MICROGLIAL-NEURONAL INTERACTIONS

A thesis submitted for the Degree Doctor of Philosophy at the Faculty of Medicine, University of London.

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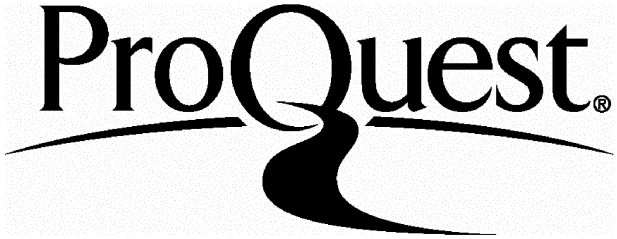

ABSTRACT

Recent evidence suggests that microglia, the resident macrophages of the CNS, play a role in neurodegeneration. In the Alzheimer's brain there are increased levels of both activated and apoptotic microglia. Chromogranin A (CGA), is a peptide upregulated in Alzheimer's disease and may be a physiological activator of microglia. The signalling pathways involved in the response to CGA were investigated in cell cultures of microglia as well as the subsequent effect of secreted factors on neuronal cultures. Following exposure to CGA, cultured microglia displayed apoptotic changes – pyknotic nuclei and DNA fragmentation. Activated microglia produced nitric oxide prior to a collapse in mitochondrial membrane potential ($\Delta\psi_m$) which could be blocked with cyclosporin A suggesting the involvement of the mitochondrial permeability transition. Nitric oxide synthase inhibitors prevented both the fall in $\Delta\psi_m$ and downstream apoptosis whereas the caspase inhibitor, YVAD-CHO, only prevented the latter. The translocation of cytochrome c from the inner mitochondrial membrane to cytosol was not necessary for caspase activation. CGA activated microglia also released glutamate, attenuation of which prevented the microglial death as did the metabotropic glutamate receptor antagonist, MSPG. Cultured neurones treated with conditioned medium from CGA activated microglia underwent apoptosis which was blocked by z-DEVD-fmk, a different class of caspase inhibitor. Neuronal death was in part mediated by activation of ionotropic glutamate receptors and also by proteins released from activated microglia. Exposure of neuronal cultures to cathepsin B, which was secreted by CGA activated microglia, resulted in neuronal apoptosis which could also be blocked with z-DEVD-fmk. Single cell fluorescence imaging of intracellular calcium ($[Ca^{2+}{}_i]$) in neurones revealed a small elevation in $[Ca^{2+}]_i$ following the addition of conditioned medium which may constitute an early step in the apoptotic pathway. The discovery of these distinct signalling pathways could allow for new therapeutic strategies in the treatment of disorders such as Alzheimer's disease.

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ACKNOWLEDGEMENTS

I would like to thank my Principal supervisor, Dr Jennifer Pocock, for her guidance and continual enthusiasm throughout the course of these studies. I am also grateful to Prof. Louise Cuzner. Thanks also to Gareth and Amanda for fun times both in the lab and at the Flyer and Goose, Anne for those all too frequent trips to "the dogs" and Sarah for her proof reading of this thesis.

ABBREVIATIONS

$\Delta\psi_m$:	mitochondrial membrane potential
$[Ca^{2+}]_i$:	intracellular free calcium
AD:	Alzheimer's disease
AIF:	apoptosis inducing factor
AMT-HCl:	2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride
ANT:	adenine nucleotide transporter
Apaf-1:	apoptosis protease activating factor
APC:	antigen presenting cells
AraC:	cytosine arabinoside
ATP:	adenosine 5'-triphosphate
BBB:	blood brain barrier
BDNF:	brain derived neurotrophic factor
bFGF:	basic fibroblast growth factor
BM:	physiological bathing medium
BSA:	bovine serum albumin
CGA:	chromogranin A
CGC:	cerebellar granule cell
CNQX:	6-cyano-7-nitroquinoxaline-2,3-dione
CNS:	central nervous system
CsA:	cyclosporin A
CSF:	cerebrospinal fluid
DAB:	diaminobenzidine tetrahydrochloride
DCG4:	(2S, 2'R, 3'R)-2-(2',3'-dicarboxycyclopropyl) glycine
DD:	death domain
DIV:	days *in vitro*
DMEM:	Dulbecco's modified eagle medium
EAA:	excitatory amino acid
EAE:	experimental allergic encephalomyelitis
ECL:	enhanced chemiluminescence
ECM:	extracellular matrix
EGF:	epidermal growth factor
EGTA:	ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetra acetic acid
FACS:	fluorescence activated cell sorting
FADD:	fas-associated death domain
Fak:	focal adhesion kinase
FCCP:	carbonyl cyanide p-(tri-fluoromethoxyl)phenylhydrazone
FCS:	foetal calf serum
Fura-2:	fura-2 acetoxymethyl ester
GalC:	galactocerebroside
GFAP:	glial fibrillary acidic protein
HRP:	horse radish peroxidase
ICE:	interleukin 1β converting enzyme
IF:	interferon
IL:	interleukin
iNOS:	inducible nitric oxide synthase
INT:	iodonitrotetrazolium
JC-1:	5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolcarbocyanine iodide

KGDHC: α-ketoglutarate dehydrogenase complex
LDH: lactate dehydrogenase
L-NNA: N^G-Nitro-L-arginine
LPS: lipopolysaccharide
MNCM: microglial non activated conditioned medium
MACM: microglial activated conditioned medium
MCSF: macrophage colony stimulating factor
MEM: modified eagle medium
mGluR: metabotropic glutamate receptor
MHC: major histocompatability complex
MK-801: (+)-5-methyl-10,11,-dihydro-5H-dinezo[a,d]cyclohepten-5,10-imine hydrogen maleate
MSPG: (RS)-α-methyl-4-sulphonophenylglycine
NGF: nerve growth factor
NMDA: N-methyl-D-aspartate
NO: nitric oxide
NOS: nitric oxide synthase
NSE: non specific esterase
PAF: platelet activating factor
PAGE: polyacrylamide gel electrophoresis
PARP: poly-(ADP-ribose)-polymerase
PBS: phosphate buffered saline
PD: Parkinson's disease
PDGF: platelet derived growth factor
PT: permeability transition
PVDF: polyvinylidene difluoride
ROS: reactive oxygen species
SBTI: soybean trypsin inhibitor
SNAP: S-nitrosopenicillamine
TGF: transforming growth factor
TMRE: tetramethylrhodamine ester
TNF: tumour necrosis factor
TTBS: tween tris buffered saline
TUNEL: terminal transferase in situ end labelling
VDAC: voltage dependent anion channel
YVAD-CHO: Tyr-Val-Ala-Asp-aldehyde
z-DEVD-fmk: benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone

1. INTRODUCTION

1. General introduction

The central nervous system (CNS) consists of a network of approximately 10^{12} neurones, the sole purpose of which is to transmit the impulses responsible for allowing an organism to communicate with the external environment (Alberts *et al.*, 1989). Since the neuronal cell plays such an active role, the other 90% of cells present in the CNS known as glia have been considered as the passive elements of the nervous system. More recently, evidence has been accumulating to suggest that the neurone-glia unit may determine neuronal function and signalling. Three principal types of glia exist; the star shaped astrocytes, the myelinating oligodendrocytes and microglia (Nissl, 1891 as cited in Barron, 1995). Microglia share many common characteristics with macrophages and have come to be considered the resident macrophage of the CNS (Del Rio-Hortega, 1932 as cited in Barron, 1995). In the normal brain, microglia assume a resting/ramified process bearing state but they have the ability to become activated and adopt a form that resembles the mature macrophage. During this transition, factors may be released which can affect neuronal signalling and survival. Such processes may underlie many neurodegenerative diseases. The aim of this study has been to investigate these microglial-neuronal interactions using a tissue culture system. Some of the important considerations are now discussed.

1.1. The CNS as an immunologically privileged site

The existence of the blood brain barrier (BBB) allows the exclusion of leukocytes from the brain and suggests that immune reactions are unlikely to occur in the CNS (Fabry *et al.*, 1994). However, recent evidence suggests that regions of the CNS can connect directly to lymph nodes thus allowing the possibility of CNS antigen presentation to take place peripherally (Cserr and Knopf, 1992). Furthermore, it is now known that

cells of CNS have the capacity to act as antigen-presenting cells (APC) since they express the Major Histocompatability Complex (MHC) antigens necessary to activate lymphocytes and produce an immune response. *In vitro* data suggested that astrocytes were responsible for this but the observations could not be confirmed *in vivo* (Fontana *et al.*, 1984). Instead it was found that microglia and perivascular cells expressed MHC *in vivo* (Matsumoto and Fujiwara, 1986; Hickey and Kimura, 1988; Streit *et al.*, 1989). In particular, a subset of microglia known as peri-vascular microglia are the more effective antigen presenters (Ford *et al.*, 1995) and these have been shown to be associated with blood vessels (Graeber *et al.*, 1989a) thereby providing a link between the CNS and external environment. In addition, the other parenchymal microglia can exhibit MHC II antigens but this expression is probably less constitutive and more inducible. This suppression of immune activity in the brain probably functions as a protective mechanism since such tissue is more sensitive to immune mediated cellular damage. For instance under pathological conditions MHC class II expression becomes apparent on microglia thus allowing them to become effective APCs (Fabry *et al.*, 1994) and parenchymal microglia are likely to mediate autoimmune diseases such as multiple sclerosis (Hayes *et al.*, 1987). Thus, since microglia have a similar immunological profile to other macrophages they have formed the basis of neuroimmunological studies.

1.1.2 Phenotypic characteristics of microgiia and their origin

Microglia were first decribed in the 1930's using a silver carbonate staining technique (Del Rio Hortega, 1932). With subsequent technical advances in microscopy the structure of this cell has been examined in detail. The resting or ramified microglia found in the adult brain have large dark oval shaped nuclei and minimal cytoplasmic

surround. At the surface there are many cell processes which become highly branched giving a distinct morphology to that of resting macrophages in other tissues. It is these resting microglia which constitute approximately 10% of the glial population in the mature brain (Vaughan and Peters, 1974). Though the numbers vary greatly in different regions of the brain they are more predominant in gray matter than white (Lawson *et al.*, 1990). Ameboid microglia are observed during development and may also be actively phagocytic. They have a more abundant transparent, cytoplasm containing numerous vacuoles and large dense bodies (lysosomes). The surface membrane emits many fine filopodia. These cells may also show increases in the expression of immuno-markers such as MHC antigens (Streit *et al.*, 1989). Also under conditions of brain injury the resting microglia may convert into a reactive macrophage in which the "activated" microglia shows upregulated levels of these macrophage proteins but do not undergo a change in morphology (Graeber *et al.*, 1988a). The opposite can also occur (see section 1.1.4). Thus the context in which the term "activated microglia" is used should always be defined.

The origin of ramified microglia has been highly debated (extensively reviewed in Cuadros and Navascues, 1998). It has been proposed that either microglia are derived from the neuroepithelium as with neurones and other glia cells, or they are of haematopoietic origin i.e. derived from monocytes which is more consistent with the mononuclear phagocyte system. There is evidence for both theories but it is the latter one that is more generally accepted. During development there is a decrease in blood-derived ameboid cells which correlates strongly with increases in ramified microglia (Ling and Wong, 1993). However, there has been mixed success in attempts to show this transition in adult brains. There may be a low turnover rate of ramified microglia

(Hickey *et al.*, 1992) with as few as 10% of the cells derived from bone marrow transplants (De Groot *et al.*, 1992) whereas other reports showed a continuing influx of genetically tagged haematopoietic cells into the brain (Eglitis and Mezey, 1997). In contrast, *in vitro* studies show that microglia and astroglia may have a common progenitor cell and thus microglia may be of neuroectodermal origin (Fedoroff *et al.*, 1997). Despite this, microglia contain most known macrophage specific antigens (Flaris *et al.*, 1993). Furthermore, there is a subset of microglia known as peri-vascular microglia that are associated with blood vessels (Graeber *et al.*, 1989a) and these are regularly replaced with cells from the bone marrow (Hickey and Kimura, 1988; Hickey *et al.*, 1992).

1.1.3 Microglial cell markers

Initial studies by Rio-Hortega used a silver staining technique to identify microglia which is now known to be non specific. Thus techniques have been developed in an attempt to selectively identify microglia but it is impossible to distinguish between macrophages and microglia. Instead only quantitative differences in the expression of antigenic markers between these different cell populations can be made. These can be examined using fluorescence activated cell sorting (FACS). For instance, microglia have been shown to have immunophenotypes which are $CD45^{low}CD11b/c^{+}$ and other macrophages are $CD45^{high}CD11b/c^{+}$ (Sedgwick *et al.*, 1991; Ford *et al.*, 1995). Microglia can be visualised in culture or sections using either immunohistochemistry, enzymatic labelling or lectin binding techniques. Monoclonal antibody staining is the most commonly used but there is still no antibody that can distinguish between monocytes/macrophages and microglia (Williams *et al.*, 1992). Furthermore, there are reports of differences in the labelling of microglia from the neonate versus adult CNS

(Tsuchihasi *et al.*, 1981; Hume *et al.*, 1983). This may be due to the activation states of the cells. Since many antibodies are raised to macrophage specific antigens they are likely to preferentially label, active, ameboid microglia with only faint staining of the ramified forms e.g. ED1-3 (Dijkstra *et al.*, 1985; Graeber *et al.*, 1989a). However, microglia have been shown to express CR3 complement receptors throughout development and this allows the identification of resting cells in rat (OX-42), mouse (MAC-1) and human (EBM11) (Graeber *et al.*, 1989a; Matsumoto *et al.*, 1985; Hayes *et al.*, 1987). The F4/80 antibody will also stain both ameboid and ramified microglia (Perry *et al.*, 1985). Additionally, antibodies directed against Fc receptor may also be useful in identifying microglia (Perry *et al.*, 1985).

Marker	Ligand/Monoclonal Antibody	Microglial form labelled
Silver carbonate	Not specific	Ramified and Ameboid
Enzyme histochemistry	Non-specific esterase (NSE)	Ameboid
Sugar/glycan receptors	Lectins I-B_4 and RCA-1	Ramified and Ameboid
Monoclonal antibody against		
97 Kd cytoplasmic antigens	ED1(rat)	Ameboid
Unknown membrane antigen	ED2, ED3 (rat)	Ameboid
Complement receptor (CR1)	Ebm11 (human)	Ramified and Ameboid
Complement receptor (CR3)	OX-42 (rat), MAC-1 (mouse)	Ramified and Ameboid
Unknown antigen	F4/80 (mouse)	Ramified and Ameboid
Fc receptor	2.4G2 (mouse), MAb10.1(human)	Ramified and Ameboid

Table 1.1.3.1 Cellular markers for microglia

Non-specific esterase (NSE) activity is absent in resting cells but associated with ameboid microglia (Hayes *et al.*, 1987). However, this marker should be used with caution when identifying cultured microglia as it is a characteristic of blood monocytes (see section 1.1.5). A number of lectins including *Griffonia simplicifolia* I-B_4 isolectin

which do not bind to neurones or other glial cells can also be used to label all microglial cell morphologies (Streit and Kreutzberg, 1987). Nevertheless since they are directed against D-galactose they will also stain macrophages. In conclusion, the presence of many macrophage specific antigens in microglia suggests a common origin but it may also be the result of the similar phagocytic role of these cells functioning in specific areas (Thomas, 1990).

1.1.4 The Facial Nerve Lesion to study microglial-neuronal interactions

Activation of microglia following CNS injury occurs as the result of signalling molecules released by dying neurones. Evidence from ischaemic lesions suggests that neuronal damage does not have to be lethal to elicit a microglia response since the penumbric region contains surviving neurones in association with reactive glial cells (Morioka *et al.*, 1993). Once activated, microglia may secrete either toxic or neurotrophic factors depending on the nature of the injury. During irreversible injury microglia could produce neurotoxic molecules to kill the neurones in a rapid manner whereas they may produce neurotrophins in situations where recovery is possible. Such reactions have been studied *in vivo*.

An *in vivo* microglial reaction can be elicited following disruption to the blood brain barrier. However, in this situation it is impossible to distinguish the events mediated by resident microglia and those occurring as the result of macrophages derived from the bloodstream. Thus, a model known as facial nerve transection is used, in which neuronal damage is evoked at a site distant to the region of injury (Streit *et al.*, 1988) thus overcoming the problem of damage caused by surgery. It is characterised by proliferation of resident microglia during which time they begin to express or up-

regulate the macrophage antigens of CR3 receptors and MHC and also the intermediate filament protein vimentin (Graeber *et al.*, 1988a and b; Streit *et al.*, 1989). They increase the secretion of a number of cytokines (Frei *et al.*, 1988; 1991). Activated microglia then surround the neurone and remove nerve endings from the cell body thus freeing the cell from any other neural input. The majority of the neurones survive and regrow their axons suggesting that this is a beneficial stage (Blinzinger and Kreutzberg, 1968). It may be that neuronal-microglial contact is needed for transfer of neurotrophic factors. Despite activation of the microglia they do not become phagocytic in this model. However if the motor neurone is induced to degenerate by addition of a toxic lectin from *Ricinus communis*, the microglia become more rounded and have phagocytic activity clearing neuronal debris (Streit and Kreutzberg, 1988). This step can be blocked by the addition of anti-metabolites such as adriamycin and cytosine arabinoside (AraC) (Graeber *et al.*, 1989c; Svensson and Aldskogius, 1993) suggesting that it is the resident microglia that become phagocytic rather than any invading macrophages. It is probable that this phagocytic phase is a late stage in the microglial activation cascade and is unnecessary for axon stripping.

