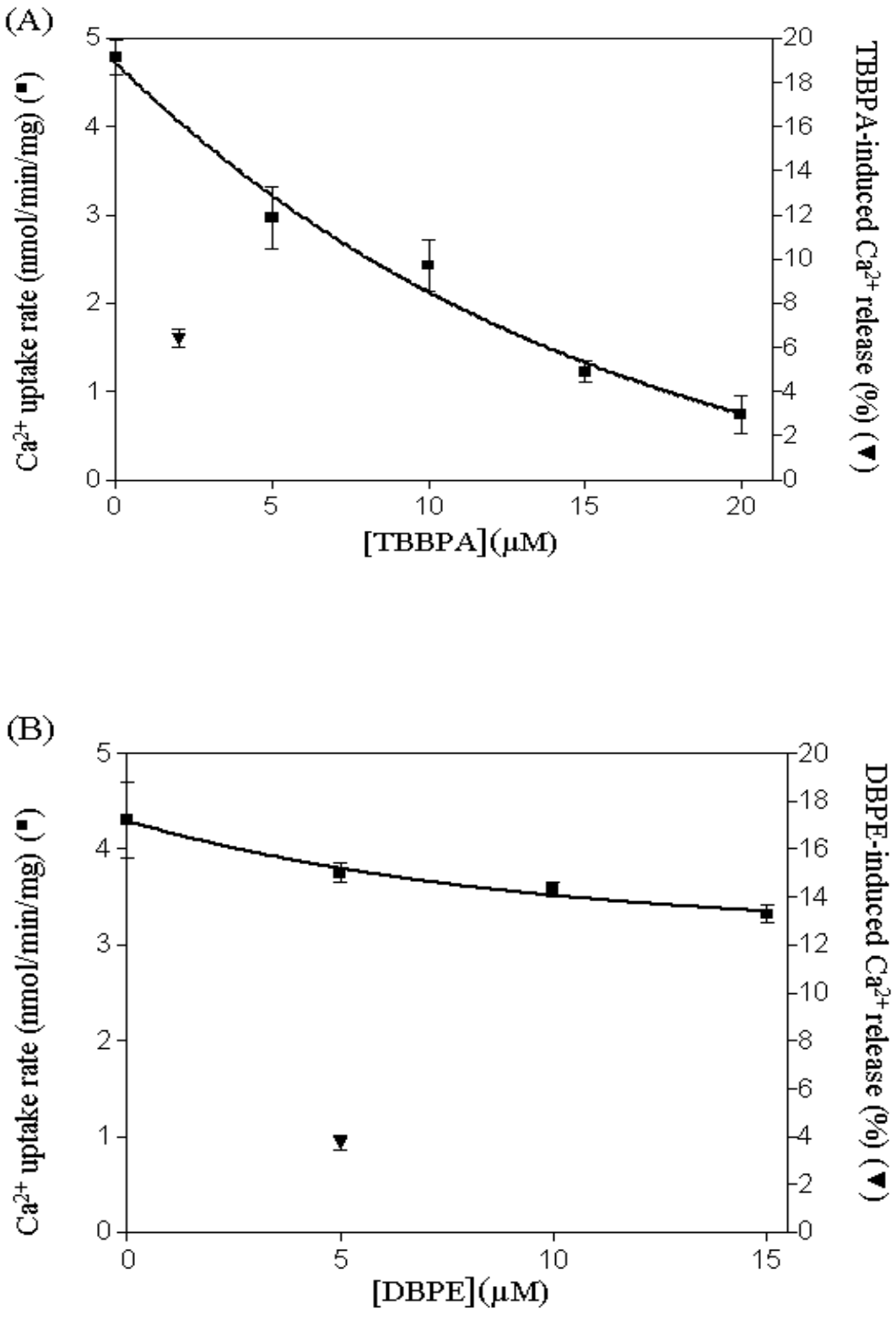


Figure 4.2.3.1; effects of HBCD on intracellular Ca^{2+} transport proteins. (A) shows the effects of HBCD on SH-SY5Y microsomal membranes Ca^{2+} uptake. SH-SY5Y membranes were suspended in 2 ml of 40 mM Tris/phosphate buffer, 100 mM KCl, pH 7.2 at 37 °C in the presence of 10 mM phospho-creatine, 10mg/ml of creatine kinase, and 1.25 mM Fluo-3. After pre-incubation with HBCD, Ca^{2+} uptake was initiated by the addition of 1.5 mM Mg-ATP. The data points represent the mean ± SD of 3-4 determinations. (B) Shows the effect of HBCD at inducing on Ca^{2+} release from Skeletal muscle SR in the absence (■) or presence (▼) of the RyR inhibitor tetracaine (1mM). Pre-treatment with 20mM caffeine followed by the addition of 3μM HBCD is shown by (●). The extent of release is expressed as percentage of that released by 12.5μg/ml A23187.

Figure 4.2.3.2


(A)
Ca2+ uptake rate (nmol/min/mg) (■)
TBBPA-induced Ca2+ release (%) (▼)
[TBBPA](μM)
0
5
10
15
20
(B)
Ca2+ uptake rate (nmol/min/mg) (■)
DBPE-induced Ca2+ release (%) (▼)
[DBPE](μM)

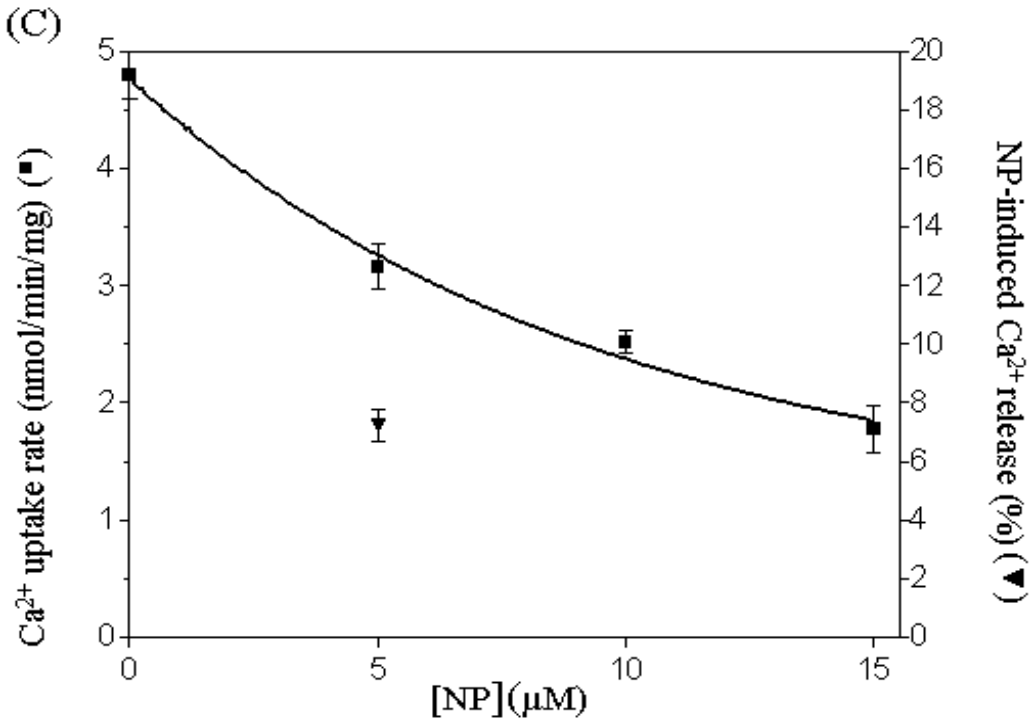


Figure 4.2.3.2; shows effects of TBBPA, DBPE and NP on intracellular Ca^{2+} transport proteins. Ca^{2+} uptake from SH-SY5Y microsomal membrane (■) and release from SR (▼) were monitoring change in fluorescence of the Ca^{2+} indicator dye Fluo-3. SH-SY5Y membrane was suspended in 2 ml of 40 mM Tris/phosphate buffer, 100 mM KCl, pH 7.2 at 37°C in the presence of 10 mM phospho-creatine, 10 mg/ml of creatine kinase, and 1.25 mM Fluo-3. Ca^{2+} uptake was initiated by the addition of 1.5 mM Mg-ATP. The data points represent the mean ± SD of 3-4 determinations. Ca^{2+} release from skeletal muscle SR by the addition of 2μM of TBBPA, and 5μM for both DBPE and NP, is shown by (▼). The extent of release is expressed as percentage of that released by 12.5μg/ml A23187.

4.2.4- The effects of HBCD on SR Ca^{2+}-ATPase (SERCA1a) activity as a function of [Mg^{2+}], [Ca^{2+}] and [ATP]

4.2.4.1-The effects of HBCD on ATPase activity as a function of [Mg^{2+}]

HBCD at concentrations, which caused approximately 50% inhibition, were pre-incubated for 10 min with SR (~ 37.5µg) in assay buffer pH 7.2 at 25°C. Ca^{2+}-ATPase activity was then measured in the presence 10µg/ml A23187 as a function of Mg^{2+}, in the absence (■) and presence (▲) of 3µM HBCD. As shown in figure 4.2.4.1, ATPase activity decreases with increasing concentrations of Mg^{2+} both in the absence and presence of HBCD. Mg^{2+} inhibits the luminal dissociation of Ca^{2+} from the phosphorylated Ca^{2+}-ATPase **(Duggleby et al., 1999)**. The IC_{50} of Ca^{2+}-ATPase activity in the absence and presence of HBCD is 1.63 ± 0.08 mM and 4.30 ± 0.82 mM, respectively, whereas the V_{max} is decreased from 9.5 ± 0.3 IU/mg to 3.46 ± 0.36 IU/mg (goodness-of-fit: chi^2 was 0.99). The data is fitted into Eq.5 (found in chapter-2).

This suggests that HBCD decreases the apparent affinity for Mg^{2+} binding to the vacated Ca^{2+} binding sites of the E2 state. This could be due to the sites not being vacated by Ca^{2+} in this state, especially if trapped in an E2 state.

Figure 4.2.4.1

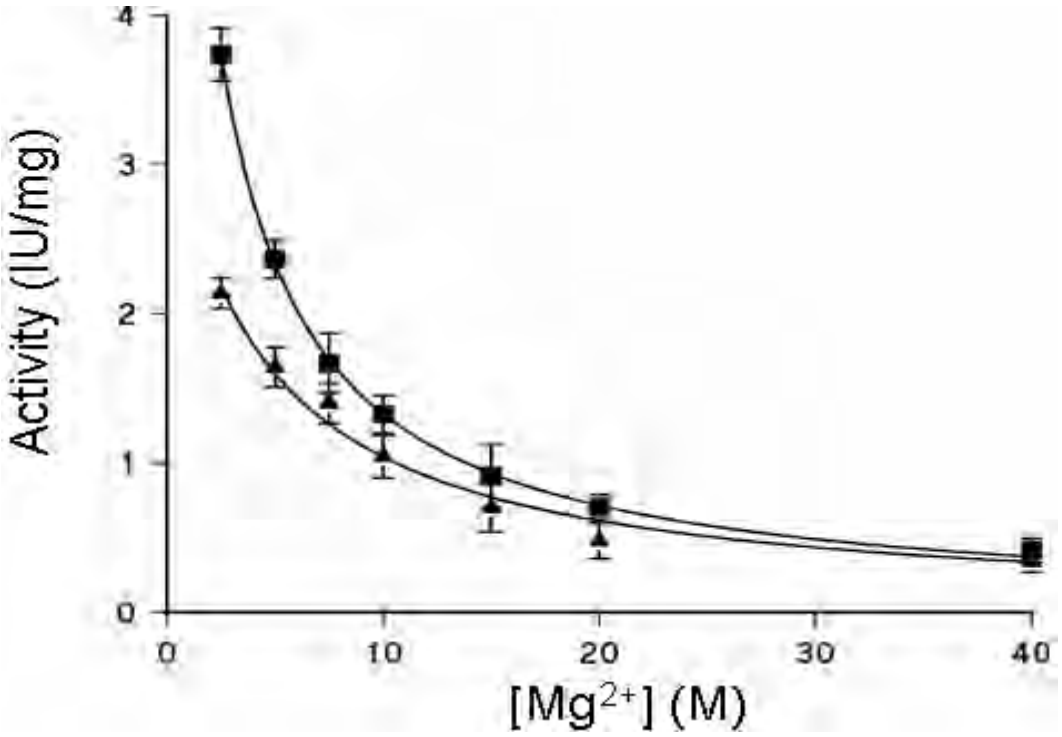


Figure 4.2.4.1 shows the effect of HBCD on ATPase activity as function of [Mg^{2+}]. The effects of varying [Mg^{2+}] on Ca^{2+}-ATPase activity measured at pH 7.2 and at 25°C, in the absence of HBCD (■) and presence (▲) of 3µM HBCD both occurred Ca^{2+}-ATPase activity at 2.5mM $Mg^{2+}SO_4$. The data points represent the mean ± SD of between 3-5determinations.

4.2.4.2- The effects of HBCD on ATPase activity as a function of free [Ca^{2+}]

Figure 4.2.4.2 shows the effects of Ca^{2+} on ATPase activity at pH 7.2 and 25°C, in the absence and presence of 3µM HBCD as a function of free Ca^{2+} concentrations. The Effects of [Ca^{2+}] on Ca^{2+} ATPase activity shows a typical bell-shaped profile. The stimulatory phase of the curve, at low Ca^{2+} concentrations reflects Ca^{2+} binding to the E1 form (denoted by Ks), while the inhibition part at higher Ca^{2+} concentrations reflects, in part, Ca^{2+} binding to the E2 conformation (denoted by Ki). In the absence of HBCD the ATPase had a V_{max} of 5.26 ± 0.41 IU/mg, with the K_s for the stimulatory phase of $6.1x10^{-7}$ ± 1.3 $x10^{-7}$ M, and a K_i value of $1.2x10^{-4}$ ± $3x10^{-5}$ M for the inhibitory phase. While, in the present of HBCD (3µM), the data is fitted into Eq.3 (found in chapter-2) and showed that V_{max} was 2.79 ± 0.26 IU/mg, and stimulatory K_s and inhibitory K_i values $5.5x10^{-7}$ ± 1.5 $x10^{-7}$ M and 1.8 $x10^{-4}$ ± $6x10^{-5}$ M, respectively (goodness-of-fit: chi^2 was 0.97).

These results suggest that HBCD is likely to have little effect on the affinity for Ca^{2+} binding to the E1 form and E2 form of the Ca^{2+}-ATPase. Only the V_{max} is affected.

Figure 4.2.4.2

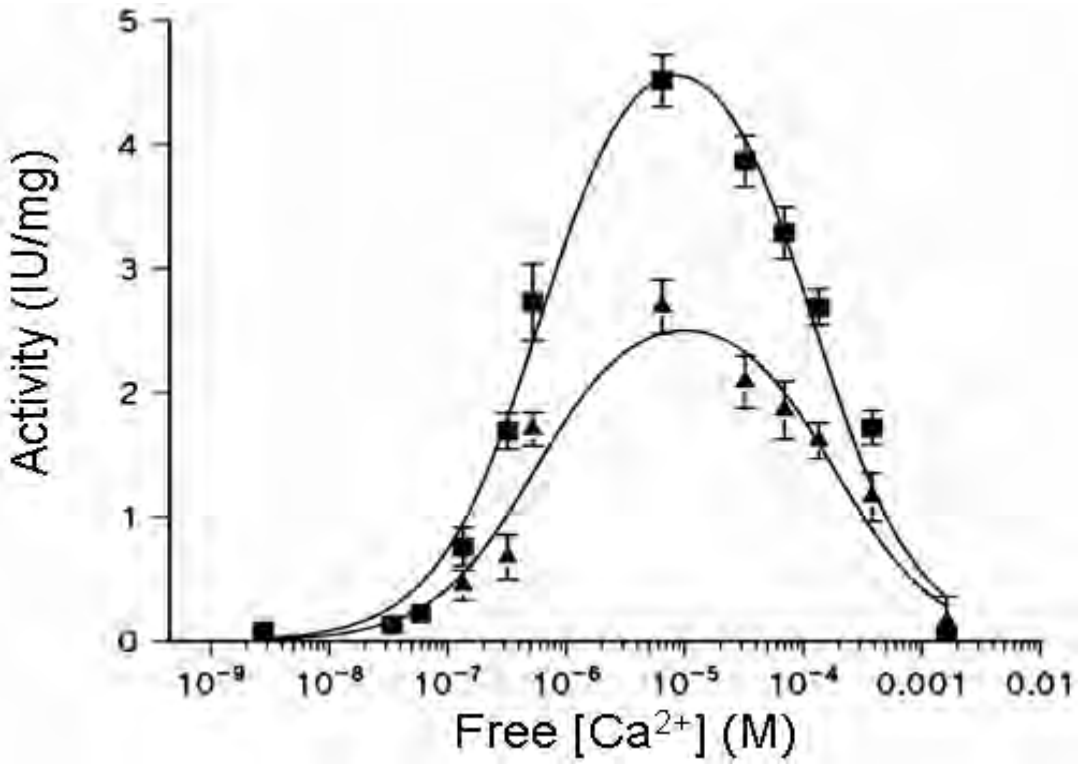


Figure 4.2.4.2 the effects of HBCD on SERCA activity as function of [Ca^{2+}] using the coupled enzyme assay, at pH 7.2 and 25°C in the absence (■) or presence (▲) of 3µM HBCD. The data points represent the mean ± SD of between 3-5 determinations.

4.2.4.3- The effects of HBCD on ATPase activity as a function of [ATP]

Figure 4.2.4.3 shows the activity of ATPase activity at varying concentrations in the absences and presence of 3µM HBCD. The data for the SR Ca^{2+}-ATPase activity in the presence and absence of HBCD shows a biphasic profile and was fitted using a bi-Michaelis-Menton Equation **(Dupont et al., 1985; Coll and Murphy, 1991)**, assuming two independent ATP binding sites designated the high affinity catalytic site and the lower affinity regulatory site. The data was fitted to Eq.4 (found in chapter-2)

The kinetic parameters for best fit; in the absence of HBCD was for the catalytic K_m = $6.5x10^{-7} \pm 2.5x10^{-7}$M with a V_{max} 1.42 ± 0.13 of IU/mg and a regulatory K_m and V_{max} of 0.00162 ± 0.009M and 4.52 ± 1.34 IU/mg, respectively. In presence of 3µM HBCD, the data could be fitted assuming, the K_m for both catalytic and regulatory sites were $1.50x10^{-6} \pm 6x10^{-7}$ M and $7.62\ x10^{-4} \pm 3.6\ x10^{-4}$ M, respectively. The V_{max} values, however, were 2.11 ± 0.31 IU/mg for the regulatory and catalytic V_{max} value were 1.03 ± 0.13 IU/mg IU/mg, respectively (goodness of fit for the data; Chi^2 was 0.98). These results suggested that HBCD has a small decrease on affinity for ATP binding (i.e., increasing the catalytic K_m, but decreasing regulatory K_m). However, the main affects are the decreases in V_{max} for both catalytic site and regulatory site.

Figure 4.2.4.3

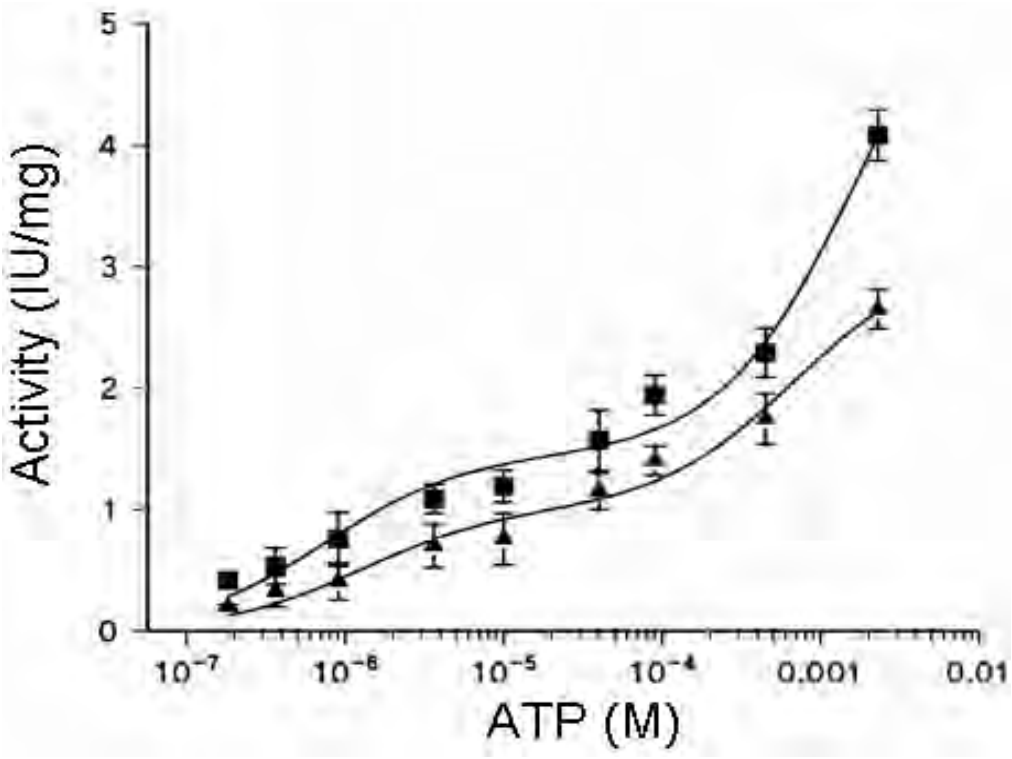


Figure 4.2.4.3 the effects of HBCD on ATPase activity as a function of [ATP] using the coupled enzyme assay, at pH 7.2 and 25°C in the absence (■) or presence (▲) of 3µM HBCD. The data points represent the mean ± SD of between 3-5 determinations.

4.2.5- The effects of HBCD and DBPE on the phosphorylation of ATPase by [γ-^{32}P]-ATP

In order to assess the possible effect of HBCD and DBPE on the phosphorylation step of Ca^{2+}-ATPase by ATP, ^{32}P-ATP phosphorylation experiments were performed with range ATP concentrations (0, 1.3, 5, 10 and 20µM) in the absence (■) and presence of 10 µM HBCD (▼) and 30 µM DBPE (●), measured at pH 7.2 and 25 °C. As shown in figure 4.2.5, the data fitted a single-site binding curve, which gave an E-P_{max} and K_d for the control data of 6.26 ± 1.7 nmol/mg and 0.143 ± 0.13 µM, respectively, whereas in the presence 10 µM HBCD, the data gave an E-P_{max} and K_d values of 6.53 ± 1.4 nmol/mg and 3.19 ± 0.78 µM, respectively. For 30 µM DBPE, the data gave an E-P_{max} and K_d values of 6.68 ± 1.21 nmol/mg and 5.19 ± 1.08 µM, respectively. These results suggest that both HBCD and DBPE cause a decrease in the ATP binding affinity to the Ca^{2+}-ATPase.

Figure 4.2.5

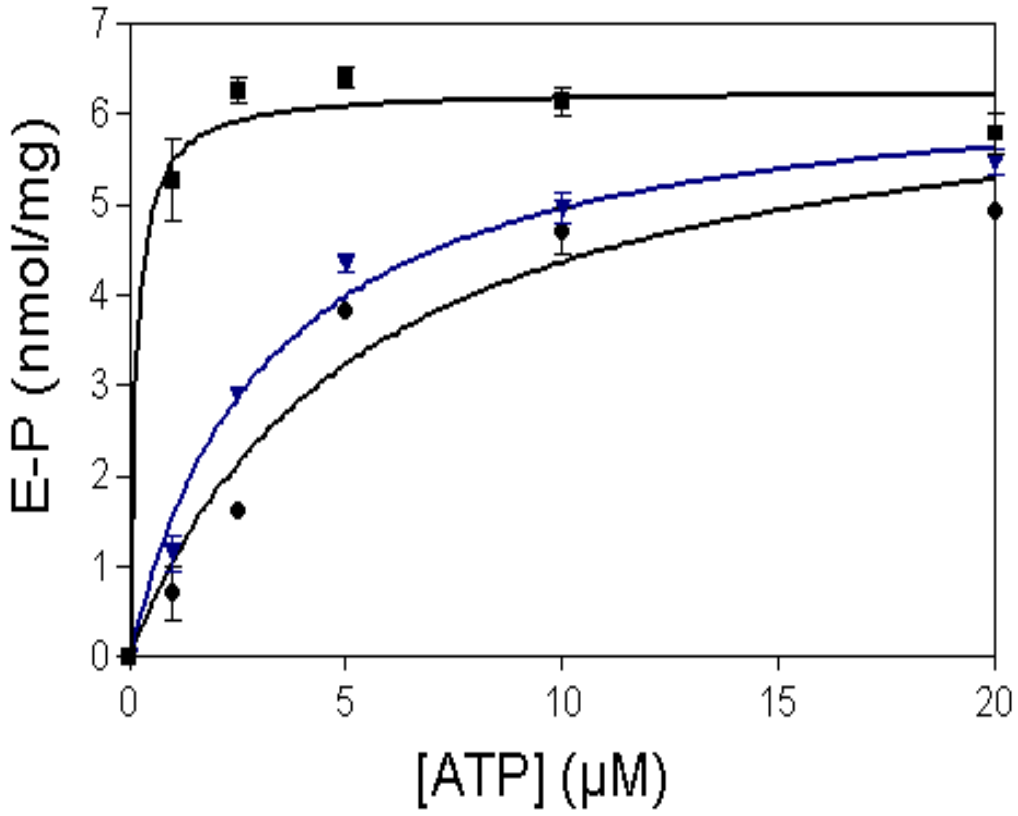


Figure 4.2.5; shows the ATP-dependent phosphorylation of Ca^{2+}-ATPase over a range of [γ -^{32}P]-ATP concentration (0-20 μM) in the absence (■) and presence of 10 μM HBCD (▼) and 30 μM DBPE (●). The data represent the mean ± SD of 3 determinations.

4.2.6-The measurement of E2-E1 conformational change using FITC-labelled Ca^{2+}-ATPase

To determine whether HBCD affects the E2→E1 transition of the ATPase, the conformational changes of the E2→E1 was determined by monitoring the Ca^{2+}-induced changes in fluorescence of Ca^{2+}-ATPase labelled with FITC **(Michelangeli et al., 1990)**. This was measured upon the addition of 100μM Ca^{2+} at pH 6, 25 °C where the enzyme is predominately in the E2 form **(Bilmen et al., 2002)**. The figure 4.2.6B-C shows the effects of HBCD on FITC-labelled Ca^{2+}-ATPase when Ca^{2+} added. The addition Ca^{2+} leads to decrease in fluorescence of 7% in the absence of HBCD, whereas in presences of 2 and 10μM HBCD, the fluorescence change was reduced to 4.6% and 0.8%, respectively. Also the rate of change in the presence 2μM HBCD was considerably reduced compared to control. Theses results indicate that HBCD binds to Ca^{2+}-ATPase and stabilizes it in the E2 state.