There are differences in how microglia respond to neuronal damage in the developing and mature CNS. The intensity of microglial activation is lower after neonatal injury with correspondingly reduced MHC expression (Milligan *et al.*, 1991; Morioka and Streit, 1991). This may be due to differences in the immunological markers found at this stage in microglial development (Ling *et al.*, 1990) but also to the fact that the microglia are likely to be responding to apoptotic rather than necrotic neuronal cell death (Ashwell *et al.*, 1989). Apoptosis occurs after nerve transection in the new-born animal as neurones are deprived of vital neurotrophins. Microglia respond to changes in cell surface receptors and require specific cell contact rather than responding to

diffusible factors released lytically by dying adult neurones (Savill *et al.*, 1993). In this instance microglia show minimal proliferation and the inflammatory response is limited.

Thus activation of the microglia is not a simple one step process but occurs in a graded fashion involving multiple signals (Kreutzberg, 1996). It was originally proposed that microglia would progress from a ramified resting morphology through reactive phenotype to the phagocytic stage but this may also involve an intermediate step during which microglia become hyper-ramified, a morphology often observed in the aged brain (Streit *et al.*, 1988). Each stage occurs in a defined period of time with associated biochemical changes i.e. cell enlargement (hypertrophy) and cytokine production is an early event within 24 h whereas proliferation and upregulation of MHC II antigens occurs over a time scale of weeks suggesting different factors are required to signal each stage. Determining the nature of these molecules and their regulation of microglial activation may hold the key to controlling the CNS immune response. Many candidate factors have been hypothesised from studies of cultured microglia.

1.1.5 Isolation and cell culture of microglia

The signal transduction pathways and molecules involved in microglial activation can be studied by using *in vitro* models of microglia. Microglia may be isolated from other cells of the CNS in a number of ways following mechanical and/or enzymatic disruption of brain tissue. After plating, the purity of the culture can be monitored using immunocytochemical markers such as the aforementioned macrophage specific monoclonal antibodies along with those to detect cells expressing glial fibrillary acidic protein (GFAP) and galactocerebroside (GalC) respective indicators of astrocytes and oligodendrocytes, the most likely contaminants of microglial cultures (Debus *et al.*,

1983; Benjamins *et al.*, 1987). Any method of isolation can be subject to contamination from circulating monocytes which cannot be distinguished from microglia with antibodies. One way of reducing such contamination is to perfuse the animal prior to removal of the brain. The initial step in any of the techniques is dissection of the tissue and subsequent removal of the meninges and any visible blood vessels. It may be first necessary to mechanically disrupt the tissue. Next a mixture of enzymes, trypsin and collagenase is used digest the tissue and free the cells, and DNase to prevent the solution becoming gelatinous. Alternatively gentle homogenisation can be used to free the cells, overcoming the need for enzymatic disruption to surface membrane molecules. Next the microglia need to be separated from the mixed population of cell types and three methods have been reported for this.

The original method described, exploits differences in the adherence of glial cells to tissue culture flasks (McCarthy and de Vellis, 1980). Simply the suspension is plated onto flasks and the glia allowed to proliferate for 7 days. The loosely attached microglia along with oligodendrocytes are then shaken off leaving behind astrocytes. This mixture of cells is then transferred to new flasks and using different rotation speeds the oligodendrocytes removed leaving only microglia attached to the flasks. These can then be removed by trypsinisation and re-plated. The cultures are typically maintained at 37°C in an atmosphere of 5%CO_2/95% air. Each shaking stage needs be monitored immunocytochemically to ensure the correct population of cells detaches. This is especially important when using cells from different stages of development since foetal microglia are less adherent and adult microglia more adherent than astrocytes. The disadvantage with this technique is the length of time it takes to obtain the microglia (2-3 weeks) and the fact that they have been exposed to factors released from other glial

cells meaning the phenotype may not be a true reflection of cells freshly isolated. Furthermore with numerous stages involved the yields obtained may be as low as 10% of the total microglial population.

Other methods use discontinuous Percoll gradients to separate the microglia from the mixed glia suspension (Sedgwick *et al.*, 1991; Havenith *et al.*, 1998). Cells may need to be isolated from myelin on an initial gradient and then microglia can be separated from other cells by using different density layers of Percoll. The cell yield from this technique is approximately 5 x 10^5/rat brain. Finally the last method takes advantage of the fact that microglia express the Fc receptor. Mixed glia can be incubated with erythrocytes to generate rosetted microglia. These are then separated from myelin and other cells by centrifugation through a single layer of Percoll and the microglia freed from erythrocytes using a protocol of hypotonic lysis (Hayes *et al.*, 1988). Cell yields are similar to those for the discontinuous gradient method. All three techniques have the inherent problem that they result in some activation of these cells. However, once in culture, microglia adopt the ramified and ameboid morphological forms seen *in vivo*. Thus *in vitro* studies can be used to investigate the functions of microglia.

1.1.6 Microglial activators and the channels and receptors involved in microglial function

Cultured microglial are seen to undergo the morphological change from resting state to ameboid form when exposed to a number of factors. The most favoured activator for studying microglia *in vitro* is lipopolysaccharide (LPS). However, whether this functions as an endogenous activator is debatable since this molecule would not be present in situtions of injury without infection. Thus other potential microglial activators and the receptors/channels involved in the activation process are reviewed.

1.1.6.1 Cytokines

Like other cells of the immune system, microglia can respond to cytokines. These soluble polypeptides include interleukin (IL), interferon (IF), tumour necrosis factor (TNF), macrophage colony stimulating factor (M-CSF) and transforming growth factor (TGF). Each of these classes may contain two or more molecular forms. Such cytokines are generally believed to control the processes of microglial activation and proliferation (reviewed in Gehrmann *et al.*, 1995). Furthermore, they affect both the immunological profile (e.g. increased MHC class II molecule expression) and the functional characteristics of these cells. Interferon-γ and IL-4 are two T-cell derived cytokines that can up-regulate Fc receptors and MHC class II expression and also cause proliferation of microglia (Colton *et al.*, 1992). Colony stimulating factors (CSF) such as granulocyte macrophage-CSF (GMCSF) and macrophage-CSF (MCSF) may be released by astrocytes and promote microglial proliferation as well as maturation and differentiation during development (Giulian and Ingeman, 1988). These ligands bind to tyrosine kinase receptors which are upregulated in activated pre-mitotic microglia (Raivich and Kreutzberg, 1994). There is also evidence of an increase in receptor number in the facial nerve model where the presence of ligand influences the expression of surface adhesion molecules necessary for the microglial reaction. Other cytokines including TGF-β1 and IL-10 are known to down-regulate microglial activation. TGF-β1 may also be produced by microglia themselves and thus serve to control the activation process (Kiefer *et al.*, 1993).

1.1.6.2 Ion channels

Patch clamp studies on cultured microglia and tissue slices suggest that microglia express a large number of ion channels (reviewed in Eder, 1998). The best studied of these are the K^+ channels and it is now known that six different types exist including an inward rectifier, delayed rectifier, HERG-like, G-protein activated and voltage dependent and independent Ca^{2+}-activated K^+ channels. In comparision with macrophages, the inward and delayed rectifiers are likely to play a role in regulating membrane potential (Gallin and Sheehy, 1985). This is important for the functional state of the microglia since hyperpolarisation may be a requirement for initiating microglial functions such as proliferation and secretion of molecules. Microglia also contain H^+ channels which may be important in maintaining membrane potential and pH of the cell following phagocytosis (DeCoursey and Cherny, 1994; Henderson and Chappell, 1996). Cl^- channels are thought to play a role in proliferation induced by M-CSF treatment (Schlichter *et al.*, 1996) and the ramification of microglia (Eder *et al.*, 1998). Microglia also express Na^+ channels and voltage gated Ca^{2+} channels, the functions of which are unknown (Korotzer and Cotman, 1992; Colton *et al.*, 1994). Microglial ion channels may be modulated by stimulation with LPS and thus the pattern of expression may be dependent on the functional state of the cell.

1.1.6.3 Microglial activators signalling through $[Ca^{2+}]_i$

Cultured microglia undergo a morphological change when treated with adenosine 5'-triphosphate (ATP) suggesting it could be a physiological modulator of activation (Ferrari *et al.*, 1997). ATP has long been known to act as neurotransmitter in the CNS by binding to purinoreceptors. These can either be coupled to adenylate cyclase or

phospholipase C (P_1 receptors) or ATP specific (P_2 receptors) which may be either ionotropic (P_{2x} and P_{2z}) or metabotropic (P_{2y}, P_{2u}, P_{2t}, and P_{2d}) (Zimmermann, 1994; Burnstock and Wood, 1996). Evidence exists that immune cells such as microglia also contain the ionotropic receptors (Walz *et al.*, 1993; Haas *et al.*, 1996) and they are responsible for the direct influx of Ca^{2+} into the cell following ATP treatment (Ferrari *et al.*, 1996). The activation of these receptors leads to microglial apoptosis (Ferrari *et al.*, 1997). In contrast the activation of metabotropic receptors probably instigates $InsP_3$ induced Ca^{2+} release from internal pools and it has been suggested might result in capacitative Ca^{2+} entry (Moller *et al.*, 1997a).

Another activator of microglia is platelet activating factor (PAF). Originally identified for its action on platelets (Benveniste *et al.*, 1979) it has a diverse role in the CNS (Feuerstein *et al.*, 1990; Bazan, 1994). Specific receptors for this molecule have been found in both primary cultures of microglia (Mori *et al.*, 1996) and immortalised microglial cells (Rhigi *et al.*, 1995). PAF treated microglia release arachidonic acid, suggesting this might be an important physiological activator of microglia (Mori *et al.*, 1996). Furthermore, PAF can induce $[Ca^{2+}]_i$ elevation in these cells through both internal Ca^{2+} release and Ca^{2+} influx. Other factors such as endothelin and complement have been shown to cause changes in intracellular Ca^{2+} in microglia but whether they are involved in the transition from a resting phenotype to active state remains to be determined. Endothelin receptors were first reported in astroglia (Hosli and Hosli, 1991) but have more recently been studied in microglia (Moller *et al.*, 1997b). Both endothelin 1 and endothelin 3 cause an increase in intracellular Ca^{2+} and probably work through the ET_B receptor since BQ788 can block their effects. These endothelin receptors may play a role in pathology especially during glial proliferation and have been implicated in Alzheimer's disease (Zhang *et al.*, 1994). The complement

fragments C5a and C3a trigger a transient rise in $[Ca^{2+}]_i$ (Moller *et al.*, 1997a) which probably arises from an initial stimulation of the metabotropic receptor followed by release of Ca^{2+} from internal stores and consequent capacitative influx. Also, recently glutamate has been shown to have an effect on microglia acting through the metabotropic receptors (Biber *et al.*, 1999). The peptides β-amyloid and chromogranin A (CGA) can cause changes in Ca^{2+} and thus proposed as activators of microglia (Silei *et al.*, 1999; Taupenot *et al.*, 1996). Since CGA was used throughout the course of these studies it will now be reviewed in greater detail.

1.1.7 Chromogranin A, activation of microglia and role in disease states

Chromogranin A (CGA) was first discovered in the catecholamine containing chromaffin granules of the adrenal medulla but has subsequently been found to exist in the secretory, large dense core vesicles of neuronal and neuroendocrine cells (Eiden *et al.*, 1987). There is a complex mechanism controlling the transcription of the CGA encoding gene (located on chromosome 14) thus restricting its expression to these cells (Wu *et al.*, 1991; Nolan *et al.*, 1995). The primary structure of the protein has been resolved and indicates a very hydrophilic protein with an abundance of charged amino acids (Benedum *et al.*, 1986; Helman *et al.*, 1988). The corresponding molecular weight is 48-52 kDa but this may be proteolytically cleaved to smaller peptides, some of which have biological activity. Cleavage is likely to occur at highly conserved regions where there are two or more adjacent, basic, amino acids (Benedum *et al.*, 1986; Helman *et al.*, 1988). A number of functions for CGA in the neuroendocrine system have been proposed. It may act as the precursor for pancreastatin, a peptide which inhibits insulin and glucagon secretion from the endocrine pancreas (Tatemoto *et al.*, 1986). The entire peptide sequence of pancreastatin is found in CGA and is bordered

by sites for proteolytic cleavage (Iacangelo *et al.*, 1988). Other peptide derivatives of CGA have been found, including vasostatin (Aardal *et al.*, 1993) and chromostatin (Galindo *et al.*, 1991) suggesting processing of CGA to be important. In addition, CGA may exert a regulatory function within the neuroendocrine cell, directing peptides to defined secretory pathways (Seidah *et al.*, 1987).

Using immunochemical technqiues it has been shown that CGA is widely expressed throughout the CNS and found in a variety of both neurones and glia (O'Connor *et al.*, 1984; Munoz *et al.*, 1990). Detection of CGA mRNA in various brain regions, with a particularly high expression in pyramidal neurones of the human cortex has further confirmed these findings (Mahata *et al.*, 1991). The glycosylated form of CGA is more widespread in the CNS than the periperhal neuroendrocine system suggesting that CGA may have distinct functions in the different systems (Schober *et al.*, 1989). For instance, it is known that CGA can directly influence synaptic activity in the dopinergic system (Brudzynski and Munoz, 1994). However, secreted CGA may also play a role in neuropathology. CGA can be detected in the cerebrospinal fluid originating from the CNS (O'Connor *et al.*, 1993). In this study, it was observed that CGA levels were stongly decreased in the CSF of Parkinson's disease (PD) patients but were unaffected in Alzheimer's disease (AD). In contrast, other studies show there are changes in the levels and processing of CGA in the CSF from AD (Sekiya *et al.*, 1994; Blennow *et al.*, 1995) and increases in CGA from PD patients (Eder *et al.*, 1998). Thus CGA may be important in these diseases. In the case of PD this is further suggested by the fact that CGA is found concentrated in the Lewy bodies of the substantia nigra (Nishimura *et al.*, 1994). CGA has also become a focus of AD reseach since it can accumulate in the senile plaques (Munoz, 1991). Furthermore, since there is synaptic pathology in AD one could speculate that CGA might be responsible (Lassmann *et al.*, 1992). This could

be a direct effect or mediated through effects on other cell types. In particular, increased numbers of activated microglia are seen in AD (see section 1.1.9.1) and recently it has been shown that CGA can activate microglia in culture, resulting in the release of NO (Taupenot *et al.*, 1996). Furthermore during the course of these PhD studies, the same group showed that conditioned medium from CGA activated microglia could cause cell death in neuronal cultures (Ciesielski-Treska *et al.*, 1998). However, the nature of the molecules responsible was not resolved. Thus CGA provides a novel, physiological activator of microglia that can be used to study microglial-neuronal interactions.

1.1.8 Microglia secrete mediators of cell function and survival

Treatment of cultured microglia with inflammatory stimuli has allowed insights into the nature of some of the molecules released by activated microglia and their role in mediating neuronal survival and function. A number of candidate factors are discussed below and the relevance of the *in vitro* observations is correlated with those seen *in vivo*.

1.1.8.1 Nitric oxide

Nitric oxide (NO) is an inorganic free radical gas generated from L-arginine through an enzymatic reaction catalysed by nitric oxide synthase (NOS). Three isoforms of the enzyme exist, two of which are constitutive (cNOS or type I NOS) and found in neurones (nNOS) or endothelial cells (eNOS) and one which is inducible (iNOS or type II NOS) and expressed in numerous cell types including macrophages and microglia (Nathan, 1992; Vincent, 1994). As the name suggests iNOS is only expressed only after exposure of the cells to factors such as cytokines and the release of NO is independent

of Ca^{2+} in contrast to cNOS (Nathan, 1992; Vincent, 1994). The NO produced by cNOS is involved in the regulation of blood pressure and neurotransmission (Lowenstein and Snyder, 1992) whereas that released by iNOS may play a role in cytotoxicity since levels remain elevated for long periods. NO can react with free radical species such as superoxide (O_2^-) to generate toxic molecules such as peroxynitrite. Microglia are probably the main contributors of NO in the CNS and this may be responsible for mediating neuronal degeneration occurring in ischaemic and neurodegenerative disorders (Dawson *et al.*, 1991; Boje and Arora, 1992; Chao *et al.*, 1992; Meda *et al.*, 1995). Expression of the iNOS protein in macrophage/microglia is upregulated in conditions such as EAE (Van Dam *et al.*, 1995) and increased NOS mRNA is associated with demyelinating regions in MS (DeGroot *et al.*, 1997). It may be that microglial released NO damages oligodendrocytes in these diseases (Merrill *et al.*, 1993). The relevance of NO in human disease remains controversial. Despite the fact that human macrophages express the iNOS gene (Reiling *et al.*, 1994) there are many reports showing that cultures of human microglial cells fail to release significant quantities of NO upon stimulation with cytokines (Lee *et al.*, 1993; Peterson *et al.*, 1994; Janabi *et al.*, 1996). This may be due to differences between the species in the signal transduction pathways mediating iNOS gene transcription. In addition to LPS and other cytokines, chromogranin A and β-amyloid protein can induce NO release from microglial cultures (Taupenot *et al.*, 1996; Meda *et al.*, 1995; Goodwin *et al.*, 1995). Finally it may be that the NO causes the release of other toxic molecules such as glutamate from microglia that may be responsible for neuronal death (Pellegrini-Giampietro *et al.*, 1990; Dawson *et al.*, 1991; Banati *et al.*, 1993).

1.1.8.2 Glutamate and NMDA receptor agonists

Glutamate is the principal excitatory neurotransmitter in the CNS and is toxic at high concentrations. This excitotoxicity may be involved in the neuronal damage observed in diseases such as Alzheimer's. Macrophages are able to produce large quantities of glutamate (Newsholme *et al.*, 1987) and therefore microglia may play a role in this process. Glutamate can be released through a low affinity Na^+ independent transporter, x_c^-, which is able to exchange extracellular cystine for intracellular glutamate (Watanabe and Bannai, 1987). Furthermore, in culture, microglial cells have been shown to release large quantities of glutamate by this mechanism and this can cause NMDA receptor mediated toxicity to neurones *in vitro* (Piani *et al.*, 1991; Lees *et al.*, 1993; Piani and Fontana, 1994; Klegeris *et al.*, 1997; Klegeris and McGeer, 1997). In addition, microglia may secrete other NMDA receptor agonists. Microglia release prostaglandins and arachidonic acid following treatment with LPS (Gebicke-Haerter *et al.*, 1989; Minghetti and Levi, 1995). Arachidonic acid has been shown to potentiate NMDA receptor currents and may thus contribute to neurotoxicity (Dumuis *et al.*, 1988) and prostaglandin levels are elevated in the CSF of patients with neurological disorders (Griffin *et al.*, 1994). Quinolinic acid levels are also increased in the CSF. This has been shown to be released from microglia (Espey *et al.*, 1997) and acts as an NMDA receptor agonist to induce cell death (Brew *et al.*, 1995). Furthermore, microglia treated with zymosan can secrete an unidentified heat stable, proteinase resistant molecule of low molecular weight (Giulian *et al.*, 1994). Whilst the neurotoxic action of this molecule can be blocked by the administration of NMDA receptor antagonists such as APV it appears to be functionally and structurally distinct from glutamate or other agonists such as quinolinic acid. Similar observations have been made in HIV-infected mononuclear cells (Giulian *et al.*, 1996).

1.1.8.3 Cytokines and other neurotoxic proteins

Microglia can secrete a number of cytokines which are able to induce apoptosis by binding to death ligands and inducing caspase activation (see section 1.2.4). TNF-α was initially identified for its toxic effect on tumour cells (Imamura *et al.*, 1987) but can cause damage to other cells. In culture microglia activated with LPS release a cytotoxic factor that is inactivated by anti-TNF-α antibody (Frei *et al.*, 1987) and it has subsequently been shown that TNF-α may kill oligodendrocytes *in vitro* (Merrill, 1992). This suggests that microglial derived TNF-α may play a role in demyelinating lesions. TNF-α also works in conjunction with IL-1 enhancing its production from macrophages thereby creating a cytokine cascade. IL-1 is produced by macrophages and microglia and is associated with many diseases of the CNS including Alzheimer's and multiple sclerosis (Morganti-Kossmann *et al.*, 1992). In the case of the latter, IL-1 acts to inhibit oligodendrocyte proliferation whilst TNF-α has a direct cytotoxic effect on the oligodendrocytes (Merrill, 1992). IL-1 released by microglia may also act as a astrocytic mitogen and stimulate reactive gliosis in brain trauma (Giulian *et al.*, 1986). Additionally, IL-6 and the anti-inflammatory cytokines, TGF-β and IL-10, are released by immunostimulated microglia (Kiefer *et al.*, 1993). Other molecules found in conditioned medium from activated microglia include elastase like proteases and urokinase plasminogen activator which are responsible for degradation of myelin basic protein in culture (Nakajima *et al.*, 1992). Microglia can also secrete metalloproteases (Qiu *et al.*, 1997) and the cysteine proteinase cathepsin B (Ryan *et al.*, 1995) which may have implications for neurodegeneration and other unidentified proteins which can induce neuronal apoptosis in culture (Flavin *et al.*, 1997).

1.1.8.4 Growth factors/neurotrophins

Besides the aforementioned cytotoxic molecules, *in vitro* studies suggest that microglia can secrete growth factors including platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin like growth factors and basic fibroblast growth factor (bFGF) which may enhance survival and neurite outgrowth of CNS neurones (Rappolee *et al.*, 1988; Shimojo *et al.*, 1991; Araujo and Cotman, 1992). Microglial conditioned medium has also been shown to stimulate myelination in culture (Nagata *et al.*, 1993). Activated microglia can also release nerve growth factor (NGF, Mallat *et al.*, 1989) which may be beneficial, though one study suggests it causes cell death via p75 in the developing retina (Frade and Barde, 1998). Different populations of microglia may exist expressing different members of the NGF family (Elkabes *et al.*, 1996). NT-3 can induce phagocytic activity in microglia and it has been proposed that the release of the neurotrophins, BDNF and NT-3, is responsible for a continuing cycle of microglial activation. Thus many of these growth factors may aid regeneration. In normal circumstances the CNS cannot regenerate following injury which is in part due to oligodendrocytes which produce molecules that inhibit nerve elongation. However, through the secretion of growth factors and the release of neurotoxic molecules such as NO that kill oligodendrocytes, regeneration may be possible (Thanos *et al.*, 1989).

In conclusion, data from *in vitro* experiments tend to indicate that when microglia are activated they are more likely to release cytotoxic than beneficial factors. This is likely to mirror the process of activation *in vivo* since the morphological changes that occur are similar. In addition to eliciting neuronal toxicity some of these factors may be responsible for causing microglial cell death. This may function to limit the degree of microglial activation. For instance, apoptotic microglia have been observed in post

mortem studies of Alzheimer's disease and HIV encephalitis (Lassmann *et al.*, 1995; Petito and Roberts, 1995). However, one recent study does suggest that cultured microglia may exist in a super-activated state compared with ameboid microglia *in vivo* and the authors advise caution in deciphering too much from cell culture models (Hurley *et al.*, 1999).

1.1.9 Role of microglia in disease states: *in vivo* observations

Activated microglia have been demonstrated in Alzheimer's disease (Itagaki *et al.*, 1988; McGeer *et al.*, 1988), Parkinson's disease (McGeer *et al.*, 1988), multiple sclerosis (Woodroofe *et al.*, 1986) and the animal model, EAE, (De Groot *et al.*, 1989; Matsumoto and Fujiwara, 1986), the dementia associated with AIDS (Dickson, 1986) and other diseases such as Pick's, Huntingdon's and amyotrophic lateral sclerosis (Banati and Graeber, 1994). In many instances the conclusion that microglia were activated in these conditions was assumed from the increased expression of MHC class II antigens normally only weakly expressed in ramified microglia. However, this observation should be treated with caution since neurological damage may produce non specific up-regulation of MHC. Nevertheless the selective involvement of microglia has been investigated in depth for a number of diseases. For instance multiple sclerosis is an autoimmune disease involving T-cell and B-cell activation. Through the secretion of cytokines, microglia have been proposed to enhance inappropriate antibody responses (reviewed in Ulvestad *et al.*, 1994). Similarly, microglia are selectively infected with the HIV virus and may thus contribute to the dementia associated with AIDS (reviewed in Gonzalez-Scarano and Baltuch, 1999). Microglial involvement in neurodegenerative diseases is complex but the basic mechanisms are illustrated by Alzheimers disease which is reviewed in the following section.

1.1.9.1 Alzheimer's disease

AD is a degenerative disorder with neuropathology spreading from the cerebral cortex to the hippocampus and amygdala (Braak *et al.*, 1994). It is the most common cause of dementia and affects 1 in 20 people over 65 years of age (Tienari *et al.*, 1996). AD is characterised histologically by neurofibrillary tangles and the formation of plaques, insoluble deposits composed of β-amyloid. The presence of activated microglia at the core of these plaques (Probst *et al.*, 1991) was initially thought of as secondary to neuronal damage but microglia may be the cause of neurotoxicity. Microglia found in regions of degenerative pathology in the AD brain express elevated levels of MHC class II antigen (McGeer *et al.*, 1987) and also show significant increases in the macrophage antigens, CD11a, CD11b, CD11c, leukocyte common antigen and Fc receptor (Akiyama and McGeer, 1990; Peress *et al.*, 1993; Eikelenboom *et al.*, 1994; Akiyama *et al.*, 1994). The presence of these receptors may be associated with their role in phagocytosis and actively phagocytosing microglia are found at the centre of senile plaques. Also it was originally proposed that the elevated MHC class II antigens serve as receptors for T-lymphocyte interactions (McGeer *et al.*, 1987). Furthermore, T-lymphocytes are known to release IL-2 and it has been reported that there are increased numbers of IL-2 receptors in the AD brain (Itagaki *et al.*, 1988). However, the classic T-cell mediated immune response is not always observed in the plaques of AD brain. Thus it has been proposed that microglia are there purely to phagocytose and remove the neurotoxic β-amyloid plaque. The presence of early proteins of the complement sequence in AD lesions (McGeer *et al.*, 1993) may serve to opsonize tissue in preparation for phagocytosis. β-amyloid may be internalised by microglia through the scavenger receptor or alternatively it could be degraded via secreted matrix

metalloprotease (Qiu *et al.*, 1997). In contrast to these studies suggesting that microglia are present to prevent further neurotoxicity, it has been suggested from cell culture studies that microglia may be cytotoxic. Synthetic β-amyloid can recruit and activate microglia (Bargon and Harmon, 1997; Meda *et al.*, 1995) and induce the secretion of cytotoxic molecules (Giulian *et al.*, 1996). Also senile plaques stimulate cultured microglia to release neurotoxins (Giulian *et al.*, 1995). Elevated levels of TNF-α are associated with AD (Filit *et al.*, 1991) and studies of the CSF from AD patients identified antibodies that bind to rat brain derived ameboid microglia (Ling *et al.*, 1992). Microglia can also produce and secrete β-amyloid (Wisniewski *et al.*, 1994) and may be under the influence of β-amyloid itself (Bitting *et al.*, 1996) suggesting that microglia may excacerbate neuronal damage induced by β-amyloid. Further evidence for the toxic role of microglia comes from clinical data which suggests that treatement with anti-inflammatory agents may reduce the progression of AD (McGeer *et al.*, 1996; Breitner, 1996). Thus suppression of microglial activation could provide a therapeutic opportunity in this and other neurodegenerative diseases.

1.1.10 Summary

The microglial cell has become recognised as an important component of the nervous system and shown to be one of the main players in brain injury and disease. As the resident macrophage of the CNS, they may respond to a variety of stimuli by proliferating, becoming active phagocytes and producing many biochemical mediators. By understanding the signalling pathways responsible from activation of microglia through to neuronal cell death, it may be possible to devise new therapeutic strategies to affect the survival of neurones following CNS insult. The next section reviews the

process of neuronal cell death focusing on the pathways of apoptosis which are becoming increasingly implicated in neurodegenerative diseases.

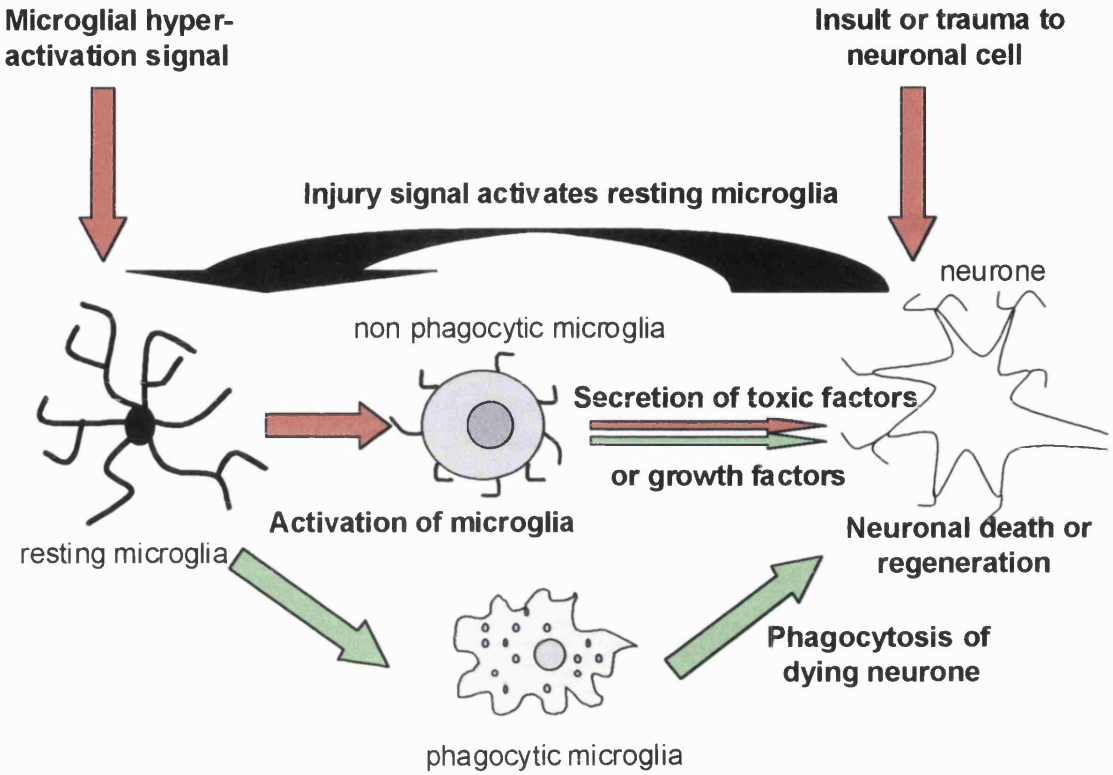


Fig. 1.1.10.1 A summary of microglial reactions. Dying neurones may signal to resting microglia which may either bring about a beneficial response (green arrows) or harmful response (red arrows). In disease states microglia may themselves become activated and release neurotoxic factors that contribute to degeneration.

1.2. Cell death in the CNS - Apoptosis or necrosis?

Two distinct forms of cell death, known as apoptosis or necrosis, may be responsible for the neurodegeneration observed in diseases of the CNS. The nature of the cell death may depend on the mechanism of induction. Typically necrosis is induced by lethal biochemical events such as abrupt anoxia or physical stimuli like rapid heating or exposure to detergents, which result in swelling and the lysis of the cells' contents to the intercellular milieu (Gores *et al.*, 1990). This may create a toxic environment for neighbouring cells and exacerbate tissue damage either directly or via the recruitment of

pro-inflammatory cytokines (Harmon *et al.*, 1991; Haslett, 1992). Thus in order to avoid inflammation and thereby limit injury, it may be preferential for cells in the CNS to undergo apoptosis during disposal of cells. In this instance, removal of cells involves a highly regulated, catabolic pathway during which the cell remains intact and generates sealed portions of cell known as apoptotic bodies (Thompson, 1995). *In vivo* observations and *in vitro* studies suggest that both apoptosis and necrosis can occur simultaneously in tissue exposed to the same insult. Thus it may be that the definition of apoptosis and necrosis only represent two extremes of possible pathways to cell death (Leist *et al.*, 1997a). An intense insult leads to cell lysis and this may activate an apoptotic "default" pathway to complete the cell death programme (Ankarcrona *et al.*, 1995). Whilst any one stimulus may activate the same intracellular pathways it appears that metabolic status determines whether a cell is directed towards apoptosis. Intracellular energy levels fall rapidly during necrosis but are maintained during apoptosis (Dypbukt *et al.*, 1994; Ankarcrona *et al.*, 1995). Cell culture work shows that a typical mediator of apoptosis such as staurosporine, can be induced to cause necrotic cell death through inhibition of ATP generation (Leist *et al.*, 1999). These observations might have significance in pathological conditions where apoptosis and necrosis coexist. Brain damage caused by an ischaemic insult results in a core region of necrotic death with an outer zone of apoptotic neurones in areas where energy depletion is likely to be less extreme (Li *et al.*, 1995a; Choi, 1996). Thus, cellular demise involves two stages; the initiation level which can activate identical intracellular pathways and the execution phase which is directed by energy requirements and may be dependent on the intensity of the initial insult.

1.2.1. Characterisation of apoptosis: morphological features

Apoptosis plays an important role in cellular homeostasis controlling normal cell turnover and during embryonic development and metamorphosis (Arends and Wyllie, 1991; Ellis *et al.*, 1991). As such, every cell in an organism possesses the ability to undergo apoptosis even in the absence of *de novo* protein synthesis implying that every aspect of the apoptotic machinery is ubiquitously present (Weil *et al.*, 1996) and is likely to be controlled at the gene level (Cotter *et al.*, 1990). Thus any resistance to apoptosis may result in excessive proliferation of cells leading to cancers and autoimmune disease and conversely an enhancement of apoptosis may underlie chronic neurodegenerative diseases such Alzheimers (Thompson, 1995). Apoptosis is associated with distinct morphological and biochemical changes (Kerr *et al.*, 1972). In contrast to necrosis where the whole cell swells, minute changes occur to the plasma membrane. This blebbing results in the flip of phosphatidylserine residues from the inner to the outer leaflet of the plasma membrane (Martin *et al.*, 1995). These early events in the apoptotic cascade act as a signal to phagocytes in order that the cell be eliminated (Savill, 1998). Other changes at the gross structural level include cell shrinkage accompanied by a reduction in intracellular potassium levels (Bortner and Cidlowski, 1996; 1999). Apoptotic cells also exhibit changes at the nuclear level including chromatin condensation (pyknosis) and DNA fragmentation. This later process involves DNA cleavage into large segments of approximately 50 kb and then progressively smaller or oligonucelosomal sized fragments (Walker *et al.*, 1993; Wyllie, 1980). This can be observed as a "DNA ladder" using the technique of agarose gel electrophoresis and is commonly used to confirm apoptosis though DNA cleavage is not always observed during apoptosis (Schulze-Osthoff *et al.*, 1994). Nevertheless, the fact that different cell types display similar features when undergoing apoptosis suggests

that no matter what the signal to trigger the death process, all cells must have the capacity to execute a common death pathway.