Figure 4.2.6

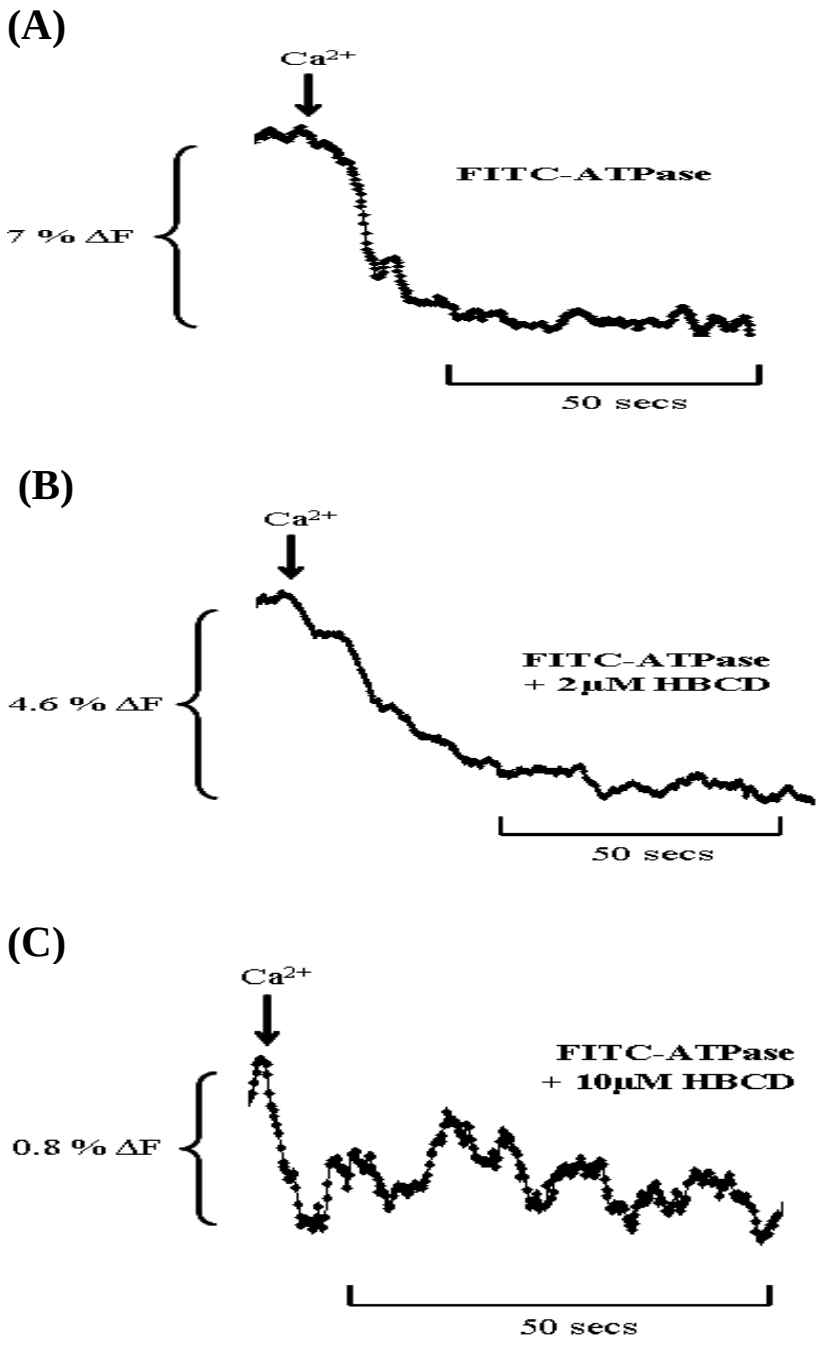


Figure 4.2.6; shows the effects of HBCD on the fluorescence intensity of FITC-labelled Ca^{2+}-ATPase upon the addition of Ca^{2+} in the absence (A), or presences of 2µM HBCD (B), or 10µM HBCD (C). Measurements were made in buffer containing 2.3 ml of 50mM Tris, 50mM maleate, 5mM $MgSO_4$, 100mM KCl, at pH 6 and 25 °C. These traces are representative of duplicate experiments.

4.3- Discussion

Toxicological studies have shown a number of BFRs and APs are toxic at low micromolar concentrations for a range of cell types **(Reistad et al., 2007; 2005; Ogunbayo et al., 2008; Hughes et al., 2000; Michelangeli et al., 2008)**.

Based on the Ca^{2+}-ATPase activity data from this study, HBCD, TBBPA and NP proved to be the most potent inhibitors of both SERCA1a (which is found in fast-twitch skeletal muscle) and SERCA2b (found in both cerebellular microsomes and SH-SY5Y cells). This is consistent with the previously reported effect of TBBPA on Ca^{2+} pump activity in both skeletal muscle SR membranes and cerebellular microsomes **(Ogunbayo et al., 2008)** and that reported for NP **(Michelangeli et al., 2008)**. Moreover, it appears that HBCD, TBBPA and NP inhibit SERCA1a better than SERCA2b. For the polybrominated diphenyl ethers (DBPE, PBPE, and OBPE), they were reasonably effective inhibitors of SERCA Ca^{2+} pumps ($IC_{50;}$ between 30 to 110 µM), but there appeared to be little correlation between number of bromines and potency. All the other BFR's tested were poor SERCA inhibitors.

SERCA are involved in Ca^{2+} uptake into organelles that also contain RyR Ca^{2+} channels **(Michelangeli et al., 2005)**. SH-SY5Y neuronal cells contain two types of intracellular Ca^{2+} channels associated with SERCA Ca^{2+} pumps; IP_3-sensitive Ca^{2+} channels (IP_3R, type I) **(Wojcikiewicz, 1998, Mackrill et al., 1997)** and Ryanodine receptor Ca^{2+} channels (RyR, type 2) **(Mackrill et al., 1997)**. In this study an investigation to assess whether BFR's (HBCD, TBBPA & DBPE) and APs (NP) affected both Ca^{2+} pumps and channels was undertaken. Data presented here show that HBCD, TBBPA, and NP are able to inhibit Ca^{2+} uptake rate in SH-SY5Y microsomal membranes and also possibly activate the RyR in SR vesicles (which contains a RyR1, **Treves et al., 2005**) at low concentrations. The effects of TBBPA and NP were comparable to that which has been found for both TBBPA **(Ogunbayo et al., 2007; 2008)** and NP **(Michelangeli et al., 2008)**. However, further studies are required to fully confirm that these BFRs and NP are able to activate the RyR.

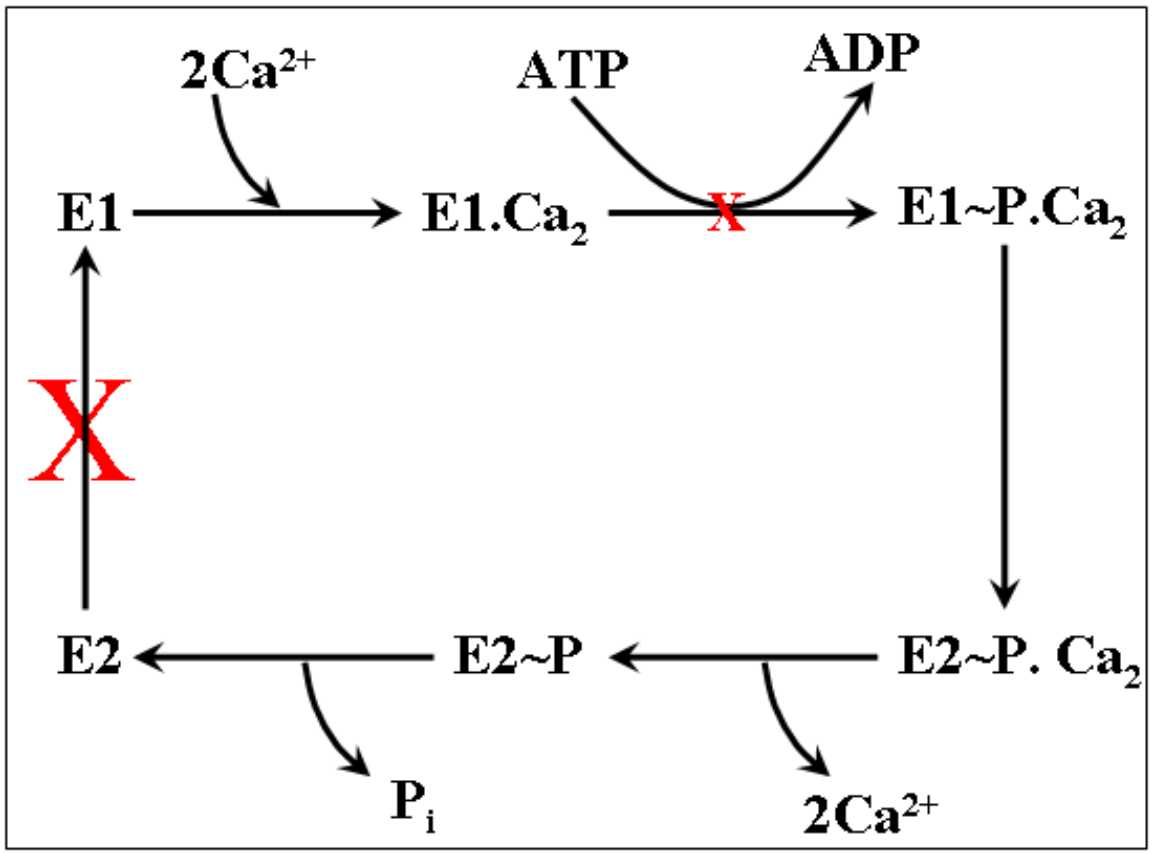
The catalysis and transport mechanism of SERCA involves alternation between two major conformational states, known as E1(which is a high affinity Ca^{2+} binding state)

and E2 (which is a low affinity Ca^{2+} binding state) **(de Meis and Vianna, 1979; Danko et al., 2004)**. The transition between the two states (E1 and E2) is dependent on a number of factors, including the presence of Ca^{2+} and Mg^{2+}, in addition to pH and temperature.

From the activity versus [ATP] (data in fig. 4.2.4.3) it shows that HBCD has a small effect on affinity for ATP. This is further supported in fig. 4.2.5 by ^{32}P-ATP phosphorylation studies. Additionally, HBCD is likely to have little effect on the affinity for Ca^{2+} binding to the E1 form and E2 form of the Ca^{2+}-ATPase (fig. 4.2.4.2). However, HBCD decreases the apparent affinity for Mg^{2+} binding and this could be due to the sites not being vacated by Ca^{2+} in this state, especially if trapped in an E2 state.

The E2-E1 equilibrium was therefore studied by monitoring Ca^{2+}-induced changes in the fluorescence intensity of FITC-labeled ATPase to assess whether HBCD affects the E2$\rightarrow$E1 transition of the ATPase. In fig.4.2.6 A, B&C the data shows that the addition Ca^{2+} to FITC-labeled ATPase at pH 6, 25 °C, leads to decrease in fluorescence at 7%, whereas in presence of 2 and 10µM HBCD, the fluorescence change was reduced to 4.6% and 0.8%, respectively. Observations have been reported previously which indicate that addition of Ca^{2+} to FITC-labeled ATPase at pH 6 caused the enzyme to undergo a transition from an E2 state to an E1 state **(Bilmen et al., 2002)**. These observations can be explained by the model, displayed in scheme1. These results suggest that HBCD binds to Ca^{2+}-ATPase and stabilizes it in the E2 form. This observation has also been reported for Bisphenol, NP and Tg in previous studies **(Brown et al., 1994; Michelangeli, et al., 1990; Toyoshima and Nomura, 2002)**.

Scheme 1



Scheme1: proposed model of inhibition by HBCD on Ca^{2+}-ATPase activity. E, Ca^{2+}-ATPase (enzyme). HBCD binds to Ca^{2+}-ATPase and stabilizes it in the E2 state and also causes some inhibition by changing the ATP binding affinity to the Ca^{2+}-ATPase. (steps are marked by **X**).

CHAPTER 5

SERCA over-expression in SH-SY5Y cells protects against HBCD-induced cytotoxicity

5.1- Introduction

Ca^{2+} is an essential signaling ion involved in range of cellular responses, prolonged or exaggerated elevation of intracellular [Ca^{2+}] levels may lead to a number of unwanted effects such as inappropriate activation of cell signaling pathways or even apoptosis. Ca^{2+} homeostasis is, however, vital to cell viability and function **(Ogunbayo and Michelangeli, 2007; David et al., 2000)**. To avoid such harmful effects, intracellular [Ca^{2+}] levels must be stringently controlled by Ca^{2+}-ATPases to maintain low cytosolic [Ca^{2+}] levels in cells, when they are at rest.

The fact that BFRs such as HBCD have been shown to induce cell death by increasing intracellular [Ca^{2+}] levels and inhibiting Ca^{2+}-ATPases, has led to an investigation of whether over-expressing SERCA within cultured SH-SY5Y cells provides protection against HBCD-induced cell death. Over-expressing SERCA would allow cells to overcome the cytotoxic effects of HBCD-induced by elevation of intracellular [Ca^{2+}] levels, thereby preventing cell death by reducing exaggerated increases in cytosolic [Ca^{2+}].

In this study, HBCD was tested in SH-SY5Y cells over-expressing SERCA1-EGFP to determine whether cytotoxic protection of these cells can be observed. At the time of this study SERCA1-EGFP was used, as it was the only SERCA isoform tagged with fluorescent GFP protein plasmid available. SERCA1-EGFP was used in order to help determine its expression levels with transfected cells.

5.2- Results

5.2.1- Over-expression of SERCA

Previous data suggest that the mechanism by which these compounds and related chemicals cause cell death is via inhibition of Ca^{2+} pumps **(Ogunbayo et al., 2007; Michelangeli et al., 2008)**. In the experiments described in this results chapter, an investigation was undertaken to over-express SERCA1-EGFP in order to see whether this could afford some degree of protection against treatment with HBCD. HBCD was used since it was shown to be the most potent BFR, at both killing SH-SY5Y cells and inhibiting SERCA Ca^{2+}- ATPase activity in these cells.

5.2.1.1- Restriction digest and gel electrophoresis of maxiprep

Figure 5.2.1.1 shows that the restriction digest of the plasmid (SERCA1-EGFP in pcDNA3.1 +) and confirmed that it was correct (lanes 3&4), DNA sequencing of this plasmid by Dr. Michelangeli's group also confirmed that it contained the SERCA1-EGFP gene.

5.2.1.2- Optimization of the transfection efficiency in cells SH-SY5Y

To determine optimal conditions for transfection of SH-SY5Y cells with the SERCA1-EGFP plasmid, the cells were grown in a 6-well tissue culture plates (until 40-50% confluent). Four concentrations of metafectene (transfection reagent) and DNA (SERCA1-EGFP plasmid were tested as shown in tables 5.2.1.1. Metafectene and plasmid DNA for each condition were mixed and incubated at 25 °C for 20 min to allow the formation of the DNA-metafectene complex. Media from the wells was replaced with DMEM without FBS and antibiotics and the metafectene-DNA mixture was added to the medium. Cells were incubated for 24h at 37 °C and 5% CO_2. The medium was then exchanged with DMEM full media with FBS and antibiotics and cells were grown for an additional 24h; transfection was complete after 48 h. Finally, the expression of SERCA1-EGFP within the cells was analyzed with a Nikon TS100F microscope in epi-fluorescence mode by counting the number of fluorescent cells from an average of the two repeats for each transfection condition, and this was used to calculate percentage of transfection efficiency (% of SH-SY5Y cells transfected with plasmid DNA). The pictures of optimal conditions for transfection is shown in figure 5.2.1.2

Figure 5.2.1.1

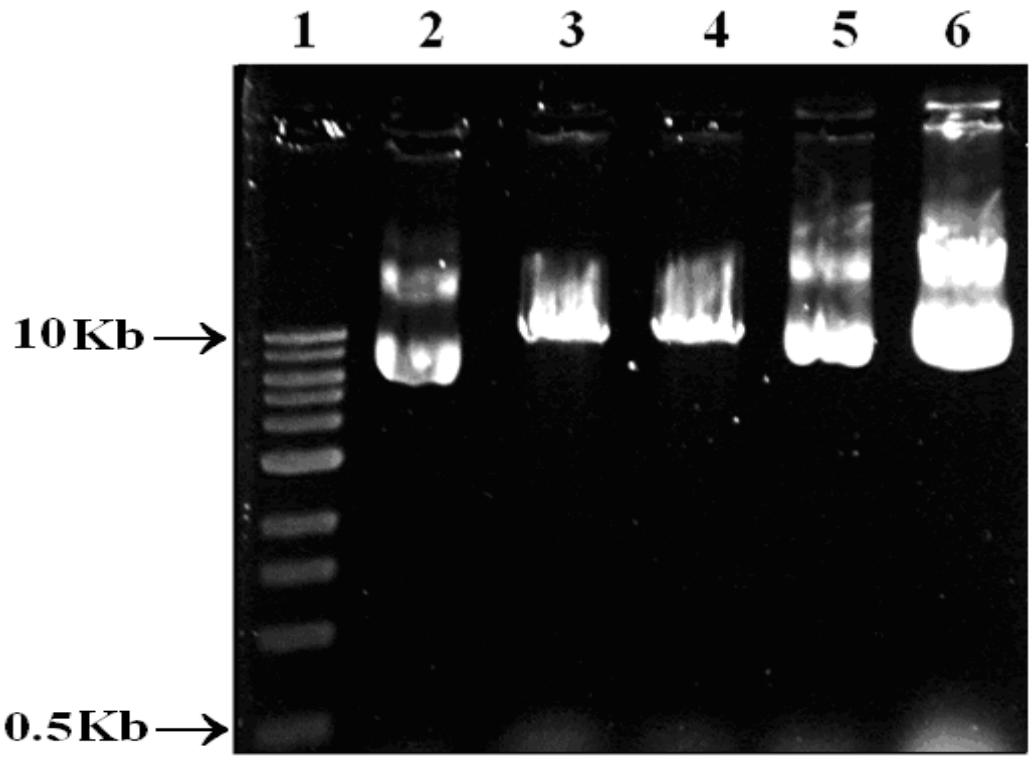


Figure 5.2.1.1; restriction digest and gel electrophoresis of plasmid containing the SERCA1-EGFP gene. A restriction digest of the plasmid DNA obtained from plasmid maxiprep Kit (Sigma). Three repeats were carried out corresponding to three overnight cultures. Samples cut with NdeI were loaded into lanes 3 (diluent 1:5) and 4 (diluent 1:10), while, uncut plasmid DNA samples (not cut with NdeI) were loaded into lanes 2 (diluent 1:5), 5 (diluent 1:10), and 6 undiluent). Uncut samples produced two bands as expected. Since NdeI only cuts once, electrophoresis of the cut plasmid DNA produced one band at ~ 9 Kb, corresponding to the fact that pcDNA3.1 (+) containing an EGFP-tagged SERCA1 sequence is approximately 9.428 bp. This suggests that the plasmid DNA obtained is the correct plasmid containing SERCA1-EGFP gene. A 10 Kb DNA marker was loaded into lane 1. This plasmid was also confirmed by DNA sequencing (undertaken by the Dr. Michelangeli's group).

Figure 5.2.1.2

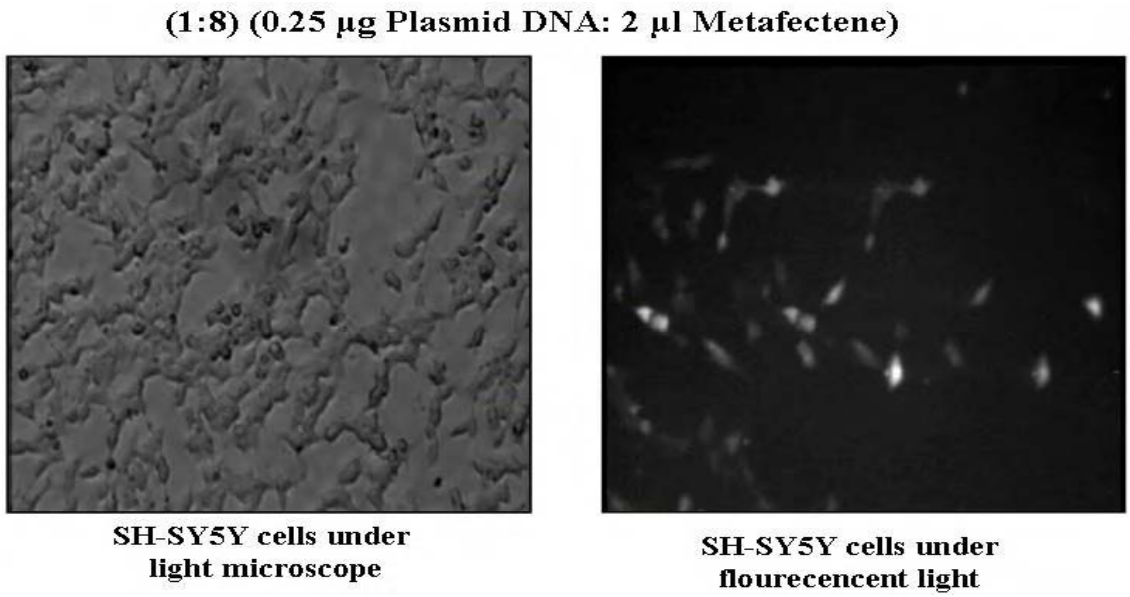


Figure 5.2.1.2; optimal conditions for transfection in SH-SY5Y human neuroblastoma cells. The optimum transfection condition (1:8) (0.25 µg Plasmid DNA: 2 µl metafectene). SH-SY5Y cells were grown in a 6-well plate until ~ 40-50 % confluent. Cells were visualized under visible and fluorescent light and EGFP-fluorescent cells were used to calculate % transfection efficiency (% of SH-SY5Y cells transfected with plasmid DNA) from an average of two repeats. Fluorescent microscope pictures were taken 48 h after transfection. Cells were viewed photographed at about 200× magnification, using an Astrovid StellaCam-EX camera connected to a Hauppauge USB TV live video capture device for viewing on a PC.

Table 5.2.1.1

Table: 5.2.1.1 transfection-efficiency of SH-SY5Y cells using different amounts of metafectence and plasmid DNA

Plasmid DNA (µg) : Metafectene (µl)	1:2	1:4	1:6	1:8
Transfection efficiency (%)	12	15	29	44

5.2.1.3- Western blotting of SERCA1-EGFP in SH-SY5Y cells with anti-SERCA antibody (Y/1F4)

Figure 5.2.1.3; shows a Western blot of whole SH-SY5Y cells transfected SERCA1–EGFP. This blot confirms the presence of SERCA1-EGFP as it gaves a band of the correct size 142kDa using anti-SERCA antibody (Y/1F4). It also shows that the antibody can detect the endogenously present SERCA2b (97kDa) also present within these cells. The intensity of the SERCA1-ECFP band compared to the endogenous bands suggests that transfected cells are expressing about 35% more SERCA Ca^{2+} pumps than non-transfected SH-SY5Y cells.

Figure 5.2.1.3

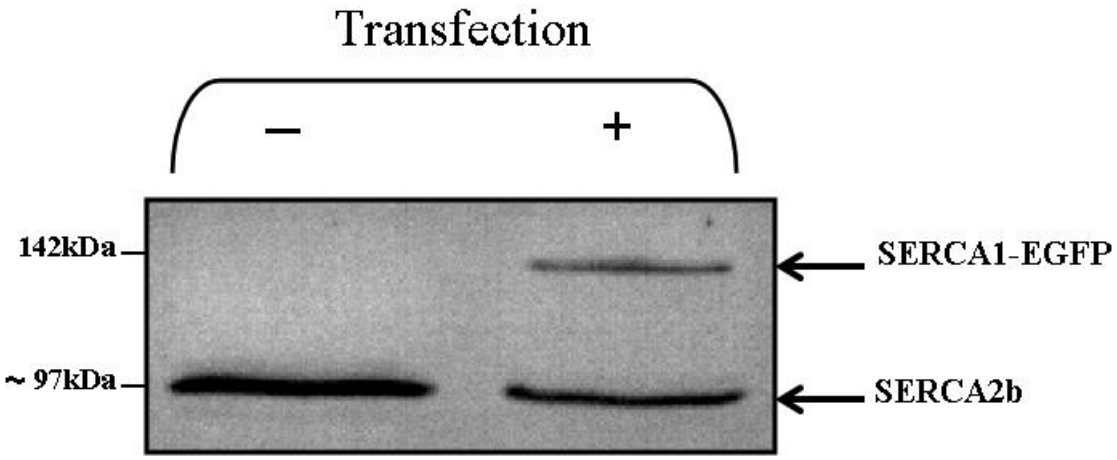


Figure 5.2.1.3; Western blotting of SERCA in SH-SY5Y cells (30 µg) was resolved on a 7.5% SDS-PAGE gel before being transferred onto nitrocellulose. The nitrocellulose was incubated with anti-SERCA (Y/1F4) antibody diluted at 1 in 500 for 1.5 h at room temperature and then with a secondary anti-mouse IgG conjugated to alkaline phosphates, for 1 h at room temperature, according to the supplier's instructions. The blot was incubated for 5-10 min with the BCIP/NBT ((5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolume): Sigma, UK) and two positive bands were detected within transfected cells, compared to one band in cells not transfected. Blots were photographed on a Kodak digital science camera (DC120).

5.2.2- Measurement of cell viability of HBCD-treated cells in SERCA1-EGFP expressing cells

Since HBCD is able to induce cell death by causing a rise in $[Ca^{2+}]_i$ levels, partly by inhibition of SERCA activity, the over-expression of SERCA Ca^{2+} -ATPase was undertaken using SERCA1-EGFP to see if this protected against cell death via Ca^{2+}-dependent cytotoxicity. After 48 h from start of transfection, the cells were treated with HBCD for about 22-24 h. Cell viability was then determined by MTT assays and flow cytometry analysis.

5.2.2.1- MTT cell viability assays in non- transfected and transfected EGFP-tagged SERCA1 in SH-SY5Y cells

To observe whether over-expression of SERCA in cultured SH-SY5Y cells provided protection against HBCD-induced cell death, a MTT cell viability assay was carried out on the cells after transfection. Once the transfection was completed, the cells were treated with HBCD for 22-24 h over the same concentrations undertaken previously (see chapter 3). MTT assays were undertaken using the same method as before and % cell viability was calculated for each HBCD concentration used.