1.2.2 Cell death in *Caenorhabditis elegans*: a model for mammalian apoptosis

All mammalian cells constitutively express the machinery required to elicit apoptosis (Jacobson *et al.*, 1994). The same is true for metazoan cells and it is from studies of the nematode, *Caenorhabditis elegans*, that our understanding of the molecules involved has advanced greatly (Shaham and Horvitz, 1996; Steller, 1995). A dozen cell death genes (*ced*) control cell death processes in this worm of which three are essential for apoptosis (Ellis *et al.*, 1991). The products of these genes can either promote apoptosis in the case of CED-3 and CED-4 or act in an inhibitory manner, CED-9 (Hengartner and Horvitz, 1994). Genetically ced-9 is upstream of ced-3 which in turn is downstream of ced-4 (Shaham and Horvitz, 1996). Thus in terms of function, CED-9 acts as a regulator of the cell death pathway by binding CED-4 and preventing the coupling of CED-4 to CED-3 which is a requirement for activation of CED-3 and promotion of apoptosis (Chinnaiyan *et al.*, 1997a,b; Wu *et al.*, 1997; Seshagiri and Miller, 1997). An apoptotic stimulus acts by dissociating CED-9 from its complex with CED-4 and CED-3. Cloning studies revealed that vertebrates have evolved similar genes to those found in *C. elegans*. The CED-3 protein contains significant homology with a mammalian protease known as interleukin-1β (IL-1β)-converting enzyme or ICE (Yuan *et al.*, 1993) and CED-4 with apoptosis activating factor (Apaf-1, Zou *et al.*, 1997). Similarly, CED-9 may be related to the Bcl-2 family of genes members of which, can either inhibit or promote mammalian apoptosis (Yang and Korsmeyer, 1996; Adams and Corey, 1998). These studies suggest the basic apoptotic machinery evolved

early in metazoan development has been highly conserved and is relevant to human pathways of cell death.

1.2.3 Caspases: structure and function

The morphological and biochemical events that take place during apoptosis occur as the result of proteolytic activity mediated by caspases. These are cysteine proteases that cleave substrates on the carboxyl side of aspartate residues thus the name - cys-asp-ase (Alnemri *et al.*, 1996). Following the discovery that ICE, now known as caspase-1, an enzyme that is responsible for the proteolytic cleavage of pro-interleukin 1β, was a homologue of CED-3, as many as 14 different caspases have been discovered which may play a role in apoptosis and/or inflammation (Cohen, 1997; Nicholson and Thornberry, 1997). The evidence for caspase involvement in apoptosis is strong. Activation of caspases correlates well with the onset of apoptosis and inhibitors of these proteinases can significantly reduce apoptosis. Mutations in the *C. elegans* gene, ced-3, prevent cell death (Ellis and Horvitz, 1986) and similarly transgenic animals with deletions in caspases show aberrations of apoptosis (Varfolomeev *et al.*, 1998).

All caspases are synthesised as inactive proenzymes which contain three domains; an NH_2 terminal domain and a large and small subunit. The pro-domain which can vary in length is cleaved, followed by the association of large and small subunits to form a heterodimer (Walker *et al.*, 1994) and initiate activation of the caspase. Two broad classes of caspase exist based on the length of the N-terminal prodomain (Wolf and Green, 1999). It has been suggested that a long prodomain is involved in targeting and regulating activation whilst caspases which contain short prodomains play a role in the execution of proteolysis. The members of each class are summarised in Table 1.2.3.1.

Groups of caspases contain differences in their preferred tetrapeptide substrate recognition sequence (Thornberry *et al.*, 1997) and these are also categorised in Table 1.2.3.1. Caspases may either have a consensus substrate sequence of WEHD, DExD, or (IVL)ExD where x denotes broad amino acid specificity.

Caspase	Prodomain length	Substrate preference
Caspase-1 (ICE)	Long	WEHD
Caspase-2 (Nedd2, ICH-1)	Long	DExD
Caspase-3 (CPP32, apopain)	Short	DExD
Caspase-4 (ICH-2)	Long	WEHD
Caspase-5	Long	WEHD
Caspase-6	Short	(IVL)ExD
Caspase-7	Short	DExD
Caspase-8 (FLICE)	Long	(IVL)ExD
Caspase-9	Long	(IVL)ExD
Caspase-10	Long	Unknown
Caspase-11	Long	Unknown
Caspase-12	Long	Unknown
Caspase-13	Long	Unknown
Caspase-14	Short	Unknown

Table. 1.2.3.1 Characteristics of the caspase family of proteins. (Adapted from Wolf and Green, 1999)

Thus based on structural homology and sequence similarities the caspases can be divided into three subfamilies (Kidd, 1998);

1. ICE-like caspases (including caspases 1, 4, and 5)
2. CPP32-like caspases (including caspases 3, 6, 7, 8, 9, 10)
3. ICH-1 subfamily (caspase 2)

A member of each of the first two families, which are significant to the work described in this thesis will now be reviewed, highlighting similarities and differences.

1.2.3.1 Caspase-1

Initial studies of caspase-1 showed it was responsible for cleaving the pro-form of interleukin 1-β (IL-1β) to yield an active 17 kDa mature form of IL-1β, a cytokine involved in inflammation (Black *et al.*, 1989; Kostura *et al.*, 1989). Caspase-1 has a molecular weight of 45 kDa (p45) and is composed of an 11 kDa prodomain and 2 kDa linker region both of which are cleaved to generate active ICE consisting of the two catalytic subunits of 20 kDa and 10 kDa, known as p20 and p10 respectively (see Fig. 1.2.3.1; Thornberry *et al.*, 1992; Cerretti *et al.*, 1992). The activation of caspase-1 is likely to be self determined since all four cleavage sites in p45 contain the recognition sequence asp-Xaa and thus caspase-1 is found predominantly as the inactive form in the cytoplasm of cells (Ayala *et al.*, 1994). Based on the substrate specificity of this enzyme, agents have been developed to identify caspase-1 activity. Acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) is a potent inhibitor and Ac-YVAD-7-amino-4-methylcoumin (Ac-YVAD-AMC) a fluorimetric substrate (Thornberry *et al.*, 1992). The three dimensional structure of caspase-1 has been elucidated and by using specific inhibitors the active enzyme is shown to consist of a tetramer of two p20 subunits surrounding two adjacent p10 subunits (Walker *et al.*, 1994; Wilson *et al.*, 1994). The p20 and p10 subunits contribute to the active site so both are therefore needed for catalytic activity. The active site pentapeptide sequence QACRG is completely conserved between CED-3 and caspase-1 (Yuan *et al.*, 1993) suggesting the involvement of the latter in mammalian apoptosis. However, the role of caspase-1 in apoptosis is not definitive. For instance, mice deficient in caspase-1 exhibit a normal pattern of development (Kuida *et al.*, 1995; Li *et al.*, 1995) but there are differences in the susceptibility of certain cells to undergo apoptosis in this system. Thymocytes were more resistant to apoptosis mediated by anti-CD95 (Fas) antibody whereas

macrophages were equally susceptible to ATP induced apoptosis. Thus the involvement of caspase-1 in apoptosis may be both cell type and stimulus dependent. Furthermore, inhibition of caspase-1 with either inhibitory peptides or by using anti-sense oligonucleotides can prevent CD95 mediated cell death (Enari *et al.*, 1995; Los *et al.*, 1995). Apoptosis in both epithelial cells and T-lymphocytes may also be mediated by caspase-1 (Boudreau *et al.*, 1995; Tamura *et al.*, 1995). In conclusion, caspase-1 may have a dual role in both mediating inflammatory reactions and controlling cellular apoptosis whilst other caspases function purely in the later process.

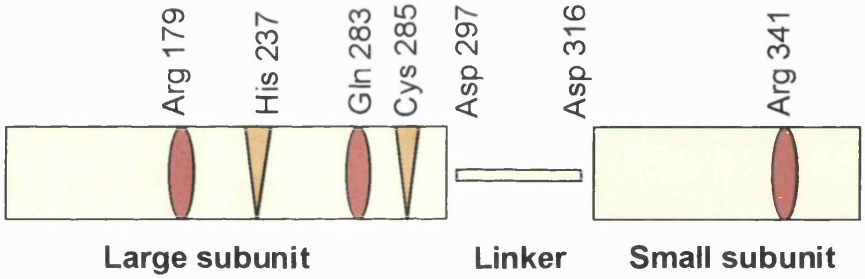


Fig. 1.2.3.1 Schematic of processed caspase-1. A linker region combines both a large and small subunit which are released from the precursor following proteolysis. The catalytic and substrate specific sites are shown in orange and red. Adapted from Stennicke and Salvesen, 1998.

1.2.3.2 Caspase 3 (CPP32/apopain)

In contrast to caspase-1, evidence exists that caspase-3 is involved in almost all apoptotic pathways suggesting it may act as an executioner of cell death (Chinnaiyan and Dixit, 1996). Furthermore, the DNA sequence of human caspase-3 shares a greater homology with CED-3 than caspase-1 (Fernandes-Alnemri *et al.*, 1994). Caspase-3 encodes a 32 kDa cysteine protease and was initially known as CPP32 (Fernandes-Alnemri *et al.*, 1994) or apopain (Nicholson *et al.*, 1995). The protein contains a short prodomain and is devoid of linker peptide. Activation of caspase-3 involves cleavage of aspartate residues in the proenzyme to yield two subunits of 17 kDa and 12 kDa

(Nicholson *et al.*, 1995). The overall three dimensional structure of caspase-3 resembles that of caspase-1 but differs in the active site region where the peptide chain of the substrate binds. This is a shallow groove which contains pockets that can differentially bind side chains – in the case of caspase-1 this site is receptive to tyrosine side chains whereas caspase-3 prefers an aspartate side chain (Rotonda *et al.*, 1996). As a consequence specific substrates (Ac-DEVD-AMC) and inhibitors (Ac-DEVD-CHO) have been developed for caspase-3. Thus, caspase-3 binds a DXXD-like substrate whereas caspase-1 prefers a YVAD-like substrate. This manifests itself in the role that caspase-3 plays during cellular apoptosis. One of the best studied reactions of caspase-3 is the proteolytic cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) which often accompanies apoptosis. The cleavage site (↓) of PARP is DEVD↓G and other natural substrates of caspase-3 contain the Asp-Xaa-Xaa-Asp (DXXD) motif (Lazebnik *et al.*, 1994). It is generally believed that once caspase-3 is activated, apoptotic cell death is irreversible and the cell is committed to die, though this has recently been disputed by *in vitro* experiments showing it can be inactivated rapidly following activation which prevents apoptosis (Marks *et al.*, 1998). Nevertheless there is both strong *in vivo* and *in vitro* evidence for caspase-3 involvement in apoptosis. Mice deficient in caspase-3 are smaller than their normal counterparts and die perinatally (Kuida *et al.*, 1996; Varfolomeev *et al.*, 1998). This is due to defects in apoptosis since there is significantly less pyknosis which is an important feature during brain development. Furthermore, neuronal cultures such cerebellar granule cells may be induced to undergo apoptosis either following withdrawal of survival factors or by exposure to excitotoxins and this may involve caspase-3 activation (Du *et al.*, 1997).

1.2.4 Death signals and caspase activation

Death receptors on the cell surface act as apoptotic sensors. They contain a region known as the death domain (DD) (Tartaglia *et al.*, 1993) which can link directly to procaspases via additional adapter molecules. Following ligand binding to the death receptor a sequence of events is initiated that results in rapid activation of the caspases and subsequent cell death a few hours later. A number of death receptors have been identified which contain highly conserved sequences, especially in the death domain, and all are members of the tumour necrosis factor (TNF) receptor gene superfamily (Smith *et al.*, 1994). The best studied death receptors are Fas (CD95 or Apo1) and those that bind TNF (TNFR) (Smith *et al.*, 1994) which are summarised in Fig.1.2.4.1. The Fas receptor and Fas ligand (FasL) are involved in situations of physiological apoptosis such as the elimination of T cells (Nagata, 1997). Three molecules of FasL bind to their receptors thereby bringing their respective DD together in close proximity (Smith *et al.*, 1994). The cytoplasmically located DD can now interact with another adapter protein known as Fas-associated death domain (FADD) (Chinnaiyan *et al.*, 1995) and this contains a caspase recruitment domain (CARD) which binds procaspase-8 (Boldin *et al.*, 1996). Subsequent oligomerisation of caspase-8 results in self-activation and the activation of a downstream caspase cascade that leads to apoptosis (Muzio *et al.*, 1998). Structurally DD's, DED's and CARD's are similar.

TNF can bind to either TNFR1 or TNFR2 and cause receptor clustering in a manner similar to Fas (Smith *et al.*, 1994). Diffferent adaptors are involved including TNFR-associated death domain (TRADD) which is responsible for the pathway to caspase-8, RAIDD coupled to procaspase-2 or TNFR-associated factor 2 (TRAF2) and receptor interacting protein (RIP) which cause the translocation of NF-κB to the nucleus to

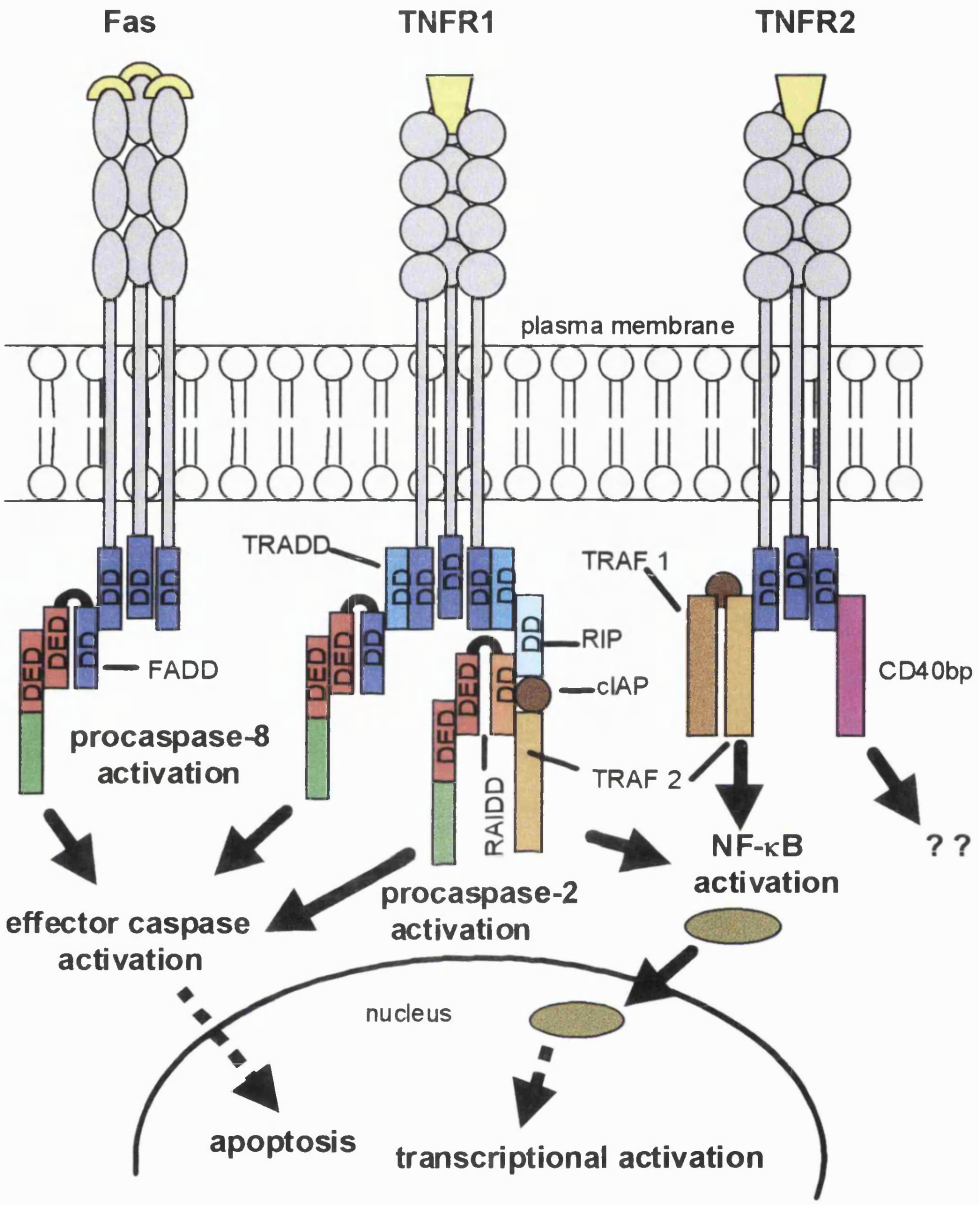


Fig. 1.2.4.1 Pathways activated by the death receptor ligands, Fas and TNF. The procaspases 8 and 2 can be coupled to death receptors via adapter molecules such as FADD, TRADD, RIP and RAIDD through regions of high sequence homology including the death domains (DD, blue) and death effector domains (DED, red). Alternatively, TNF can activate NF-κB via TRAF1 or TRAF2 and upregulate gene transcription. The function of CD40bp another TRAF family member is still uncertain. Modified from Ashkenazi and Dixit, 1998.

mediate transcription and cell survival (Rothe *et al.*, 1995; Hsu *et al.*, 1996; Duan and Dixit, 1997; Ahmad *et al.*, 1997; DiDonato *et al.*, 1997). TNFR2 binds TRAF1 and TRAF2 and is also linked to a protein known as CD40bp which may be involved in NF-κB activation.

1.2.5. Caspases and the execution of apoptosis

Following the binding of ligand to death receptor and formation of the death inducing signalling complex, initiator caspases including caspases 8, 9, and 10 are activated (Fraser and Evan, 1996). These and other as yet unidentified pathways are responsible for the early proteolytic events which may account for plasma membrane changes such as blebbing. During this stage a number of cytoskeletal proteins can be cleaved including actin (Kayalar *et al.*, 1996), vimentin (van Engeland *et al.*, 1997) and fodrin which is closely associated with the plasma membrane (Cryns *et al.*, 1996). It is proposed that caspases are responsible for the proteolysis of fodrin since it contains a number of potential caspase cleavage sites (Cryns *et al.*, 1996; Vanags *et al.*, 1996). However, calpain may also mediate some of these effects (Martin *et al.*, 1995). The caspase inhibitor, Ac-DEVD.CHO, has been shown to prevent Fas induced apoptosis but not fodrin cleavage, providing further evidence that this is an early event preceding the activation of the execution caspases (Cryns *et al.*, 1996). Additional biochemical changes such as phosphorylation of myosin complete the sequence of events resulting in overall cell shrinkage and blebbing (Mills *et al.*, 1998).