Figure 5.2.2.1 shows the dose-dependent affects of HBCD on SH-SY5Y cell viability which were ether non transfected (■) or transfected (▲) with EGFP-tagged SERCA1. Exposure to 1µM HBCD for 22-24h caused more than 25% cell death in non-transfected compared to only 12% cell death after transfection. At 30 µM HBCD again there were non viable cells compared to control cells. The calculated LC_{50} in untransfected cells for HBCD was 5 ± 0.8µM, while in transfected cells more HBCD was required to cause 50% cell death (i.e. LC_{50} = 15 ± 1.4 µM).

Figure 5.2.2.1

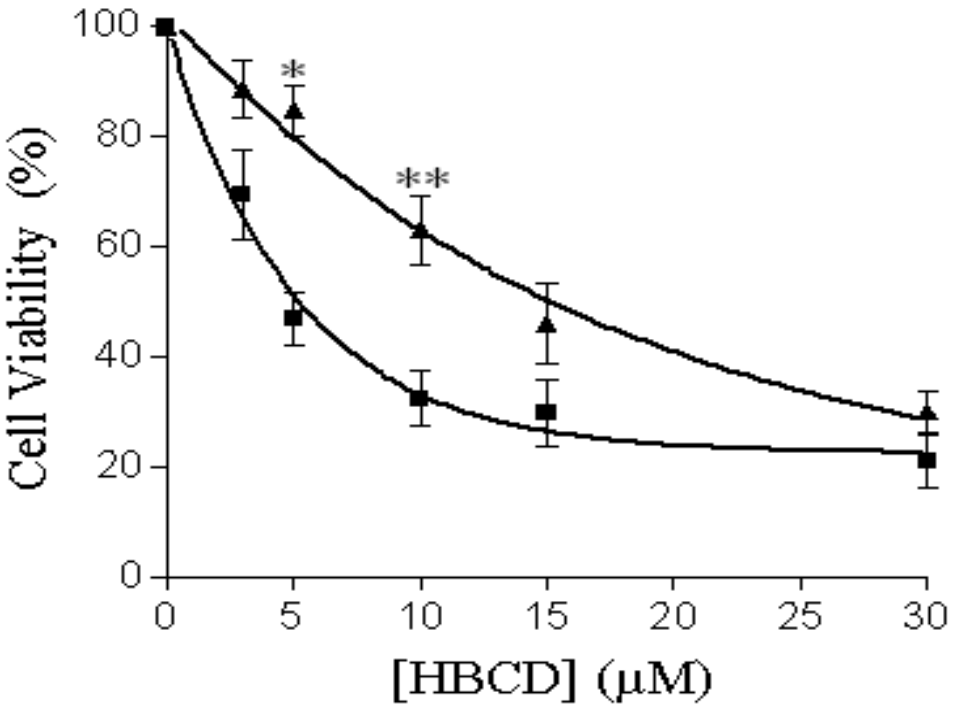


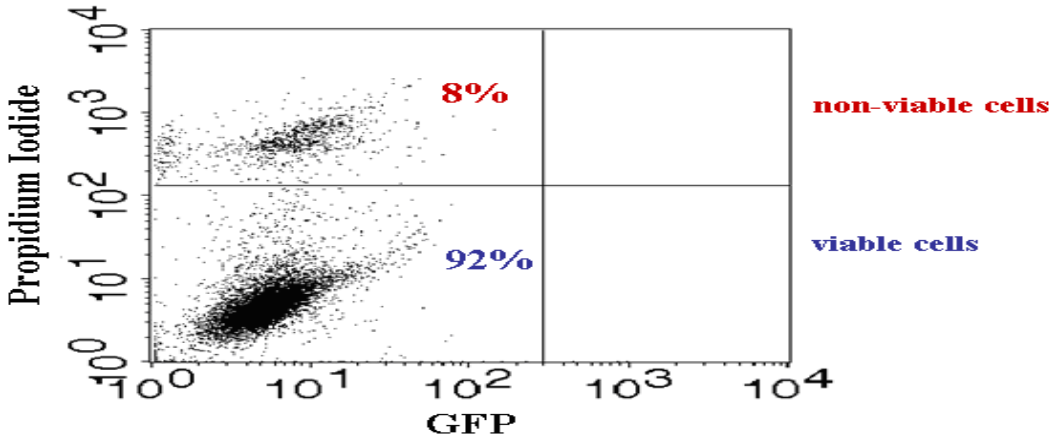
Figure 5.2.2.1; effects of HBCD on SH-SY5Y cells viability in the absence or presence of SERCA1-EGFP. SH-SY5Y cells were grown in 24-well tissue culture plates in 1ml of fresh complete medium and incubated at 37 °C in a CO_2 incubator until they were at a confluence of 40-50%. The optimum transfection condition (1:8) (0.25 µg plasmid DNA: 2 µl metafectene) was used to over-express SERCA1-EGFP in these SH-SY5Y cells. They were mixed and incubated at room temperature for 20 min. After 48 h for transfection to take place the cells were treated with HBCD for 22-24 h. Cell viability was determined by MTT assay, non-transfected cells (■) and transfected cells (▲). The data points represent the mean ± SD of 3-5 determinations. (* $p \leq 0.05$, ** $p < 0.01$, compared to their corresponding values in non-transfected cells).

5.2.2.2- Flow cytometry analysis

Figure 5.2.2.2A shows that cells that have been not transfected or treated with HBCD are mainly viable (92%) when assessed with propidium iodide (PI) staining and flow cytometry analysis. In figure 5.2.2.2B, the SH-SY5Y cells have been transfected with SERCA1-EGFP and treated with 3µM HBCD. The flow cytometry analysis shows 4-subpopulations of cells which is consistent with 2-subpopulations of cells which are not transfected and therefore show low levels of GFP fluorescence. One of these populations has low levels of PI fluorescence and therefore contains viable cells, while the other has higher levels of PI fluorescence, which indicate they are non-viable. The percentages of cells within these 2-subpopulations were similar at 17% and 14%, respectively. This would indicate that in cells which were not transfected, 3µM HBCD was causing approximately half the numbers of cells to die and consistent with previous findings. Of the 2-subpopulations which showed high levels of GFP fluorescence, about 63% of the cells were viable (i.e. low levels of PI) and only 6% of the cells were non-viable. This result would indicate that in SH-SY5Y cells over-expressing SERCA1-EGFP only about 10% of the cells are non-viable in the presence of 3µM HBCD.

Figure 5.2.2.2

(A)



(B)

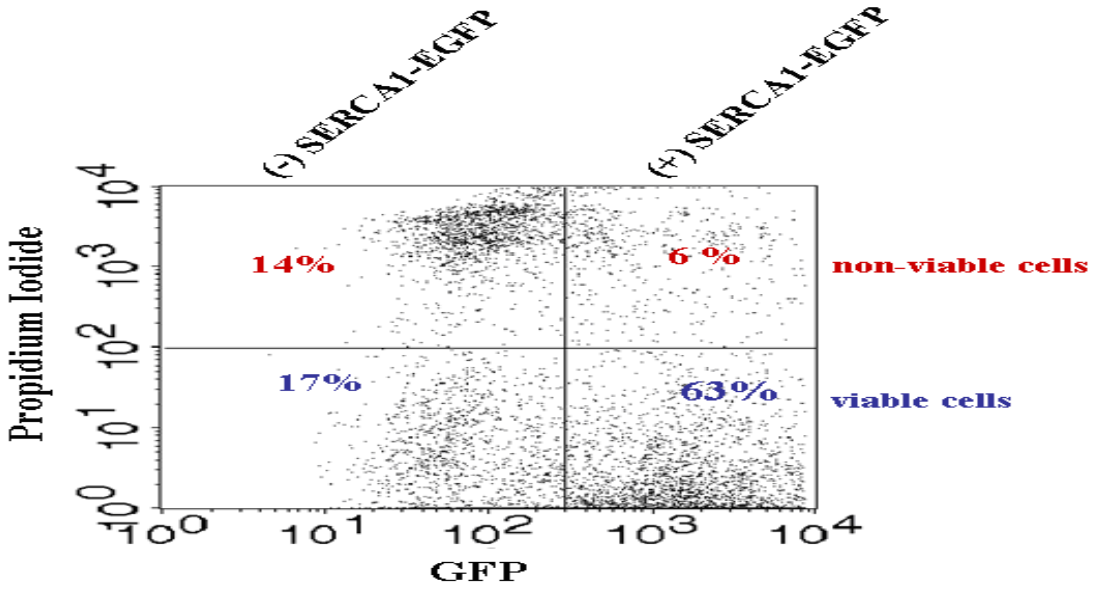


Figure 5.2.2.2 (A-B); flow cytometry measurement of cell viability in SH-SY5Y cells treated with HBCD. (A) dot plot of SH-SY5Y cells (control) treated with 2% DMSO, while (B) the cells were treated with 3 µM HBCD for 22-24h. Cells over-expressing SERCA1-EGFP, from FACS analysis indicates a significant transfection rate of 66% for the SH-SY5Y cell populations. Values represent the mean ± the SD of between 4 determinations. The quadrants were placed to distinguish the 4- subpopulations of cells i.e. untransfected (viable and non viable) and transfected (viable and non viable).

5.2.3- Measurements of Ca^{2+}-ATPase activity in SH-SY5Y cells either transfected or non-transfected with SERCA1–EGFP

In order to assess whether over-expressing SERCA1-EGFP actually produces functional SERCA protein, Ca^{2+}-ATPase activity were undertaken in SERCA1-EGFP in transfected SH-SY5Y cell microsomes and compared to SH-SY5Y cell microsomes that were not transfected. In SH-SY5Y transfected cells membranes it can clearly be seen that the Ca^{2+}-ATPase activity is substantially increased (by 32%) (fig. 5.2.3).

Figure 5.2.3; also shows the effects different concentration HBCD on untransfected and transfected SH-SY5Y microsomal Ca^{2+}-dependent ATPase activity. As can be seen the SERCA activity in the SERCA1-EGFP transfected cell membranes is consistently higher than control cell membranes upon 3µM HBCD concentrations.

Figure 5.2.3

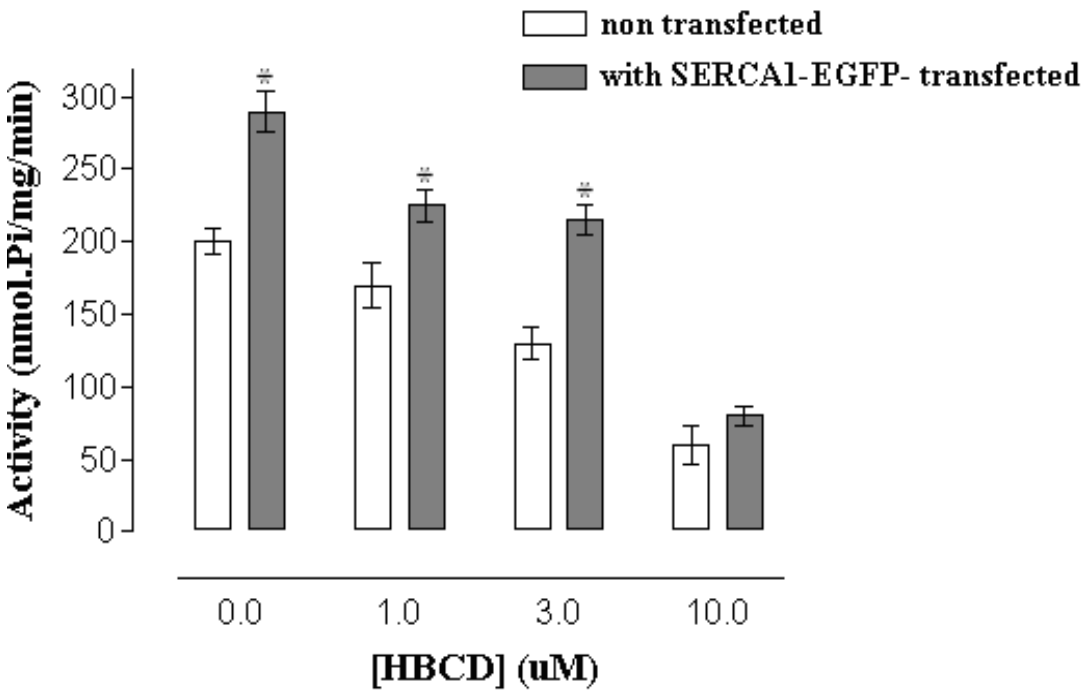


Figure 5.2.3; the effects different [HBCD] on untransfected or transfected SERCA1-EGFP SH-SY5Y microsomal Ca^{2+}-dependent ATPase activity. SH-SY5Y cell membranes microsomal were isolated from both SERCA1–EGFP transfected and non transfected cells. The Ca^{2+}-dependent ATPase activities were measured using phosphate liberation assay at pH 7.0, 37 °C. The values represent the mean ± SD of between 3 or 4 determinations. (*) indicates values that are statically significant ($p \leq 0.05$) compared to non-transfected cell membranes.

5.3- Discussion

The data presented in this study demonstrate that the commonly used BFRs, HBCD is able to induce death of cultured SH-SY5Y cells by presumably elevating [Ca^{2+}] levels and especially by inhibiting SERCA Ca^{2+}-ATPase activity. Over-expression of SERCA Ca^{2+}-ATPase activity in SH-SY5Y cells provides protection to some degree against HBCD-induced cell death, further supporting the fact that some BFRs induce cell death by a Ca^{2+}-dependent mechanism **(Ogunbayo et al., 2008)** and Ca^{2+} dys-regulation is involved in the cytotoxicity of these compounds **(Reistad et al., 2007)**.