The execution caspases can also cleave a number of proteins that maintain the cytoskeletal architecture such as the catenins (Brancolini *et al.*, 1997; Herren *et al.*, 1998) and focal adhesion kinase (Fak, Wen *et al.*, 1997; Gervais *et al.*, 1998). However, these caspases have more profound effects on proteins associated with the nucleus and are thus considered to be responsible for evoking changes such as DNA condensation and fragmentation. Of these proteins, the proteolytic cleavage of PARP, an enzyme responsible for DNA repair, is the best characterised. Whole PARP is 116 kDa but can be cleaved to 24 kDa and 89 kDa fragments (the DNA binding and

catalytic domains respectively) by a number of caspases (Gu *et al.*, 1995) to diminish DNA repair. Other DNA associated enzymes degraded by caspases include DNA-PK and U1-70 kDa (Casciola-Rosen *et al.*, 1994). DNA fragmentation occurs as the result of nuclease action and some DNase's are dependent on caspase activity (CAD) (Enari *et al.*, 1998; Liu *et al.*, 1997). DNA fragmentation can also occur as the result of the degradation of a set of structural proteins known as lamins which form the nuclear envelope (Ucker *et al.*, 1992; Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995).

1.2.6 Are mitochondria involved in apoptosis?

The mitochondrion is believed to have evolved from the endocytosis of bacteria by an ancestor of the present day eukaryotic cell (Margulis, 1996). This symbiotic relationship may have arisen to protect cells from the increasing levels of oxygen in the atmosphere. Oxygen could be used in energy production but this also generated toxic reactive oxygen species (ROS) so the balance between life or death was dependent on the newly forming mitochondria (Frade and Michaelidis, 1997). Throughout the course of evolution the genes coding for the protein that determined cell viability have been transferred to the nucleus and thus are integral components of signal transduction pathways in the present day cell. This would account for the fact that mitochondrial DNA is not required for apoptosis (Jacobson *et al.*, 1993).

However, early studies of the role of mitochondria in apoptosis focused on the production of ROS rather than these proteins. Instead of being converted to molecular oxygen, electrons transferred along the respiratory chain can be lost and converted to the superoxide anion, $O_2^{\cdot}$. During apoptosis high levels of $O_2^{\cdot}$ are produced (Bredesen, 1995) and thus it could be that mitochondrial production of ROS acts as a control point

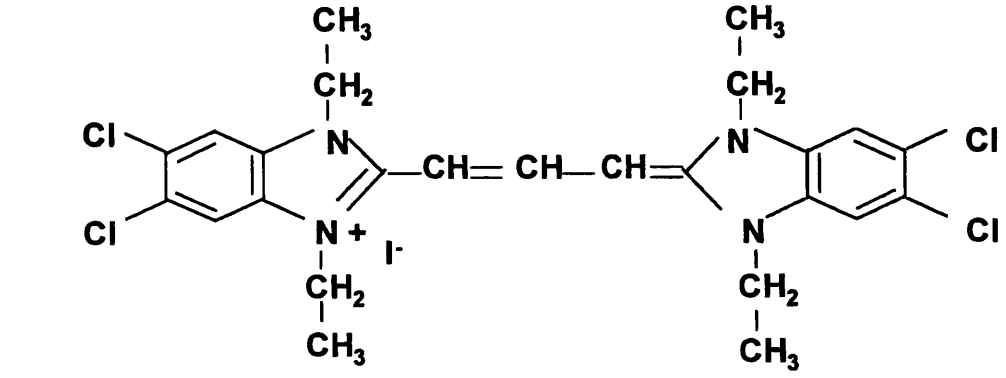
in apoptosis (Buttke and Sandstrom, 1994). However, there is more evidence to suggest that this is not the case and that ROS might only be produced late in the apoptotic cascade. For instance, cells still undergo apoptosis in the absence of oxygen (Jacobson and Raff, 1995) and a functional respiratory chain (Jacobson *et al*, 1993). In the case of Fas mediated apoptosis there is no production of ROS (Schulze-Osthoff *et al.*, 1994). Another possible cause for apoptosis is through actual disruption to electron transport. This has been reported to occur following exposure to a number of apoptotic stimuli such as ceramide (Garcia-Ruiz *et al.*, 1997) and Fas ligation (Adachi *et al.*, 1997) both of which show a disruption at the cytochrome c step. However, a dysfunctional respiratory chain might be expected to cause rapid loss of ATP which, as discussed previously, may be needed for the downstream events in apoptosis (Eguchi *et al.*, 1997). Nevertheless changes in mitochondrial function might allow mitochondrial associated proteins to interact with other external elements of the apoptotic cascade such as the caspases.

1.2.7 Mitochondrial membrane potential, permeability transition and apoptosis

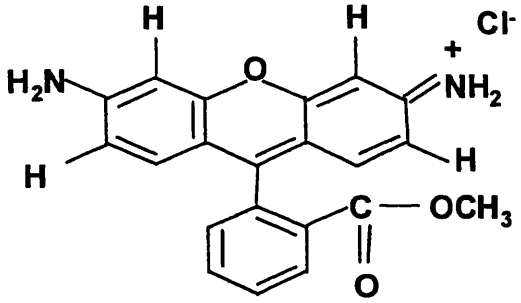
Oxidative phosphorylation involves the transfer of electrons through a series of respiratory complexes with protons being pumped out of the matrix to be used in the generation of ATP via an ATP synthetase. A voltage gradient between the inner mitochondrial membrane which is negatively charged and the outer positively charged membrane known as the mitochondrial membrane potential ($\Delta\psi_m$) is necessary to maintain this movement. Evidence exists that a decrease in $\Delta\psi_m$ might be an early event in apoptosis. The $\Delta\psi_m$ can be monitored in living cells using a number of potential sensitive dyes such as 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), rhodamine 123 or

tetramethylrhodamine ester (TMRE) the structures of which are displayed in Fig. 1.2.7.1. The lipophilic nature of these molecules allows them to easily cross membranes and enter the mitochondrial matrix in a $\Delta\psi_m$ dependent manner. JC-1 has been used to study mitochondrial changes during apoptosis (Ankarcrona *et al.*, 1995; Barbieri *et al.*, 1998). This dye has the advantage that it is a dual emission probe which when combined with a suitable optical filter set can be used to view mitochondria with both a normal potential and those with a collapsed $\Delta\psi_m$ (depolarised). At low mitochondrial membrane potential the dye exist as a monomer and fluoresces green whereas at higher potentials it forms red fluorescent "J-aggregates". Thus ratiometric measurements can be made of the green signal (emission maxima ~510 nm) and the J-aggregate (emission maxima 590 nm) when excited in the region of 485 nm (Reers *et al.*, 1991). Rhodamine 123 may be used as a general marker for mitochondria exhibiting a yellow/green fluorescence when viewed under a fluorescein filter. Though it has been used to study membrane potential (Duchen, 1992), data may be misleading as it tends to aggregate and as a consequence potential may be dependent on interactions with other intracellular components. However, these problems are overcome by using TMRE a closely related ester of rhodamine. This is more readily taken up into the matrix and exhibits a high mitochondrial specific fluorescence thus minimising the problems of aggregation and environmental interactions (Cossarizza *et al.*, 1993).

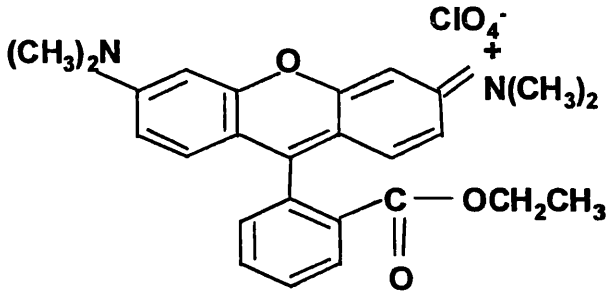
A fall in $\Delta\psi_m$ has been observed during apoptosis evoked by a variety of stimuli such as TNF (Marchetti *et al.*, 1996), dexamethasone (Petit *et al.*, 1995) and following deprivation of nerve growth factor in neuronal cultures (Marchetti *et al.*, 1996). Some studies suggest that mitochondrial depolarisation occurs as a consequence of apoptosis (Barbieri *et al.*, 1998) but there is more evidence to indicate it resides upstream of



JC-1



rhodamine 123



TMRE

Fig. 1.2.7.1 The chemical structures of potentiometic dyes used to study mitochondria.

events such as DNA fragmentation (Vayssiere *et al.*, 1994; Petit *et al.*, 1995; Zamzami *et al.*, 1995) and therefore marks a point of no return from cell death. In many situations cyclosporin A (CsA) can prevent this mitochondrial depolarisation and cell death (summarised in Kroemer *et al.*, 1998) which has lead to the notion that the opening of a large conductance channel known as the mitochondrial permeability transition (PT) pore is involved (Zoratti and Szabo, 1995). The pore is normally closed but can be opened by a variety of physiological modulators allowing molecules of <1500 Da through and thus resulting in ionic re-equilibration between matrix and cytosol leading to a reduction in $\Delta\psi_m$ (Bernardi *et al.*, 1992; Petronilli *et al.*, 1994). The

components of the PT pore are known (Fig. 1.2.7.2) but how they co-operate to form a channel is still not entirely understood (Crompton, 1999). The adenine nucleotide transporter (ANT) is found in the inner mitochondrial membrane and may bind various ligands including ADP and ATP (Le Quoc and Le Quoc, 1988). It can exist in two conformations in which the ADP/ATP binding site is localised to either the matrix side of the membrane (m state) or the cytoplasmic side (c state). This is influenced by modulators of the permeability transition such as bonkrekic acid which bind to the m state and inhibits pore opening and atractyloside which binds to the external domain of ANT and activates the protein (Crompton, 1999). In order to form a functional channel linking inner and outer membranes, ANT acts in concert with the voltage dependent anion channel (VDAC) which resides on the outer mitochondrial membrane. Additionally cyclophilin D (CyP-D) binds to the active site of ANT and thus is an integral part of the pore. It is in this region that CsA exerts its inhibitory role on PT by preventing CyP-D binding (Woodfield *et al.*, 1998). Furthermore, evidence that protoporphyrin IX a peripheral benzodiazepine receptor ligand induces PT suggests that this receptor might also be associated with the pore (Pastorino *et al.*, 1994). These core proteins are probably associated with other peripheral ones including hexokinase and creatine kinase. The fact that pharmacological inhibitors of the PT pore prevent apoptosis and that Bcl-2 and Bax, both cellular modulators of mitochondria function, also influence PT provides evidence that this phenomenon is a central controlling point for the progression of apoptosis. It is possible that molecules released through the pore could be responsible for the late events occurring during apoptosis such as DNA fragmentation.

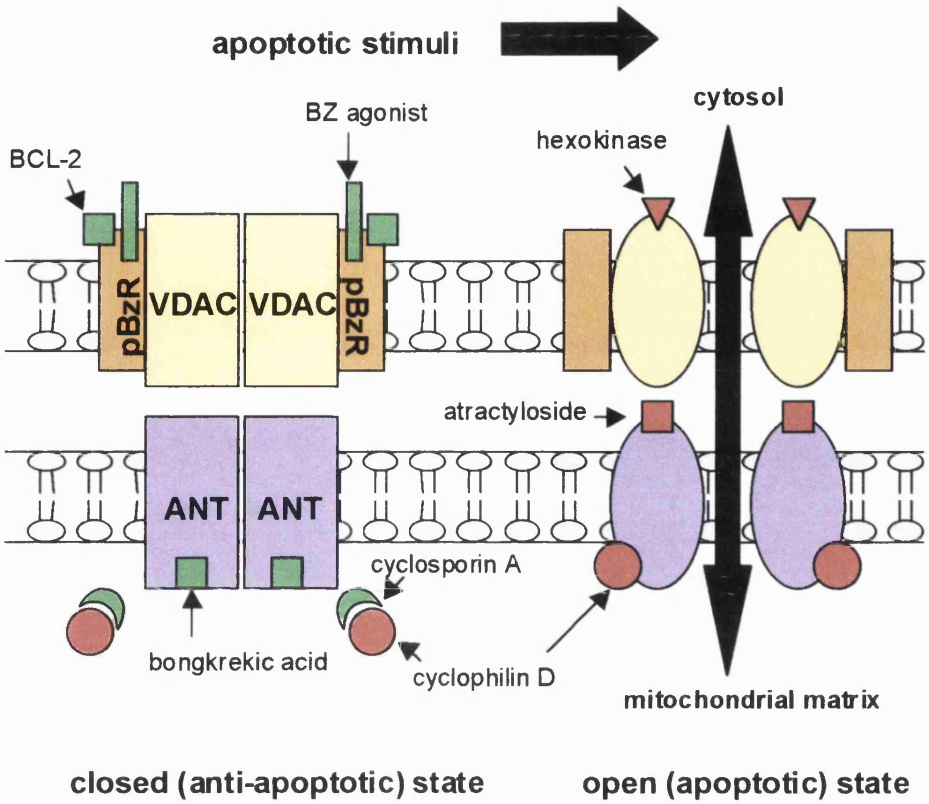


Fig. 1.2.7.2 Schematic of the components of the mitochondrial PT pore and factors that influence its opening. The core structure consists of ANT localised to the inner mitochondrial membrane and VDAC plus peripheral benzodiazepine receptor (pBzR) in the outer membrane. Apoptotic stimuli can result in the opening of the pore which may result in a fall in $\Delta\psi_m$, matrix swelling and release of apoptogenic factors. The open state may also occur in the presence of atractyloside and is associated with cyclophilin D binding to ANT and possibly hexokinase to VDAC. The pore is closed in the presence of bongkrekic acid, benzodiazepine agonist, and Bcl-2 and also cyclosporin A which prevents cyclophilin D binding. (Modified from Tatton and Olanow, 1999).

1.2.8 Mitochondria release death proteins

There are two well studied examples of proteins that might be released from the intermembrane space within the mitochondria to the cytosol where they can transmit a death signal. These include cytochrome c, a protein associated with the respiratory chain and a 50 kDa protein known as apoptosis inducing factor (AIF). Additionally there is some evidence to suggest that caspases themselves are released from the

mitochondria to the cytosol where they become active and evoke apoptosis (Mancini *et al.*, 1998).

1.2.8.1 Cytochrome c

Within the respiratory chain cytochrome c acts as electron carrier, accepting an electron from cytochrome c reductase and passing it to cytochrome c oxidase. Cytochrome c is made within the cytoplasm and then translocated to the mitochondria where it loosely attaches to the surface of the inner mitochondrial membrane, sitting in the intermembrane space. During this process the unfolded protein gains a functional haem group and forms its native structure. It is now not naturally amenable to passing through the outer membrane. The release of cytochrome c has been shown to occur following PT (Ellerby *et al.*, 1997; Kantrow and Piantadosi, 1997) but it may also be observed prior to falls in $\Delta\psi_m$ (Adachi *et al.*, 1997; Kluck *et al.*, 1997) suggesting other mechanisms are responsible. Recent evidence suggests that Bcl-2 family members may form pores in combination with VDAC to allow the release of cytochrome c (Shimizu *et al.*, 1999). Cytochrome c release from mitochondria to cytosol has been observed following stimulation of cells with a number of well known inducers of apoptosis such as Fas (Scaffidi *et al.*, 1998), staurosporine and UV irradiation (Bossy-Wetzl *et al.*, 1998). In cell free systems the microinjection of cytochrome c into the cytosol can also evoke apoptosis (Duckett *et al.*, 1998; Zhivotovsky *et al.*, 1998). It was the use of these models that determined how cytochrome c might activate caspases. Firstly, induction of apoptosis in cell free extracts was shown to be dependent on both cytochrome c and dATP (Liu *et al.*, 1996) and secondly there was also a requirement for another cytosolic factor known as apoptosis protease-activating factor (Apaf-1) to promote caspase-3 processing (Zou *et al.*, 1997). Sequencing of Apaf-1 revealed that it shared a high

sequence homology with CED-4 and furthermore it contained a caspase recruitment domain. This has led to the notion that for apoptosis to occur the formation of a tetrameric complex known as an apoptosome is necessary. Cytochrome c can interact with Apaf-1 in the presence of dATP thus enabling recruitment of specifically procaspase-9 via the CARD region in Apaf-1 and thereby leading to production of active caspase-9 which can in turn trigger a cascade of caspase activation including caspase-3 (Li *et al.*, 1997).

1.2.8.2 AIF

Treatment of purified mitochondria with atractyloside, an ANT binding ligand and promoter of PT was shown to result in the release of the flavoprotein, AIF, that could directly induce apoptotic changes in isolated HeLa cell nuclei (Zamzami *et al.*, 1996; Susin *et al.*, 1996). Therefore AIF acts in a distinct manner from cytochrome c which is dependent on downstream caspase activity. Recently the protein has been sequenced and shown to bear homology to bacterial oxidoreductases suggesting it may play a role in electron transfer (Susin *et al.*, 1999). In a similar way to cytochrome c, the initial translation of AIF occurs outside of the mitochondrion and only once it is inside does it acquire the native structure necessary for apoptogenic activity. AIF treated nuclei display only signs of chromatin condensation rather than oligonucleosomal laddering, a caspase activated deoxyribonuclease (CAD) dependent event, providing further evidence that it by-passes the need for caspase activity (Susin *et al.*, 1999). Up-stream caspase-1 activity may mediate the release of AIF from mitochondria (Susin *et al.*, 1997) and this release can be self amplifying since injection of AIF into cells causes mitochondrial depolarisation and the release of cytochrome c (Susin *et al.*, 1999).

Thus evidence exists that proteins may be released from the mitochondria in a PT dependent or independent manner. Furthermore, the release of apoptogenic proteins may not be required in all pathways to apoptosis. For instance in the case of Fas mediated apoptosis, evidence suggests that certain cell types require mitochondria whereas others do not (Scaffidi *et al.*, 1998). Caspase 8 can activate a mitochondria dependent pathway that involves cytochrome c release as well as a mitochondria independent one (Kuwana *et al.*, 1998). Similarly, in Apaf knockouts some cells retain sensitivity to Fas induced killing whereas others lose it (Kuida *et al.*, 1998; Cecconi *et al.*, 1998). Therefore it is likely that other factors can interact both upstream and downstream of mitochondria to determine whether this organelle is involved and releases apoptogenic proteins. These are likely to involve Bcl-2 family members. A series of homologous proteins with both apoptotic and anti-apoptotic properties constitute the Bcl-2 family which can now be subdivided into three subfamilies; the anti-apoptotic Bcl-2, and Bax and Bik which are pro-apoptotic (Adams and Cory, 1998). The majority of Bcl-2 family proteins are located in the mitochondrial outer membrane and they probably form ion channels (Kelekar and Thompson, 1998). Recent studies suggest that Bcl-2 family members interact with VDAC, an integral membrane protein normally associated with the PT pore, to control cytochrome c release (Shimizu *et al.*, 1999). VDAC forms a pore on its own which is normally too small to allow release of cytochrome c. However, following an apoptotic signal the Bax protein undergoes dimerisation and can be recruited to the mitochondria where it induces a conformational change in VDAC that allows the release of cytochrome c. Binding of Bcl-x_L closes the channel. Other possible sites where Bcl-2 and Bax interact are summarised in Fig. 1.2.8.1

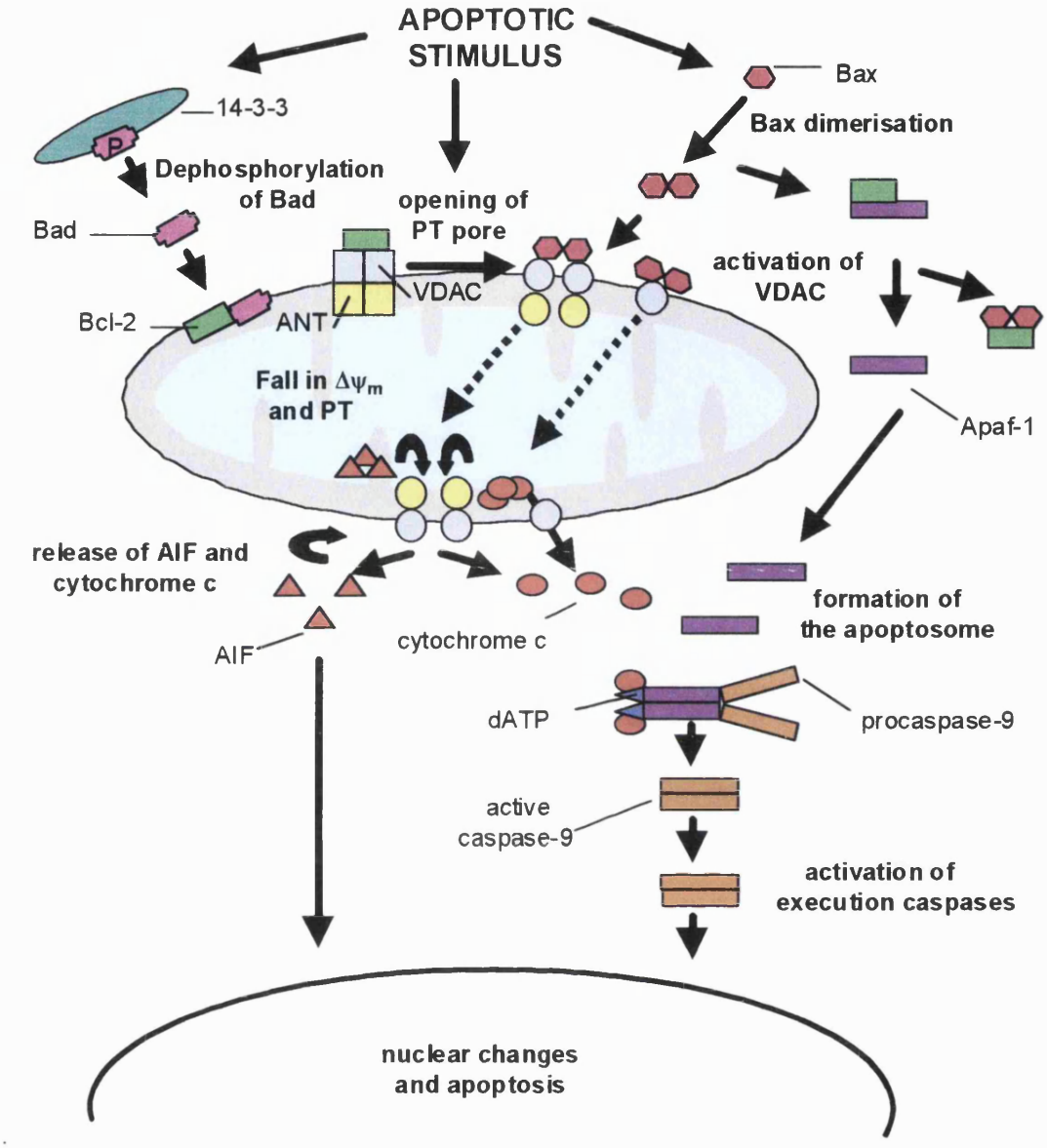


Fig. 1.2.8.1 Scheme depicting the mitochondrion at the centre of the apoptotic cascade. Apoptotic signals result in the opening of the PT pore which may be facilitated by displacement of Bcl-2 with the active Bax dimer. Bcl-2 is also sequestered by dephosphorylated Bad. Opening of the PT pore results in the release of AIF which can act directly on the nucleus to cause apoptosis. Cytochrome c may also be released via the pore but this is more likely to occur through VDAC channels following Bax activation. Cytochrome c can combine with Apaf-1 (which is freed from its inactive complex with Bcl-2 in the presence of Bax), dATP and procaspase-9 to form the "apoptosome". Procaspase-9 is activated to caspase-9 which can active further downstream execution caspases and result in apoptosis. Modified from Gross *et al.*, 1999.

1.2.9 Apoptosis and mitochondria in neurodegenerative disease

Apoptosis was originally believed to occur only in physiological conditions such as embryogenesis. For instance, during development of the CNS many more neurones are produced than required to form the necessary synaptic connections in the mature brain (Raff *et al.*, 1993). Studies have shown that these cells are removed by apoptosis when they are starved of growth factors probably following similar pathways to those observed with *in vitro* models. However, in the past few years evidence has accumulated to suggest that apoptosis might be involved in pathological conditions and play a role in neurodegenerative disorders such as Alzheimer's disease. Cell culture models are useful in elucidating the signalling pathways involved in apoptosis whereas it is only possible to monitor late events such as DNA damage in the diseased brain. The most common method is to detect the free 3'-OH end of single stranded DNA by in situ end labelling (ISEL). This can be performed immunocytochemically by adding labelled nucleotides to the ends using terminal transferase (TUNEL) (Tatton *et al.*, 1998).

1.2.9.1 Alzheimer's disease (AD)

Numerous studies have shown that there is an increase in TUNEL staining in both neurones and glia in the AD brain when compared with age matched controls (Smale *et al.*, 1995; Lassman *et al.*, 1995; Dragunow *et al.*, 1995). The cells labelled also correlate with regions in which the majority of cells are lost during disease progression, such as the neocortex and hippocampus (Troncoso *et al.*, 1996). However, there is a worry that in the majority of cases, the TUNEL positive cells do not show the classic morphological signs of apoptosis and if the preparation of tissue sections is delayed post mortem there is an increase in background TUNEL staining (Anderson *et al.*, 1996).

There is also some immunocytochemical evidence to suggest mitochondrial involvement in the cell death. The proapoptotic protein, Bax, is upregulated in both neurones and microglia in the AD brain (Su *et al.*, 1997). In contrast to this study there is a report of increased reactivity for Bcl-x_L in activated microglia (Drache *et al.*, 1996) and others suggest increased Bcl-2 expression in astrocytes but not neurones (O'Barr *et al.*, 1996) suggesting that the Bcl-2 family is an important mediator of glia cell survival. In terms of mitochondrial function complex II, III, and IV activities are reduced in the AD brain (Reichmann *et al.*, 1993). It is possible that reduced transcription of mRNA for cytochrome oxidase subunits I and III and subsequently diminished activity may account for the observed dysfunction of complex IV (Chandrasekaran *et al.*, 1997; Chagnon *et al.*, 1995). In addition, changes in the structure of the mitochondrial membrane occur in the AD brain (Mecocci *et al.*, 1997).

Thus there is strong evidence supporting the role of apoptosis in diseases such as AD. However, to date this is not conclusive due to limitations in the techniques available to assess post mortem tissue. Cell culture studies and animal models indicate that activation of caspases is central to the pathways of apoptosis. With the advent of new tools to detect specific caspase activation in situ it might be possible to confirm the role of apoptosis in neurodegenerative disease.

1.3 Models to study neuronal cell death

The nervous system comprises hundreds of thousands of neuronal pathways that link together in a complex array to give the overall biochemical and physiological profile that determines how an organism will respond to its environment. To dissect the cellular and molecular interactions that mediate these connections between neurones

and their targets it has been necessary to develop simplified systems isolating individual neurones from the extensive cell heterogeneity found within the brain. A number of models have been developed. Synaptosomes have been used to study the composition of the neuronal membrane and the process of neurotransmitter release (Whittaker, 1993). Synaptosomes are formed when neurones are homogenised and discrete cell fragments reseal and form viable particles. They are thus a representative model for events happening at the synapse. Brain slices can be prepared from various regions of the brain such as the hippocampus and cerebellum (Llinas and Muhlethaler, 1988) allowing the study of neurotransmission between different neurones. Electrophysiological analysis suggests that responses from the synapses compare well with *in vivo* data. The disadvantages of slices is that they degrade relatively quickly and developmental changes cannot be assessed. Alternatively, dissociated cell cultures can be obtained either from foetal or postnatal tissue from almost any part of the brain and these allow the dissection of multiple pathways down to a single cell type. Factors regulating survival, growth and differentiation of neurones can be examined as the cells mature. Changes in trophic activity, the biochemistry of membrane channels and receptors and synaptic connections can all be investigated *in vitro*. One difficulty in some cases has been the ability to obtain sufficient numbers of cells. To overcome this, clonal cell lines that exhibit neuronal features can be used. The experiments described in this thesis have utilised both a neuronal primary culture of rat cerebellar granule cells (CGCs) and a mouse clonal hippocampal cell line, HT22. In the following sections there follows a review of the different cell types, the region of the brain they are derived from and how they can be used as models to study neuronal degeneration.

1.3.1 The cerebellum and its circuitry

The cerebellar cortex is composed of four discrete layers. The molecular layer is followed by the Purkinje cell layer composed of Purkinje neurones with large somata in a monolayer. Next comes the granular layer (granule cells) and beneath the granular layer is the medullary layer which contains myelinated fibres from other regions of the brain. The cerebellar granule cells (CGCs) are the most numerous neurones of the cerebellar cortex. The main circuits are shown in Fig. 1.3.1.1. The granule cell axon passes vertically through the granular and Purkinje layers finally branching out to form a horizontal region known as the parallel fibre which can link to the Purkinje cell. Overall the ratio of granule cells to Pukinje cells is 449:1 in the rat and as high as 3300:1 in the human brain (Korbo *et al.*, 1993). The application of electrical current to the parallel fibres in the molecular layer is conducted to other connecting cells. All the neurones in contact with the fibres are inhibitory so the overall effect of granule cell excitation is the inhibition of discharges by their target neurones. GABAergic input to granule cells comes from the golgi cell terminals and granule cells have been shown to have GABA receptor subunits (Zheng *et al.*, 1993). The excitatory transmitter is glutamate (Kaneko *et al.*, 1987). There are also three types of glia within the cerebellar cortex. Astroglia are found in the granular layer, oligodendroglia in the medullary layer and lastly the Bergmann glia, a specialised form of astroglia, are localised in a row within or just beneath the Purkinje cell layer.

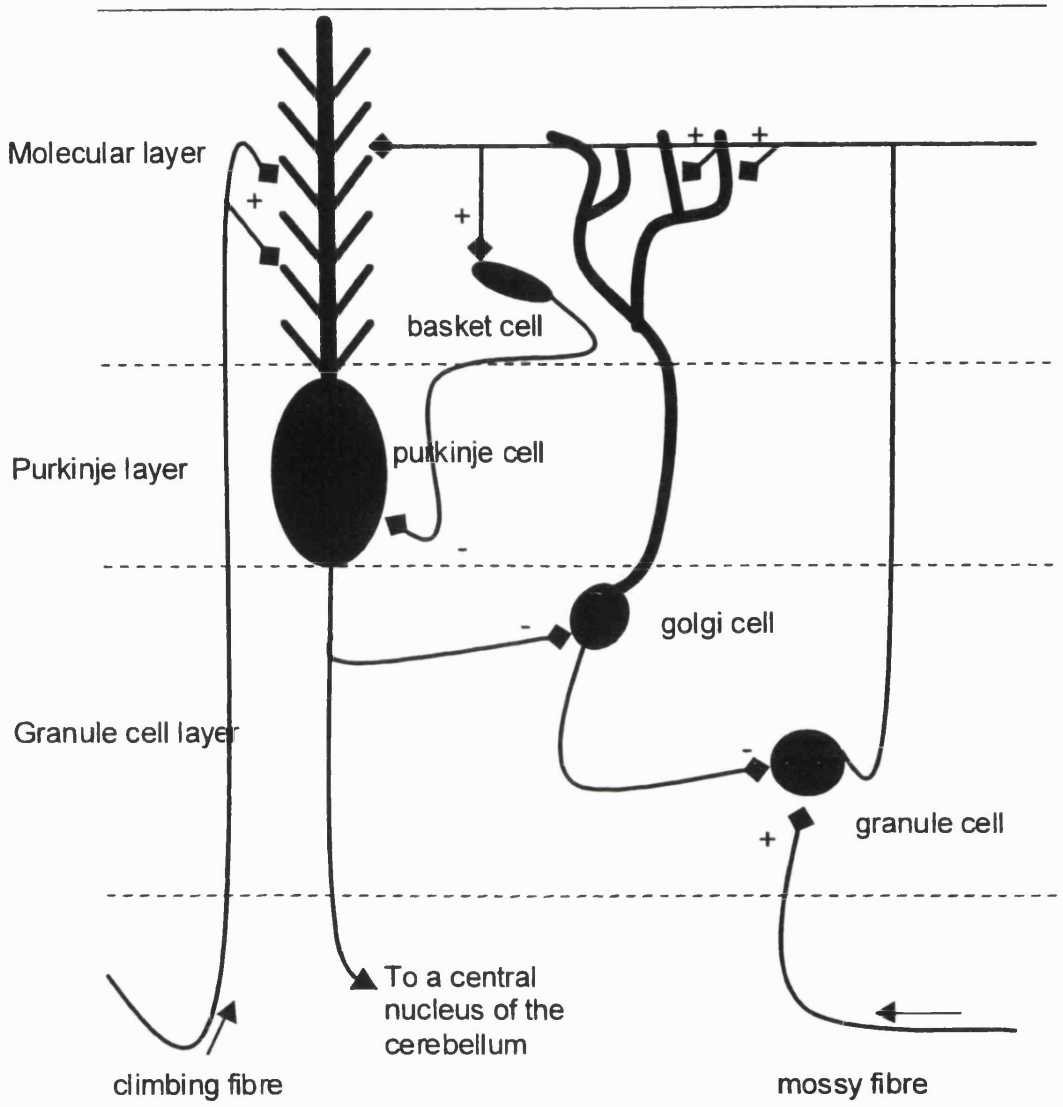


Fig. 1.3.1.1 Neurones in the cerebellum showing excitatory and inhibitory synapses. Adapted from Kiernan, Barr's The Human Nervous System, Lippincott, 1998

1.3.2 The cerebellum and pathology

The cerebellum was initially only believed to play a role in posture and voluntary movement but it may also be important in associative events enabling an adaptive response to the environment to be elicited (Thompson, 1986). This indicates therefore that the cerebellum may be necessary for cognitive as well as motor processes (Thach, 1998). A number of cerebellar disorders exist, most notably the hereditary ataxias, a

group of inherited neurodegenerative disorders (Klockgether and Evert, 1998). Friedreichs ataxia is the most common form of an autosomal recessive ataxia and may occur as the result of a breakdown of frataxin, a mitochondrial protein, which leads to a disruption of energy levels and oxidative stess (Klockgether *et al.*, 1996). Alzheimer's is one of the most common neurodegenerative disorders and there is growing evidence that pathological changes may occur in the cerebellum (Larner, 1997). Presenilins, an important component for progression of the disease have also been localised to the cerebellum (McGeer *et al.*, 1998). Mutations in presenilin genes account for 60% of the early onset familial Alzheimer's diseases cases observed. Also there is extensive cerebellar pathology with the early onset form (Fukutani *et al.*, 1997). Other diseases such as prion disease may result in cerebellar lesions (Shmerling *et al.*, 1998) and DNA fragmentation is observed in the human cerebellum following global ischaemia (Hara *et al.*, 1995). Thus cultured CGCs may provide a valuable model for studying mechanisms of neurodegeneration.