SERCA Ca^{2+}-ATPase in SH-SY5Y cells would allow excess Ca^{2+} from the cytosol to move back in to the ER, thereby preventing HBCD-induced cell death by maintaining low cytosolic [Ca^{2+}] levels. This study has shown that cell death was reduced after SERCA transfection, when cells were exposed to low µM concentration of HBCD in comparison to non-transfection cells. Cell death was further reduced by 44% when cells were exposed to 3µM HBCD (fig.5.2.2.2B). Also the data in figure 5.2.2.1; demonstrates that if SH-SY5Y cells over-express SERCA, the LC_{50} for HBCD cytotoxicity dramatically changes from 5 ± 0.8µM in normal cells to 15 ± 1.4µM in cells over-expressing SERCA1-EGFP. Therefore over-expressing SERCA1-EGFP in SH-SY5Y cells does provide protection to some extent against HBCD-induced cell death. However, it is important to state that this protection was only apparent when cells were exposed to low µM concentrations of HBCD. Once a higher concentration of HBCD was reached, over-expressing SERCA1-EGFP in SH-SY5Y cells could no longer provide protection against HBCD-induced cell death, as indicated by the fact that little improvement in % cell viability could be seen after transfection at 10µM HBCD.

Previous studies showed that SERCA over-expression has a different effect on cultured cardiac cell **(Wu et al., 2004**). This study suggested that over-expression of SERCA in neonatal cardiomyocytes induces cell death **(O'Donnell et al., 2001; Wu et al., 2004)**, while SERCA over-expression in adult cardiomyocytes does not. In contrast, these data clearly suggest that over-expressing SERCA1 improved cell viability especially in cells exposed to certain cytotoxic chemicals such as HBCD. This study is the first experiment of this type to be performed.

Additional experiments are therefore required to investigate whether SERCA can also give protection against other BFRs and APs. In addition, other experiments should be undertaken to assess whether other types of Ca^{2+} pumps (i.e. different SERCA isoforms, PMCA or SPCA) can also give some protection against their cytotoxic pollutants.

CHAPTER 6

Amyloidogenic effects of brominated flame retardants and alkylphenols in SH-SY5Y cells

6.1- Introduction

There have been a number of studies to suggest that some persistent organic pollutants (POPS) such as brominated flame retardants (BFRs) and polychlorinated biphenyls (PCBs) are neurotoxic in nature. Animal studies have shown that exposure to environmental relevant concentrations of some of these pollutants can lead to impaired neurological function. Several rodent studies have shown that exposure to environmentally relevant concentrations of BFRs caused profound neurological disturbances in spontaneous behaviour and disrupted habituation in the adult animal **(Birnbaum and Staskal, 2004)**. Some as yet identified pollutants are also believed to be a risk factor in Alzheimer disease (AD) **(Dosunmu et al., 2007; Santibanez et al., 2007)**. Furthermore, AD causes progressive destruction of neurons within the brain leading to a loss of cognitive function and dementia. The most commonly observed histological biomarkers of AD, used to confirm diagnosis, are the presence of β-amyloid plaques (Aβ) and or neurofibrillary tangles (NFT) within the brain **(Shankar et al., 2008; Jack et al., 2010)**. The accumulation Aβ is the main cause of neuronal death **(Lesne et al., 2006; Shankar et al., 2008**). Aβ peptides are produced from the cleavage of amyloid precursor protein (APP) by β- and γ-secretases **(Selkoe et al., 1996)**. γ-Secretase inhibitors are currently being extensively studied as a compound to treat AD by reducing Aβ peptide levels in the brain **(Jack et al., 2010; Wu and Zhang, 2009; Panza et al., 2010)**

Of the BFRs studied, most attention has been placed on their effects upon neurological and behavioural development and little attention has been paid to the effects that these pollutants might cause to neurodegenerative diseases by causing neuronal cell death. In fact there is a correlation between the increased incidence of AD and industrialisation, where people are more likely to be exposed to higher levels of these pollutants. In this study focused on the potential neurodegenerative effects of some commonly used BFRs and APs by investigating their effects on APP cleavage and their ability to secrete β-amyloid peptide from SH-SY5Y human neuronal cells.

6.2- Results

6.2.1- Effects of BFRs and APs on the cleavage of Amyloid Precursor Protein (APP) and the secretion of βeta-amyloid peptide (Aβ) from human neuronal SH-SY5Y cells

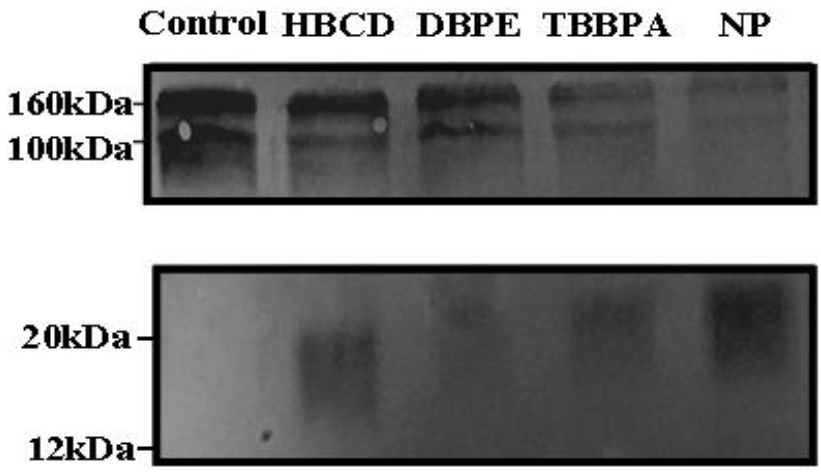
There appears to substantial evidence to indicate a link between disruption of Ca^{2+} homeostasis within neuronal cells and the initiation of AD **(Kato-Negishi, Kawahara, 2008; Celsi et al., 2009)**. One of the key molecular factors in AD is the formation of Aβ plaques which are neurotoxic. Aβ plaques are formed from the aggregation of soluble Aβ peptides which have been cleaved from the plasma membrane bound APP by the action of a number a secretase enzymes.

6.2.1.1- Immunoblotting of APP fragments

Initial studies were to investigate the potency of BFRs and APs to cause APP cleavage in neuronal SH-SY5Y cells. Figure 6.2.1.1 A-B; shows a number of Western blots of whole SH-SY5Y cells that had been pretreated with a number of BFRs and nonylphenol for 12 h. The blots were probed with an anti-APP antibody and in control cells the antibody cross reacted with proteins that were around 110kDa and 160kDa in size, which corresponded to intact APP **(Dovey et al., 2001; Shipley et al., 2005)**. Figure 6.2.1.1 A-B; also shows that upon treatment with HBCD, TBBPA, DBPE and NP, additional smaller protein bands were labelled by the antibody at molecular weights between about 12 to 24kDa. These bands probably correspond to partially cleaved APP and were not detected in control cell extracts. In figure 6.2.1.1B it can be seen that if the cells are co-treated with DAPT (30μM), which is a γ-secretase inhibitor, in addition to 3μM HBCD the 12-20kDa and the 24kDa bands becomes more intense. This would suggest a build up of the partially processed APP protein prior to the formation of soluble β-amyloid peptide by the action of γ-secretase inhibitor. This observation has also been shown in previous studies **(Dovey et al., 2001)**.

Figure 6.2.1.1

(A)



(B)

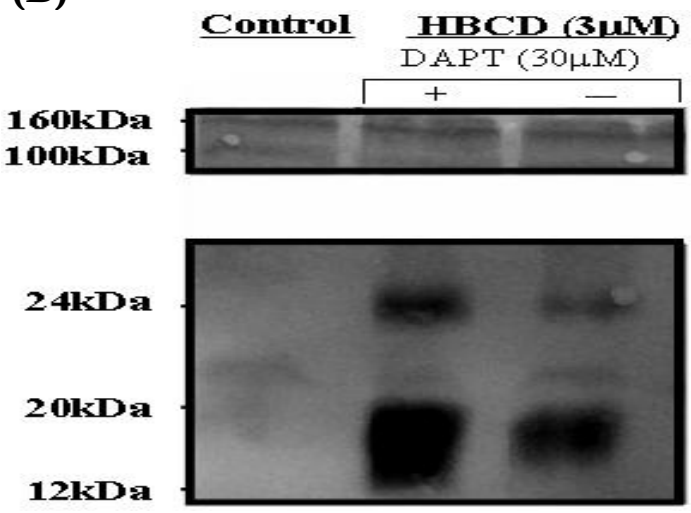


Figure 6.2.1.1; the effects of different types of BFRs and NP on APP processing in SH-SY5Y cells. (A) SH-SY5Y cells were either untreated (control), a also treated with 2% DMSO, or cells treated 10μM of HBCD, TBBPA, NP and DBPE dissolved in DMSO, then incubated for 12 h. Cell lysates were separated by SDS-PAGE on 8% polyacrylamide Tris/glycine gel, transferred into nitrocellulose membranes and immunostained using a β-Amyloid antibody (20.1: sc-53822, Santa Cruz). (B) shows the effects of 3μM HBCD in presence or absence of 30μM DAPT (γ-secretase inhibitor) on APP cleavage in SH-SY5Y cells. Blots are representative of 3 replicates.

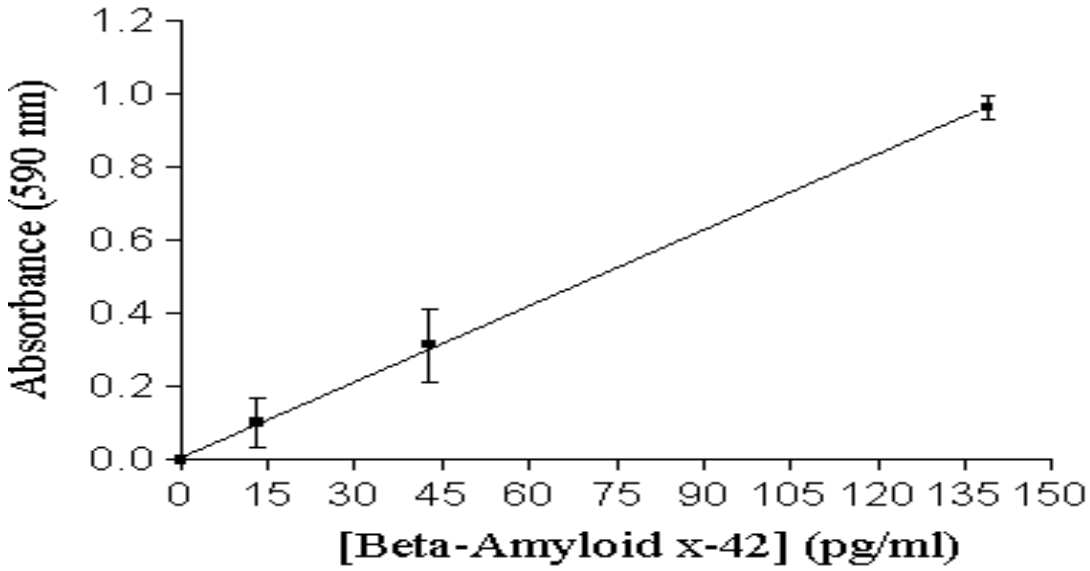
6.2.1.2- Measurements of Aβ peptide level by ELISA assay

In order to confirm that APP was cleaved to the Aβ peptide and transported out of the cell, the extracellular fluid was analysed and quantified by sandwich ELISA using a β-amyloid-42 antibody (as of the several Aβ peptides that are known to be formed, Aβ-42 is believed to be the most fibrillogenic and is thus more closely associated with AD).

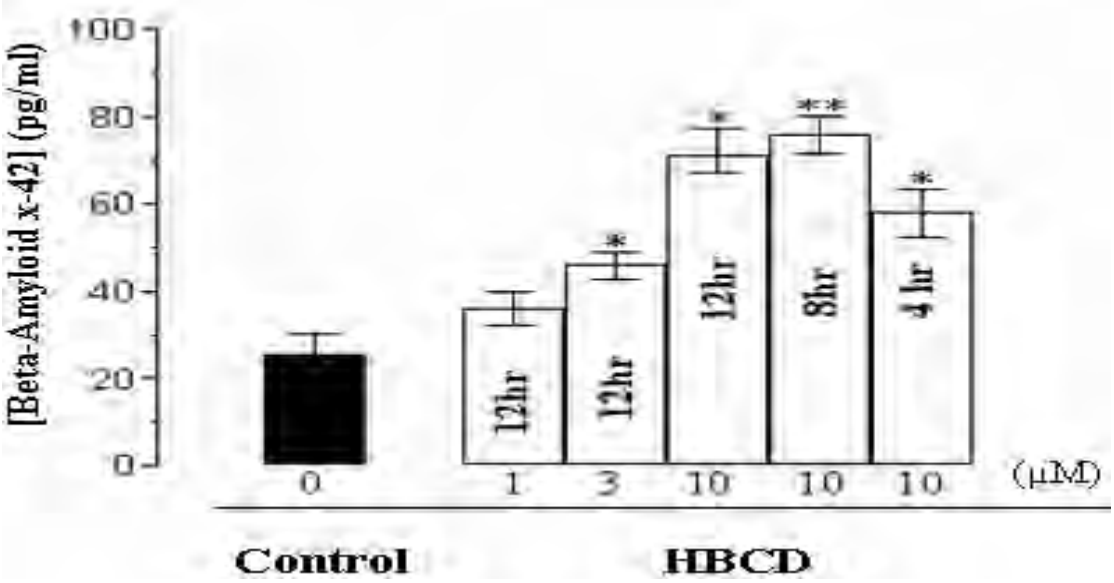
Figure 6.2.1.2 A; shows the calibration curve with Aβ x-42 standard peptide which was used to determine the concentration of Aβ x-42 of unknown samples. Figure 6.2.1.2B; shows that HBCD causes an increase in the extracellular presence of Aβ-42 in both a time dependent and dose-dependent fashion. Significant levels of Aβ-42 peptide were detected, compared to control, in cells exposed for 12h with 3μM HBCD and also increased in a time-dependent fashion. Figure 6.2.1.2C; shows the effects of TBBPA, NP and DBPE on secreted Aβ 1-42 levels from SH-SY5Y cells for 12 h. Secreted Aβ-42 levels from SH-SY5Y cells increased above those of control with 3 and 10μM of TBBPA and NP, while only 10μM of DBPE and BPA increased the Aβ-42 levels of above those of control (fig. 6.2.1.2C).

Figure 6.2.1.2

(A)



(B)



(C)

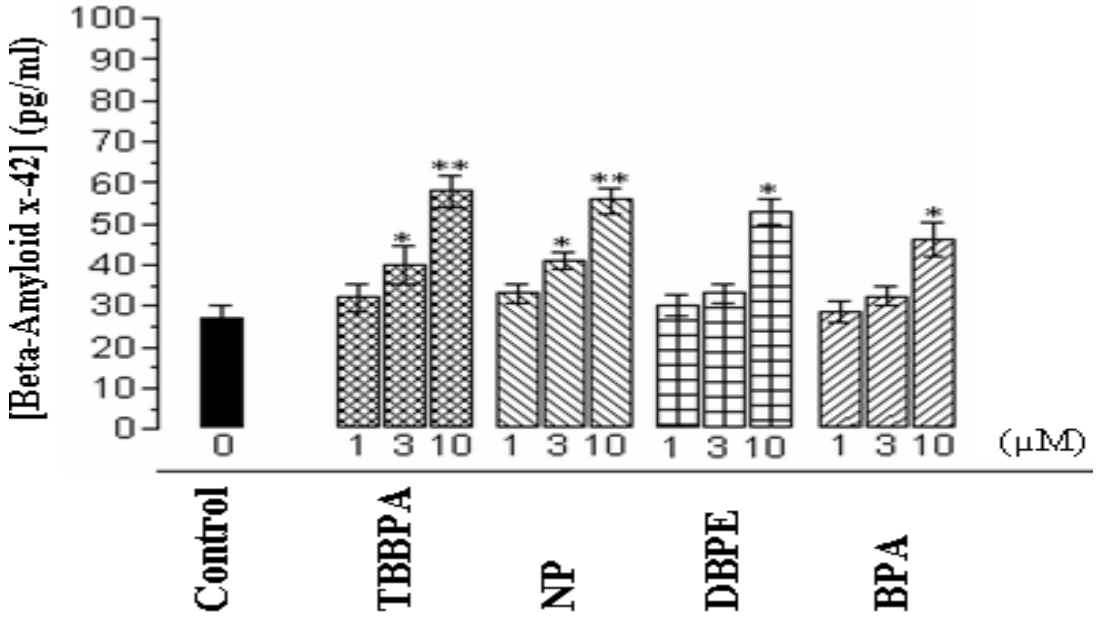


Figure 6.2.1.2; measurements of Aβ peptide level by ELISA. (A) the calibration curve with Aβ x-42 standard. (B) shows the effects of different HBCD concentration on secreted Aβ 1-42 levels from SH-SY5Y cells and also its time-dependent effects. (C) shows the effects of different concentrations of NP, TBBPA, PBPE and BPA on the secreted Aβ 1-42 levels from SH-SY5Y cells, when exposed for 12h. (* data points where the value were statistically significant from control; (a value * $P \leq 0.05$, ** $P \leq 0.001$)). Each bar represents the mean ± SD of 3 experiments.

6.3- Discussion

Over recent years much focus has been given to the potentially neurotoxic effects of BFRs with particular attention being paid to impaired neurological function. Several rodent studies have shown that gestational or neonatal expose to environmental relevant concentrations of BFRs caused effects on spontaneous behaviour and disrupted habituation in the adult animal and also had profound effects on learning and memory. Interestingly, memory loss and diminished learning skills were also reported in humans that had been occupationally exposed to high levels of dioxins (which are chemically related to PBDEs). AD is a neurological disorder characterized by an accumulation of Aβ1-42 plaques in the brain **(Glenner and Wong, 1984; Roher et al., 1993; Iwatsubo et al., 1994; Jin et al., 2004)**. Aβ is a protein fragment snipped via the amyloidogenic processing pathway by the β-secretase which generates a soluble N-terminal fragment (β-APPs), and a ~12 kDa C-terminal fragment of APP (CTF or C99). This remaining C99 fragment is then further cleaved to release the free 40 to 42 amino acid Aβ peptide by γ-secretase **(Citron et al., 1996)**.

The data in fig 6.2.1.1A; shows that levels of full length APP decline in human neuronal SH-SY5Y cells after treated with HBCD, TBBPA and NP. The amount of a C-terminal 12-24 kDa APP fragment which contains the Aβ sequence appears to increase in treated cells. This suggests that these compounds induced cleavage of APP and might lead to increased generation of Aβ. The data presented here shows that release Aβ 1-42 into culture medium was increased by exposure of SH-SY5Y cells to HBCD, TBBPA, NP, DBPE and BPA (see fig. 6.2.1.2A&B).

The process of directly reducing Aβ production is by the inhibition of the specific secretases. DAPT (γ-secretase inhibitor) was used to reduce Aβ levels in vivo and is being used as a potential treatment for AD **(Micchelli et al., 2003)**. Furthermore, our data also shows that SH-SY5Y cells exposed to 3μM HBCD in the presence of DAPT (30 μM) leads to increased levels of 12 to 24 kDa bands (Aβ is cleaved from ~12 kDa C-terminal fragment of APP), therefore reducing its ability to form Aβ 1-24 (fig.6.2.1.1B). These results suggest that all compounds tested at low concentrations are amyloidgenic and could be implicated in the development of AD.

There is substantial evidence to indicate that molecular lesions associated with AD are caused by disruption of Ca^{2+} signalling and mitochondrial function **(Ferreiro et al., 2008)**. Some of this evidence has been generated using cells expressing presenilins (which have been shown to be mutated in certain forms of genetically induced familial forms of AD). Presenilins (PSs) form part of the γ-secreatase complex. Mutant presenillins have been shown to affect store-operated Ca^{2+} entry into cells, increase the activity and or expression of intracellular Ca^{2+} channels such as the Ryanodine receptor (RyR) and Inositol 1,4,5-trisphosphate receptor (IP_3 R) **(Chan et al., 2000; Landman et al., 2006)**, and modulate the function of SERCA Ca^{2+} pumps **(Green et al., 2000; Small et al., 2010)**. Furthermore, it is believed that Aβ peptides form oligiomers within the plasma membrane of neurons and this then causes excessive Ca^{2+} influx into the cells. In fact one popular current idea in regards to neuronal cell death in AD is that of the 'ER Ca^{2+} overload hypothesis', whereby ER and possibly other Ca^{2+} stores become overfilled with Ca^{2+} leading to exaggerated increases in cytosolic [Ca^{2+}] levels. Although the effects of excess cytosolic Ca^{2+} can initially be subtle and have negligible pathological effects, with increasing age (or indeed toxicological assault), the Ca^{2+} homeostatic machinery it is known to become less effective in combating such changes leading to Ca^{2+}- and mitochondrial- induced neuronal cell death **(Hensley et al., 1994; Celsi et al., 2009)**. Moreover, Aβ has previously been shown to induce cytochrome c release from mitochondria and the formation of ROS **(Lee et al., 2005)** and also shown to activate of caspase-3 **(Kim et al., 2002)**. However, there is an urgent need to determine the extent to which common environmental pollutants are a risk factor for the development of AD and to characterise their mechanism of action.

A further study could assess whether cells expressing mutant PS, are more susceptible to cellular stress / cell death. The ratio of Aβ1-42/ Aβ1-40 production could be measured, which may prove to be a more informative indicator of the potency of these pollutants to cause neuronal cells to initiate Aβ plaque formation. It could also be determined whether these pollutants also affect Tau hyper-phosphorylation, as Tau proteins also play a central role in the pathogenesis of various neurodegenerative tauopathies, including AD.

CHAPTER 7

Overall Discussion

7.1- Overall Discussion

Some persistent organic pollutants (POPS) such as brominated flame retardants (BFRs) and alkylphenols (APs) are chemicals commonly found within the environment and have human health concerns. In-vitro studies have shown that a variety of the BFRs and APs are toxic at low micromolar concentrations for a range of cell types **(Reistad et al., 2007; 2005; Ogunbayo et al., 2008; Hughes et al., 2000; Michelangeli et al., 2008)**.

The data in this study show that HBCD was the most potent at inducing cell death in the SH-SY5Y human neuronal cells (see table 3.2.1 in chapter-3). The LC_{50} for HBCD were determined to be 2.7 ± 0.7μM, and was similar in potency to that observed by **Reistad et al., (2006)** (the LC_{50} value was estimated 3μM in cerebellar granule cells).

Furthermore, these results show that HBCD, TBBPA, NP can depolarize mitochondria (see fig.3.2.5.1A and fig.3.2.5.2 in chapter-3) and are able to generate measurable levels of ROS in SH-SYSY cells at low micromolar concentration, (an observation also seen in another study in these neuronal cells **(He et al., 2008)** and in Hep G2 cells **(Zhang et al., 2008)**). This would indicate that further experiments investigating the effects of these compounds on DNA damage should be undertaken.

HBCD, TBBPA and DBPE are shown to induce the activation of both caspase-8 and -9, resulting in the activation of caspase-3 (see fig.3.2.2A, B&C); while NP appeared to specifically activate caspase-9. The apoptotic pathway through mitochondria was further confirmed by cytochrome c release (see fig.3.2.3.4).

Some BFRs have been shown to disrupt Ca^{2+} homeostasis by increasing $[Ca^{2+}]_i$ in SH-SY5Y cells which can the then activate the death receptor which induces caspase-8. This results in activation of the pro-apoptotic Bid which enters the mitochondria and blocks the functioning of Bcl-2 resulting in the release of cytochrome c. This stimulates caspase-9 which then activates caspase-3 and leads to cell death **(He et al., 2009)**. These results, at least for HBCD, indicate it unlikely that caspase -9 is

activated via caspase-8 as shown in studies **(Sprick and Walczak, 2004; He et al., 2009)**, as the results with caspase inhibitors and the time-course of induction of caspase -8 and -9 activities, would likely preclude this. The most likely mechanism is that BFRs activate both intrinsic and extrinsic pathways of apoptosis independently.

Based on the Ca^{2+}-ATPase activity data from this study, HBCD, TBBPA and NP proved to be the most potent inhibitors of both SERCA1a (which is found in fast-twitch skeletal muscle) and SERCA2b (found in both cerebellular microsomes and SH-SY5Y cells). This is consistent with the previously reported effect of TBBPA on Ca^{2+} pump activity in both skeletal muscle SR membranes and cerebellular microsomes **(Ogunbayo et al., 2008)** and that reported for NP **(Michelangeli et al., 2008)**. Moreover, it appears that HBCD, TBBPA and NP inhibit SERCA1a better than SERCA2b. For the polybrominated diphenyl ethers (DBPE, PBPE, and OBPE), they were reasonably effective inhibitors of SERCA Ca^{2+} pumps (IC_{50}; between 30 to 110 µM), but there appeared to be little correlation between number of bromines and potency. All the other BFR's tested were poor SERCA inhibitors.

SERCA are involved in Ca^{2+} uptake into organelles that also contain RyR Ca^{2+} channels **(Michelangeli et al., 2005)**. SH-SY5Y neuronal cells contain two types of intracellular Ca^{2+} channels associated with SERCA Ca^{2+} pumps; IP_3-sensitive Ca^{2+} channels (IP_3R, type I) **(Wojcikiewicz, 1995, Mackrill et al., 1997)** and Ryanodine receptor Ca^{2+} channels (RyR, type 2) **(Mackrill et al., 1997)**. Data presented here shows that HBCD is able to inhibit Ca^{2+} uptake rate in SH-SY5Y microsomal membranes and also possibly activate the RyR in SR vesicles (which contains a RyR1, **Treves et al., 2005**) at low concentrations. The effects of TBBPA and NP were comparable to that which has been found for both TBBPA **(Ogunbayo et al., 2007; 2008)** and NP **(Michelangeli et al., 2008)**. However, further studies are required to fully confirm that these BFRs and NP are able to activate the RyR.

The catalysis and transport mechanism of SERCA involves alternation between two major conformational states, known as E1(which is a high affinity Ca^{2+} binding state) and E2 (which is a low affinity Ca^{2+} binding state) **(de Meis and Vianna, 1979; Danko et al., 2004)**. The transition between the two states (E1 and E2) is dependent

on a number of factors, including the presence of Ca^{2+} and Mg^{2+}, in addition to pH and temperature.

From the activity versus [ATP] (data in fig. 4.2.4.3); it shows that HBCD has a small effect on affinity for ATP. This is further supported in fig. 4.2.5 by ^{32}P-ATP phosphorylation studies. Additionally, HBCD is likely to have little effect on the affinity for Ca^{2+} binding to the E1 form and E2 form of the Ca^{2+}-ATPase (fig. 4.2.4.2). However, HBCD decreases the apparent affinity for Mg^{2+} binding and this could be due to the sites not being vacated by Ca^{2+} in this state, especially if trapped in an E2 state. The E2-E1 equilibrium was therefore studied by monitoring Ca^{2+}-induced changes in the fluorescence intensity of FITC-labelled ATPase to assess whether HBCD affects the E2→E1 transition of the ATPase. In fig.4.2.6 A, B&C the results suggest that HBCD binds to Ca^{2+}-ATPase and stabilizes it in the E2 form (see scheme-1 in chapter-4). This observation has also been reported for bisphenol, NP and Tg in previous studies **(Brown et al., 1994; Michelangeli, et al., 1990; Toyoshima and Nomura, 2002)**.