1.3.3 Cerebellar granule cells *in vitro*

Cultured CGCs are a very popular model for studying mechansims of apoptosis and excitotoxicity. The cultures are relatively easy to prepare yielding a high number of a homogenous sample with minimal contamination from glia.

1.3.3.1 Isolation and morphological development

In brief, the protocol for granule cell culture involves the dissection of the cerebellum from the brain and subsequent removal of the meninges. The tissue is then subjected to mechanical and enzymatic disaggregation and following trituration centrifuged through a 4% BSA gradient to remove any cellular debris. This yields approximately 5-10 x 10^6

viable cells per cerebellum. Following plating, granule cells grow very quickly *in vitro* and can be identified as cells with small round cell bodies (8μm in diameter) extending neurites of up to 25-30μm. The granule cell constitutes in the region of 95% of the total cell population observed. The main contaminating cells are both type I and type II astrocytes, fibroblasts, some GABAergic interneurones and low levels of oligodendrocytes and macrophages. In order to prevent overgrowth of non-neuronal cells in serum containing medium, a mitotic inhibitor such as cytosine arabinofuranoside is included in the medium after 24h.

Optimal conditions for granule cell survival have been developed (Balazs, 1984). CGCs were shown to develop differently when grown in serum containing medium or chemically defined medium (Kingsbury *et al.*, 1985). In the presence of serum, granule cells migrated along the neurites to form clumps and by 7 DIV they had a differentiated appearance. In contrast, cells grown in chemically defined medium attached to the substratum efficiently but lacked the movement to form clumps and instead a fine mesh network of neurites was observed (Kingsbury *et al.*, 1985). Neurones in serum containing medium rapidly died after 2 weeks in culture but those in chemically defined medium survived longer. Furthermore, there appeared to be a difference in the requirement for depolarisation. Serum containing cultures had to be grown in an elevated concentration of K^+ but in chemically defined medium this was not necessary.

1.3.3.2 Developmental changes

The role of depolarisation was further investigated (Gallo *et al.*, 1987). It was found that the dependence on depolarising conditions developed in parallel with the expression of the differentiated form of the cells. The importance of Ca^{2+} came to light

as depolarisation induced transmembrane Ca^{2+} influx was necessary for maintenance of cell viability. Blocking Ca^{2+} entry with high Mg^{2+} or EGTA compromised cell integrity whereas the dihydropyridine Ca^{2+} agonists, Bay K 8644 and CGP 28392, prevented neuronal loss (Gallo *et al.*, 1987).

The observation that granule cells require chronic depolarisation using 20-40 mM K^+ for survival suggests that this might mimic potassium current expression during *in vivo* development. Since potassium channels are essential for determining membrane excitability they have a function in neuronal and synaptic development and overall plasticity (Kaang *et al.*, 1992). There are changes in K^+ current and channel expression during the development of CGCs in culture. Depolarisation of "mature" cerebellar granule cells evokes an outward potassium current (Cull-Candy *et al.*, 1989). In "immature" cells the transient current is inactivated in a monoexponential manner but by 7-9 DIV there is a strong dependence on voltage as higher depolarised conditions are necessary to inactivate it (Gorter *et al.*, 1995). Furthermore, the properties of the potassium current are dependent upon culture conditions (Gorter *et al.*, 1995) with the necessity for voltage dependent activation increasing in cells cultured under low K^+. It has been proposed that the developmental profile of the K^+ current may be a consequence of increased K^+ channel expression in the absence of high K^+ (Ficker and Heinemann, 1992) and may be responsible for the ability to release neurotransmitter. Additionally another study suggests that there may also be Ca^{2+} dependent potassium currents (Fagni *et al.*, 1991). Voltage operated Ca^{2+} channels also play a role in neurotransmitter release. Calcium uptake studies revealed the existence of a dihyropyridine sensitive pathway in CGCs (Kingsbury and Balazs, 1987) and this is now believed to be reponsible for Ca^{2+} dependent glutamate release (Pocock *et al.*, 1993; Evans and Pocock, 1999). Multiple types of Ca^{2+} channel exist including L, N

and P types of which each may exist in different forms. This is particularly the case for L-type Ca^{2+} channels, which constitute the majority of Ca^{2+} channels present in the CGC (Forti and Pietrobon, 1993). Using single cell fluorescence imaging, the effect of depolarisation on granule cells can be investigated. Upon application of KCl there is a rapid rise in cytoplasmic Ca^{2+} which is made up of a transient spike followed by a non inactivating plateau which is sensitive to dihydropyridine, the toxin Aga-GI and a residual component (Pocock *et al.*, 1993). This residual component is localised to the neurites and is coupled to the exocytotic release of glutamate (Harrold *et al.*, 1997).

1.3.3.3 Apoptotic cell death in culture

During the development of the cerebellum a large number of CGCs die via the process of apoptosis (Wood *et al.*, 1993). Primary cultures of CGCs mimic this cell death when maintained at low concentrations of potassium (5mM) whereas elevated levels of potassium (25 mM) block this programmed cell death. Thus the switching of cultures from 25 mM to 5 mM K^+ has become a tool for investigating apoptotic pathways in granule neurones (Yan *et al.*, 1994). In this model the dying neurones show the characteristic signs of apoptosis i.e. cytoplasmic blebbing, chromatin condensation and nucleosomal size DNA fragmentation. The influence of various factors on "immature" and "mature" granule cells cultured in 5 mM has been investigated. For instance, a number of cytokines including TNF-α and IL-10 can delay apoptosis in "immature" cells (de Luca *et al.*, 1996) and thapsigargin, a drug which raises intracellular Ca^{2+} can support the survival of "mature" granule cells in the absence of high K^+ (Levick *et al.*, 1995). Insulin like growth factor-I (IGF-I) has also been shown to be anti-apoptotic in this model of neuronal apoptosis (D'Mello *et al.*, 1993). Recent experiments have identified caspases that are activated during apoptosis (Eldadah *et al.*, 1997; Taylor *et*

al., 1997). Thus there may a developmental profile for factors that influence the survival of granule cells and this may be associated with changes in biochemical and electrophysiological properties during time in culture.

1.3.3.4 Glutamate: The neurotransmitter of CGCs

Physiological stimulation of CGCs *in vivo* occurs through stimulation of excitatory amino acid receptors (Levi *et al.*, 1982) such as the NMDA-R which will allow Ca^{2+} entry and the activation of further Ca^{2+} pathways. *In vitro* experiments appear to confirm this. If neurones are cultured under low K^+ conditions the addition of NMDA can dramatically increase the survival of the cells (Balazs *et al.*, 1988). The requirement for NMDA develops at between 2-4 DIV, similar to that for high K^+, and shows concentration and depolarisation dependent effects. This may mirror developmental changes in subsets of NMDA receptors. During cerebellar development there are changes in the distribution of mRNAs for the different NMDA receptor subunits. For instance, prior to synapse formation granule cells display NMDA-R which are characteristic of most neurones but the mature cells can develop an atypical form with differing kinetic properties (Farrant *et al.*, 1994). Furthermore, the activation of these ligand gated ion channels may be responsible for the cell motility necessary during migration (Rakic and Komuro, 1995). Glutamate can also activate the transcription factor, nuclear transcription factor κB (NF-κB) in cultured CGCs (Guerrini *et al.*, 1995) which may modulate gene expression during development. However, during time in culture, developing granule cells become vulnerable to the toxic effects of excitatory amino acids which is dependent upon the growth conditions. Mature CGCs are more vulnerable to the toxic effects of glutamate in 25 mM K^+ but are unaffected when grown in the presence of 10 mM K^+ and NMDA. This suggests that

25 mM K^+ can remove the voltage dependent block of Mg^{2+} within the channel (Resink *et al.*, 1994). Other studies suggest that the period when CGCs become sensitive to the toxic effects of NMDA is determined by the number of NMDA receptors available. Under conditions when cells are grown in the continuous presence of NMDA there is a significantly lower density of receptors suggesting an activity dependent down regulation that in vivo may protect the cells from excitotoxicity during development (Didier *et al.*, 1994). Besides NMDA receptors, other glutamate receptors are found in CGCs. Both kainate and AMPA can promote neuronal survival in low K^+ cultures suggesting that the ionotropic receptors have distinct functions (Pizzi *et al.*, 1994). However, AMPA receptors also have a developmental profile in culture, that switches from trophic to toxic, becoming toxic when receptor desensitisation is blocked (Hack *et al.*, 1995). Finally, CGCs express metabotropic glutamate receptors (Aronica *et al.*, 1993) and it is proposed that they may control the onset of developmental apoptosis (Copani *et al.*, 1998). There is extensive evidence that the toxicity of ionotropic glutamate receptor agonists is due to disturbances of Ca^{2+} homeostasis in a phenomenon known as excitotoxicity (Ankarcrona *et al.*, 1996; Cebers *et al.*, 1997) and this is reviewed in the subsequent sections beginning with methods available to study Ca^{2+} responses.

1.3.4 Methods in $[Ca^{2+}]_i$ imaging

The spatial and temporal distributions of Ca^{2+} signals can be dissected by the technique of single cell fluorescence Ca^{2+} imaging. It is possible to generate a computerised false colour image of a cell, that represents different concentrations of Ca^{2+} in any particular region. A continuous stream of these images can then be used to record real time changes in Ca^{2+}. These experiments require a chemical probe with optical properties

that can change relative to $[Ca^{2+}]_i$, a fluorescence microscope, a video camera and a computer system to convert the images into real $[Ca^{2+}]_i$.

1.3.4.1 Fluorescent dyes

A variety of fluorescent dyes exist that can be used to measure Ca^{2+} at the subcellular level (Cobbold and Rink, 1987). The advantage of fluorescent probes is that by using a suitable filter, background signals can be minimised. They include quin-2, indo-1, and Fura-2 (Grynkiewicz *et al.*, 1985). The latter is the most commonly used since its Ca^{2+} affinity can be used to measure concentrations in the range 50 nM to 2 μM. Furthermore, it is highly selective for Ca^{2+} over Mg^{2+} which was a problem with the orginal dyes. Fura-2 is taken up into the cell in the membrane permeable form of an acetoxymethyl ester. Once inside the cell it will be hydrolysed to its free acid form and becomes trapped within the cell. Fura-2 responds to changes in wavelength under different excitation conditions. It is possible to quantify the amount of Ca^{2+} activity by taking measurements at two wavelengths (dual wavelength method) if the dissociation constant K_D is known. Binding of fura-2 to Ca^{2+} results in an increase in fluorescence and also shifts the maximal excitation wavelength from 365 nm to 340 nm. At 361 nm there is a point where the fluorescence is independent of Ca^{2+} and it is usual to use wavelengths either side of this point for quantitative measurements of Ca^{2+} (normally at 340 and 380 nm). To work out exact levels of Ca^{2+} ratios need to be measured in the absence of Ca^{2+} (R_{min}) and presence of saturating Ca^{2+} (R_{max}). The free Ca^{2+} can then be assessed using the Grynkiewicz equation:

$[Ca^{2+}] = fK_D[(R-R_{min})/(R_{max}-R)]$ (Grynkiewicz *et al.*, 1985)

Fura-2 is commonly calibrated at the end of an experiment by exposing cells to a Ca^{2+} ionophore such as ionomycin followed by application of 1 mM Ca^{2+} with 10 mM EGTA.

1.3.4.2 Imaging systems

Imaging of cultured cells is performed by using an inverted microscope to measure the fluorescence from the cells and allow experimental manipulations to take place from above. The coverslip is placed into a steel ring and then fitted into a larger temperature controlled stage. A UV lamp provides the source of ultraviolet excitation which is linked to a monochromator. The wavelengths of 340 nm and 380 nm are selected by using a rotating wheel which is comprised of the suitable optical interference filters. The brightness can be altered using a series of adjustable slits. The light passes through a field iris and is reflected by a dichroic mirror where it passes upward via the objective lens to the cell. The fluorescence is then transmitted back via the dichroic and through a set of filters and prisms to the video camera where it is then converted to an image. The output from the camera is stored as a series of frames which are normally corrected for background. Following image processing the end result is an image which has arisen from a process of digitisation and superimposition of pictures at 380 nm and 340 nm. The setup is summarised in Fig.1.3.4.1.

1.3.5. Definition of excitotoxicity

Excitotoxicity can be defined as a neuronal phenomenon occurring when a stimulation is more intense or longer lasting such that it becomes unphysiological. Glutamate is the best studied of the excitotoxic stimulants in the CNS. It is proposed that synaptic

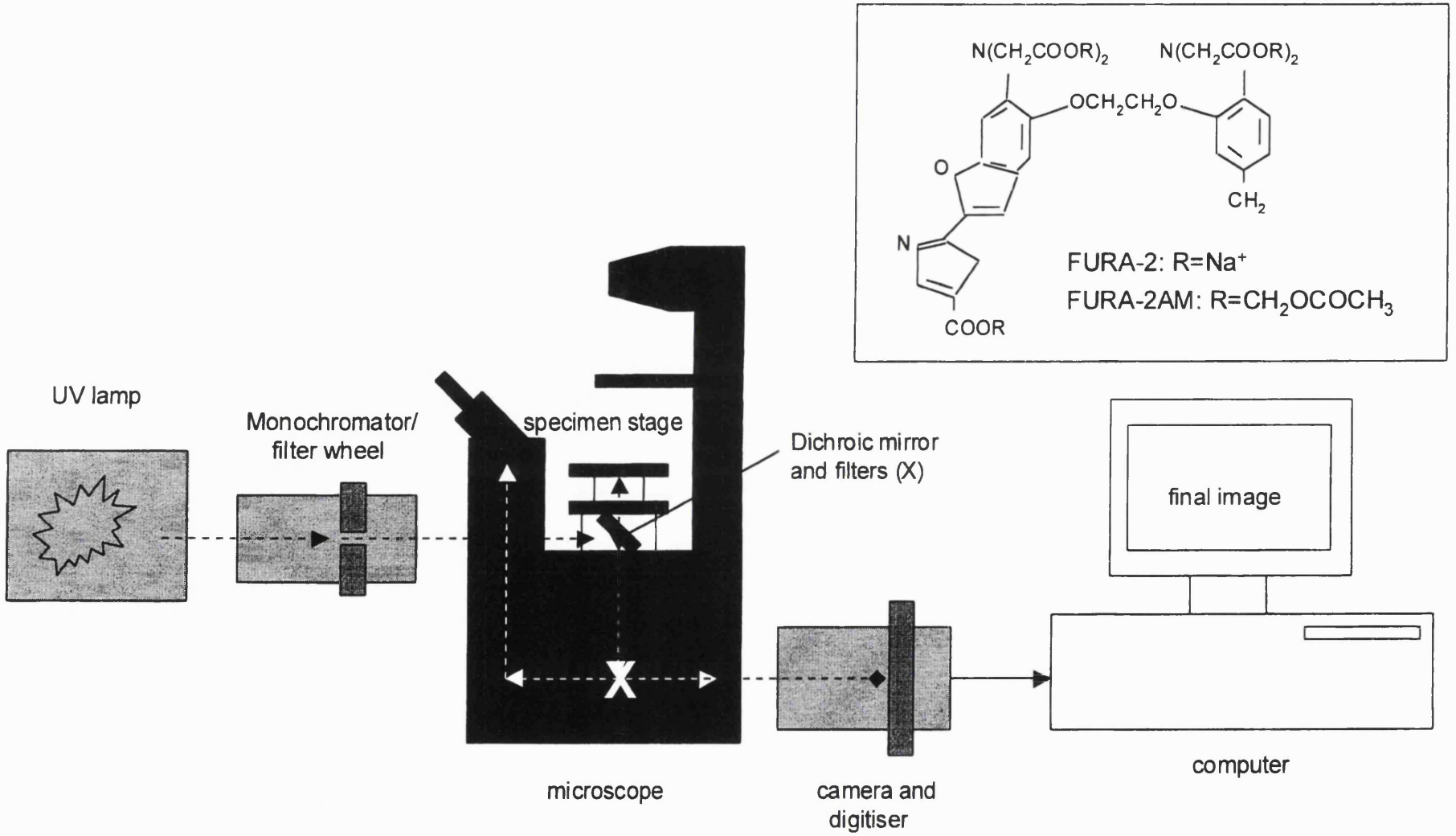


Fig. 1.3.4.1 Schematic of an imaging system and inset the structure of Fura2. Adapted from Sharif, Molecular Imaging In Neuroscience, IRL Press, 1993.

overactivity brought about by the actions of glutamate lead to increases in $[Ca^{2+}]_i$ which may be deleterious to the neurones. With both studies of excitatory amino acid (EAA) induced toxicity in cultured neurones and brain slices suggesting the presence of Ca^{2+} (Choi, 1985), and the development of fluorescent Ca^{2+} sensitive probes, there exists a basis for the calcium hypothesis which states that neuronal Ca^{2+} overload can lead to eventual neurodegeneration (Choi, 1995).