However, virtual modelling experiments suggest that HBCD can fit into the same pocket in the E2 form of SERCA as cyclopiazonic acid (CPA) **(Moncoq et al., 2007)** (see fig. 7.1). This is also suggesting that TBBPA and BHQ could bind to this in the SERCA **(Ogunbayo et al., 2007)**. It would be interesting to undertake such as modelling experiments with a range of BFRs and APs used in my work to determine whether all these molecules bind to the same site on SERCA.

The results of this research show that HBCD is able to induce death of cultured SH-SY5Y cells by presumably elevating [Ca^{2+}] levels and especially by inhibiting SERCA Ca^{2+}-ATPase activity. The results also shown that over-expression of SERCA Ca^{2+}-ATPase activity in SH-SY5Y cells provides protection to some degree against HBCD-induced cell death, further supporting the fact that some BFRs induce cell death by a Ca^{2+}-dependent mechanism **(Ogunbayo et al., 2008)** and that Ca^{2+} unregulation is involved in the cytotoxicity of this compound **(Reistad et al., 2007)**.

Figure 7.1

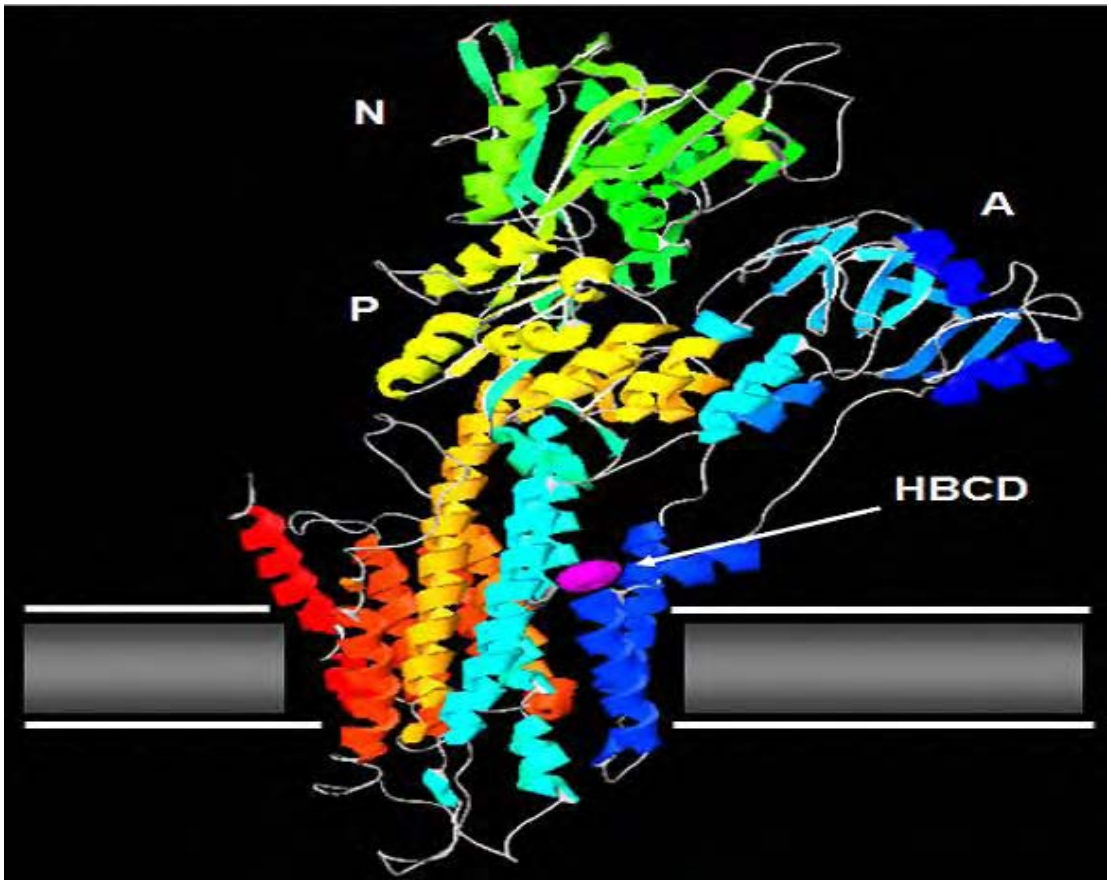


Figure 7.1: predicted binding sites for HBCD on E2 SERCA.

The three cytoplasmic domains of SERCA are colour coded accordingly: N (green), P (yellow), and A (blue). The SERCA structure was from the program Swiss Pdb-Viewer 3.7(file 2EAV). HBCD is shown as pink elliptic.

The results in this study showed that HBCD-induced cell death was reduced after SERCA over-expression, where cell death was reduced by 44% when cells were exposed to 3µM HBCD (fig.5.2.2.2B). Previous studies showed that SERCA over-expression has a different effect on cultured cardiac cell **(Wu et al., 2004**). This study suggested that over-expression of SERCA in neonatal cardiomyocytes induces cell death **(O'Donnell et al., 2001; Wu et al., 2004)**. These data clearly suggest that over-expressing SERCA1 improved cell viability especially in cells exposed to certain cytotoxic chemicals such as HBCD. This study is the first experiment of this type to be performed.

Additional experiments are therefore required to investigate whether SERCA can also give protection against other BFRs and APs. In addition, other experiments should be undertaken to assess whether other types of Ca^{2+} pumps (i.e. different SERCA isoforms, PMCA or SPCA) can also give some protection against their cytotoxic pollutants.

Over recent years much focus has been given to the potentially neurotoxic effects of BFRs with particular attention being paid to impaired neurological function. Several rodent studies have shown that gestational or neonatal expose to environmental relevant concentrations of BFRs caused effects on spontaneous behaviour and disrupted habituation in the adult animal and also had profound effects on learning and memory. Interestingly, memory loss and diminished learning skills were also reported in humans that had been occupationally exposed to high levels of dioxins (which are chemically related to PBDEs). AD is a neurological disorder characterized by an accumulation of Aβ1-42 plaques in the brain **(Glenner and Wong, 1984; Roher et al., 1993; Iwatsubo et al., 1994; Jin et al., 2004)**. Aβ is a protein fragment snipped via the amyloidogenic processing pathway by the β-secretase which generates a soluble N-terminal fragment (β-APPs), and a ~12 kDa C-terminal fragment of APP (CTF or C99). This remaining C99 fragment is then further cleaved to release the free 40 to 42 amino acid Aβ peptide by γ-secretase **(Citron et al., 1996)**.

The data in fig 6.2.1.1A; shows that levels of full length APP decline in human neuronal SH-SY5Y cells after treated with HBCD, TBBPA and NP. The amount of a

C-terminal 12-24 kDa APP fragment which contains the Aβ sequence appears to increase in treated cells. This suggests that these compounds induced cleavage of APP and might lead to increased generation of Aβ. The data presented here shows that release Aβ 1-42 into culture medium was increased by exposure of SH-SY5Y cells to HBCD, TBBPA, NP, DBPE and BPA (see fig. 6.2.1.2A&B).

The process of directly reducing Aβ production is by the inhibition of the specific secretases. DAPT (γ-secretase inhibitor) was used to reduce Aβ levels in vivo and is being used as a potential treatment for AD **(Micchelli et al., 2003)**. Furthermore, our data also shows that SH-SY5Y cells exposed to 3μM HBCD in the presence of DAPT (30 μM) leads to increased levels of 12 to 24 kDa bands (Aβ is cleaved from ~12 kDa C-terminal fragment of APP), therefore reducing its ability to form Aβ 1-24 (fig.6.2.1.1B). These results suggest that all compounds tested at low concentrations are amyloidgenic and could be implicated in the development of AD.

A further study could assess whether cells expressing mutant PS, are more susceptible to cellular stress / cell death. The ratio of Aβ1-42/ Aβ1-40 production could be measured, which may prove to be a more informative indicator of the potency of these pollutants to cause neuronal cells to initiate Aβ plaque formation. It could also be determined whether these pollutants also affect Tau hyper-phosphorylation, as Tau proteins also play a central role in the pathogenesis of various neurodegenerative tauopathies, including AD.

In summary, this study has been shown these pollutants (BFRs & APs) induce Ca^{2+} release from ER to by SERCA inhibition and RyR Ca^{2+} channel stimulation. Previous study by Dr. Michelangeli's group has shown that BFRs (TBBPA) inhibited the SERCA pump in addition to activating the RyR **(Ogunbayo et al., 2008)**. Increases in intracellular [Ca^{2+}] induce cleavage of APP which might the lead to increased generation of Aβ by activation of β & γ-secretases. Furthermore, increase intracellular [Ca^{2+}] cause mitochondrial dysfunction.

Aggregate Aβ outside the cell leads to the formation of amyloid plaques and this cause's oxidative stress by excessive Ca^{2+} entry through the plasma membrane which

then leads excessive Ca^{2+} influx in mitochondria. Ca^{2+} overload in the mitochondrial matrix can collapse mitochondrial membrane potential ($\Delta\psi$mit) and increase intracellular accumulation of ROS. Loss of $\Delta\psi$mit can also result from the opening of the mitochondrial permeability transition pore (PTP), resulting in the release of apoptogenic factors, including cytochrome c. Once in the cytosol, cytochrome c binds to the apoptosis-inducing factor (AIF) and activates caspase-9, which activates caspase-3 and leads to cell death (see fig. 7.2).

In conclusion, I feel that there is sufficient evidence from my research to indicate that both BFRs and APs could be potentially neurotoxic to humans and could also be implicated in neurodegenerative diseases. More research is, however, clearly required to be definitive.

Figure 7.2

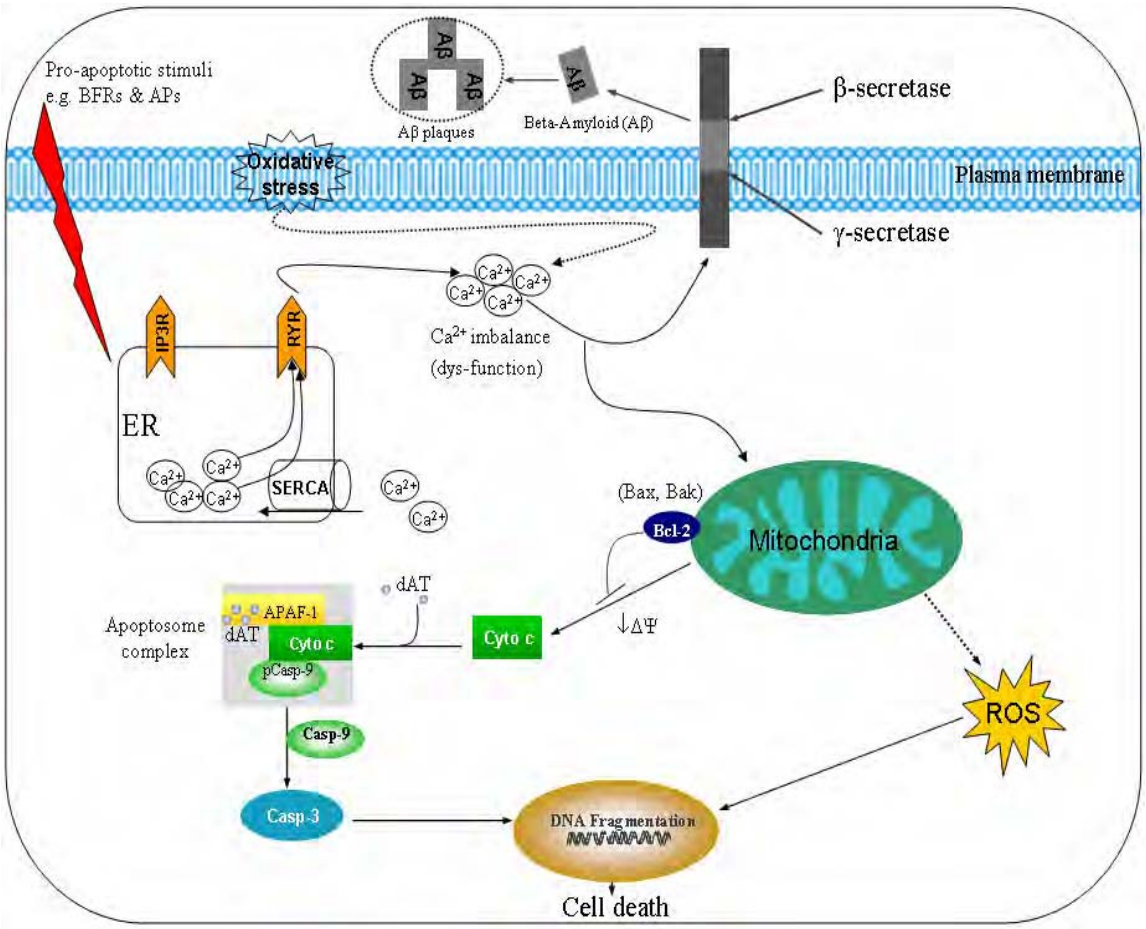


Figure 7.2: schematic showing summary of the study

CHAPTER 8

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