1.3.5.1 Glutamate and Ca^{2+} influx

Glutamate stimulated Ca^{2+} influx may occur via various routes with the best characterised through the NMDA receptor, a Ca^{2+} permeable channel (Seeburg, 1993). Both *in vivo* and *in vitro* models of excitotoxic death demonstrate increases in $[Ca^{2+}]_i$. These include the ischaemic brain (Simon *et al.*, 1984; Valentino *et al.*, 1993; Myseros and Bullock, 1995), NMDA-R agonist application to brain slices (Garthwaite and Gartwaite, 1986) and using fluorescent probes to study NMDA-R stimulation (Tymianski *et al.*, 1993). Secondly, removal of extracellular Ca^{2+} and pharmacological inhibition of NMDA-R prevents neuronal death (Garthwaite and Gartwaite, 1986; Lipton and Rosenberg, 1994; Dubinsky, 1993). Glutamate may also act at the AMPA/kainate ionotropic receptors allowing direct Ca^{2+} influx into the neurones to prolong neurotoxicity (Gu *et al.*, 1996). In addition, agonists of these receptors may result in neuronal depolarisation and thereby relieve the Mg^{2+} block of the NMDA-R (Monyer *et al.*, 1992). Metabotropic glutamate receptors also play a role in controlling the excitotoxicity elicited by ionotropic glutamate receptors (Pizzi *et al.*, 1996; Mukhin *et al.*, 1996). As well as glutamate receptors, other ion channels such as the aspartate receptor on Purkinje cells (Yuzaki *et al.*, 1996) or voltage dependent Ca^{2+} channels (Sucher *et al.*, 1991) can be important mediators of excitotoxicity. In conclusion, by

inhibiting the downstream effectors of Ca^{2+} toxicity it may be possible to prevent excitotoxic neuronal damage.

1.3.5.2 Ca^{2+} dependent effectors of cell death

Many hydrolytic enzymes can be activated by high $[Ca^{2+}]_i$ and are thus implicated in excitotoxicity. The best characterised are the Ca^{2+} dependent proteases or calpains (Saido *et al.*, 1994). These enzymes are responsible for nonlysosomal proteolysis and may function in cytoskeletal remodelling and the modulation of cell mitosis. Activation of these Ca^{2+} proteases has been implicated in excitotoxic neuronal death (Brorson *et al.*, 1994). Nuclear activated Ca^{2+} proteases may also contribute to neuronal death (Nicotera *et al.*, 1994). Recent evidence also suggests that caspases are sensitive to changes in $[Ca^{2+}]_i$ (Moran *et al.*, 1999). Phospholipases may also be responsible for neuronal damage. These enzymes are responsible for the hydrolysis of membrane phospholipids and many require Ca^{2+} for activation. They can cause the release of arachidonic acid and fatty acids from the membrane which can then be further degraded by lipoxygenases or cyclooxygenases to generate free radical species (ROS). The breakdown in membrane structure allows Ca^{2+} influx to occur more freely (Traystman *et al.*, 1991) and the accumulation of arachidonic acid inhibits glutamate uptake and thus may contribute to a more pronounced response from this excitotoxin on its receptors (Volterra *et al.*, 1992). Finally there also exists a group of Ca^{2+} dependent DNases known as endonucleases. These become activated during Ca^{2+} overload and are responsible for the DNA cleavage observed in neuronal apoptosis (Arends *et al.*, 1990).

1.3.6 Apoptotic cell death in excitotoxic conditions

The process of apoptosis occuring during neuronal development has been well studied. In the case of CGCs, caspase precursors are present and may be activated during apoptosis induced by K^+ withdrawal (Armstrong *et al.*, 1997). However, the role of apoptosis and caspases in damage elicited by excitotoxins has only recently been studied. Injection of excitotoxic mediators into the brain causes apoptosis (Portera-Cailliau *et al.*, 1995; Pollard *et al.*, 1994) and also apoptotic cell death is a feature of many stroke models (Li *et al.*, 1995b; Beilharz *et al.*, 1995). Neuronal conditions such as epilepsy or brain trauma which are associated with excitotoxicity show a degree of cellular apoptosis (Charriaut-Marlangue *et al.*, 1996; Kruman *et al.*, 1997; Rink *et al.*, 1995). In addition molecules which affect apoptosis such as Bax protein and Bcl-2 display changes in cerebral ischaemia (Chen *et al.*, 1996; Farlie *et al.*, 1995) and p53, the proapoptotic transcription factor is induced in neurones following kainate treatment (Sakhi *et al.*, 1994). Neurones *in vitro,* exposed to glutamate, exhibit signs of apoptosis (Du *et al.*, 1997;Ankarcrona *et al.*, 1995). The intensity of the Ca^{2+} overload probably determines whether cells will undergo apoptosis. Recent studies in CGCs have addressed this point (Leist *et al.*, 1997b; Bonfoco *et al.*, 1996). Nitric oxide donors were used to activate the NMDA-R and cause Ca^{2+} influx. Caspase inhibitors were able to block the cell death induced by low levels of NO (and Ca^{2+} influx) but were ineffective at higher concentrations suggesting that a more rapid disruption of Ca^{2+} homeostasis may cause caspase dependent steps to be bypassed.

Thus CGCs provide a good model for excitotoxicity and NMDA-R mediated neuronal damage. Another model for glutamate induced neurotoxicity exists which does not involve the NMDA-R and is known as oxidative stress (Ratan *et al.*, 1994). This

phenomenon is subsequently reviewed and a model system from the hippocampus described.

1.3.7 Oxidative neuronal cell death

A cell is described as being under oxidative stress when its antioxidant mechanisms cannot control the levels of free radicals. Free radicals are chemical entities with orbitals that contain unpaired electrons. They can interact with other cellular molecules by either donating their free electrons or accept new ones and in the process inactivate the normal function of these molecules thereby creating a vicious cycle of free radical production (Mark *et al.*, 1997). Reactive oxygen species (ROS) include oxygen containing atoms such as hydrogen peroxide H_2O_2 and peroxynitrite ($ONOO^-$) and these may be produced as part of normal physiology by mitochondrial enzymes, nitric oxide synthase and xanthine oxidase (Saran and Bors, 1990; Halliwell, 1997). This is because the cell contains various mechanisms to control the levels of ROS. The nervous system contains the antioxidant enzymes superoxide dismutase and glutathione peroxidase but lower levels of catalase (Shivakumar *et al.*, 1991). Also present are the antioxidants, vitamins C and E and glutathione (Meister, 1994, 1995). However, once the balance between levels of intracellular oxidants and antioxidants is perturbed neuronal death is likely to occur.

Cell culture studies reveal the involvement of ROS and oxidative stress under conditions that induce apoptosis. For example, CGCs deprived of trophic factors undergo apoptosis as the result of ROS accumulation (Atabay *et al.*, 1996) and hippocampal neurones exposed to staurosporine show increases in superoxide prior to caspase-3 activation (Krohn *et al.*, 1998). Caspases are also activated following

ischaemia and traumatic brain injury both conditions associated with oxidative stress (Yakovlev *et al.*, 1997). In addition the excitotoxin, glutamate can kill neurones by oxidative stress via mechanisms not involving the NMDA receptor. In this instance, the glutamate/cystine antiporter is inhibited which impairs the delivery of the antioxidant, cystine, to the cell (Murphy *et al.*, 1989) This leads to irregular cystine homeostasis, falls in glutathione levels and increases in ROS which results in cell death. Both glutamate and free radicals have been implicated in Alzheimer's diseases suggesting that oxidative stress may play a role (Behl *et al.*, 1994; Coyle and Puttfarcken, 1993). Furthermore, it has been shown that the hippocampal region is one of the earliest to suffer damage in AD so a hippocampal cell line was used to examine how microglial factors might affect this region.

1.3.8 The hippocampus

The hippocampus is part of the limbic system which is involved with memory. It is identified as a C shaped horn found at the base of the temporal lobe. Together with the dentate gyrus and the parahippocampal gyrus it constitutes the hippocampal formation. Extensive animal experiments have revealed the neuronal pathways of this region.

1.3.8.1 Hippocampal organisation and circuitry

The hippocampus seen in transverse section contains three areas known as CA1, CA2, and CA3 and the hippocampal cortex is divided into three cellular layers. Firstly, there is the molecular layer which consists of interacting axons and dendrites. Next is the pyramidal cell layer made up of large pyramidal neurones. The dendrites of these cells extend into the molecular layer. In addition, Schaffer collaterals pass through the outer

layer and pyramidal layers to link synapses with other pyramidal neurones. Pyramidal cells in the CA1 region are particularly sensitive to oxygen deprivation and are also the first to be affected by conditions such as Alzheimer's disease. Finally the outer region is known as the polymorphic layer and consists of a mixture of axons, dendrites and interneurones. The hippocampus engulfs the dentate gyrus which has a predominant granule cell layer in regions where pyramidal cells would exist in the hippocampus. Efferent fibres from the dentate gyrus are known as mossy fibres. The main circuitry patterns are summarised in Fig.1.3.8.1.

1.3.8.2 The hippocampus and memory disorders

The hippocampus and its connections are responsible for the consolidation of new and short term memories (reviewed in Vanderwolf and Cain, 1994). This is shown by the condition known as transient global amnesia in which the hippocampus may become starved of oxygen for a short period of time (Gaffan and Gaffan, 1991). Similar symptoms of short term memory loss occur after concussion in which the hippocampus may be damaged by haemorrhaging. The inability to form new memories in Alzheimer's disease may also be due to degenerative changes in the hippocampus as well as loss of neurones projecting into the hippocampus from the basal forebrain (Van Hoesen and Hyman, 1990).

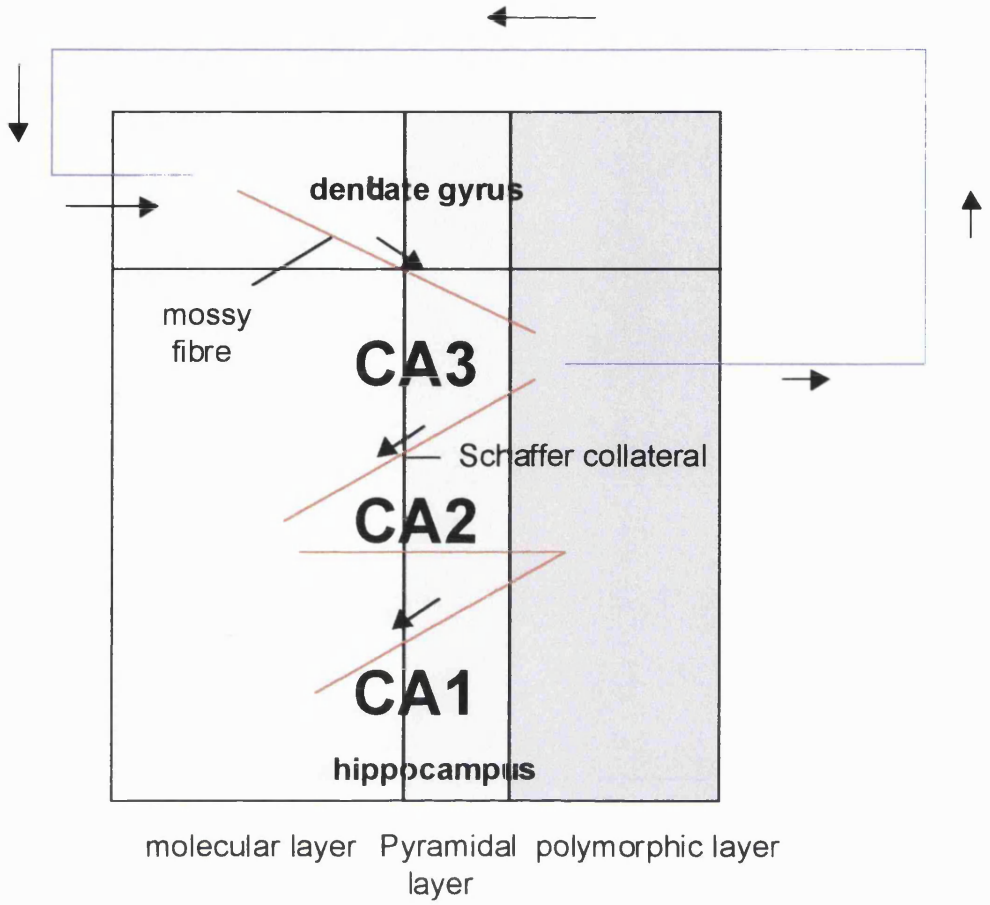


Fig. 1.3.8.1 The neuronal circuits in the hippocampal formation. Neurons of the hippocampus and dentate gyrus are in red and the axons of afferent neurones are blue. Black arrows indicate the connections formed by mossy fibres and Schaffer collaterals. Adapted from Kiernan, Barr's The Human Nervous System, Lippincott, 1998.

1.3.8.3 HT22 cells: a mouse hippocampal cell line

HT22 cells were first described as a subclone from the HT4 immortalised mouse hippocampal cell line (Davis and Maher, 1994) and selected for their particular sensitivity to glutamate induced death. Cell death is mediated via oxidative stress since the antioxidant melatonin was a significant inhibitor (Lezoualc'h *et al.*, 1996) and furthermore it required monamine metabolism as a source of free radicals (Maher and Davis, 1996). More recently, it has been shown that the cell death resembles apoptosis in some morphological respects (Tan *et al.*, 1998a) and a sequence of events involving a fall in glutathione levels followed by ROS mediated caspase activation and subsequent

large increases in ROS levels are responsible for this cell death (Tan *et al.*, 1998b). Thus this model provides an alternative system to the CGC in which to study microglial-neuronal interactions.

2. MATERIALS AND METHODS

2.1 Materials

Minimal essential medium (MEM), Dulbecco's Modified Eagle Medium (DMEM) and foetal calf serum (FCS) were obtained from Life Technologies Ltd (Paisley, UK). All tissue culture plasticware was from either Greiner (Gloucestershire, UK) or Triple Red Laboratory Technology (Oxford, UK) and coverslips from Scientific Laboratory Supplies (Nottingham, UK). Chromogranin A was purchased from Scientific Marketing Associates (Herts, UK). Rhodamine 123 and 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazole carbocyanine iodide (JC-1) were from Molecular Probes (Leiden, The Netherlands) and fluorescein diacetate (FDA), propidium iodide (PI), and Hoechst 33342 were from Sigma (Dorset, UK). Tetramethyl rhodamine ethyl ester (TMRE) was a gift from Dr Huseyin Mehmet, Hammersmith Hospital and fura-2 AM was purchased from Calbiochem (Nottingham, UK). N^G-Nitro-L-arginine (L-NNA), Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO), benzylocarbonyl-Asp-Glu-Val-Asp-fluormethyl ketone (z-DEVD-fmk), and FK506 were from Calbiochem (Nottingham, UK) and 2-amino-5,6-dihydro-6-methyl-4H-1,3,-thiazine hydrochloride (AMT-HCl) and (RS)-α-methyl-4-sulphonophenylglycine (MSPG) were obtained from Tocris (Bristol, UK). Griess reagent and nitrate reductase were from Sigma (Dorset, UK). The glutamate assay kit and ATP bioluminescence kit were purchased from Roche Molecular Biochemicals (Lewes, UK). Bradford protein assay reagent was from Pierce and Warriner (Chester, UK). Enhanced chemiluminescence (ECL) substrate and Percoll were obtained from Amersham (Amersham, UK). OX-42 (IgG1) was from Dako (Ely, UK), ED-1 (IgG1) was from Serotec (Oxford, UK) and glial fibrillary acidic protein antibody (GFAP; monoclonal clone no G-A-5) was from Sigma (Dorset, UK). The biotin-avidin peroxidase was purchased from Vector Laboratories (Oxford, UK). The anti-cytochrome c (clone 6H2.B4), anti-cytochrome oxidase (subunit V, clone 20E8),

and anti-caspase 1 (c20) antibodies were from Pharmingen UK and anti-actin antibody from Sigma (Dorset, UK). Anti-iNOS (IgG1, clone 54) antibody was from Transduction Laboratories and the anti-mouse IgG-HRP was from SAPU (UK).

2.2 Microglial cell culture

2.2.1 Method 1

Microglial cells were isolated from 7 day old Wistar rat pups by a modification of methods previously described (Slepko and Levi, 1996; Ford *et al.*, 1995). Brains were removed and transferred to ice cold specially prepared phosphate buffered saline (PBS) (140 mM NaCl, 5 mM KCl, 25 mM Na_2HPO_4, 11 mM glucose, pH 7.4), visible blood vessels (meninges) removed and the tissue minced with a McIlwain tissue chopper into 375 μm sections. The material was collected and washed with PBS by centrifugation at 400 g for 10 min. The pellet was resuspended and enzymatically dissociated with 50 U type XI collagenase and 400 U DNase I per brain diluted in enzyme buffer (20 mM $MgCl_2$,17 mM $CaCl_2$, 50 mM KCl, and 153 mM NaCl, pH 6.5). After 45 min digestion the cells were twice washed by centrifugation at 400 g for 10 min using 40 ml PBS, and then combined with 21.4 ml PBS (per 2-3 brains) and 8.6 ml of an isotonic Percoll solution (diluted 9 parts Percoll plus 1 part 10X PBS). This suspension was then centrifuged at 500 g for 20 min, the pellet resuspended, washed as before and transferred to a discontinuous Percoll gradient consisting of 10 ml steps of the following densities; 1.088 g/ml, 1.072 g/ml, 1.065 g/ml, 1.03 g/ml. The gradient was centrifuged at 1250 g for 20 min at 20°C and cells collected from the 1.065-1.072 g/ml interface. Any contaminating cells were separated from microglia at the 1.072-1.088 g/ml interface. After washing by centrifugation at 400 g for 10 min, the pellet was resuspended in a small volume of minimal essential medium (MEM) supplemented with 25 mM KCl, 30 mM glucose, 25 mM $NaHCO_3$, 1 mM

glutamine, 10% foetal calf serum and 50 U/ml penicillin plus 50 μg/ml streptomycin. The cells were counted using a haemocytometer and plated onto 13 mm coverslips in 90 μl suspensions containing 1 X 10^5 cells, allowed to adhere for 1 h, and then replenished with the same supplemented MEM. Cells were maintained in the above culture medium at 37°C with 5% constant CO_2 and used at 1-2 days *in vitro* (DIV).

2.2.2 Method 2

An alternative method to enzyme digestion (Slepko and Levi, 1996; Ford *et al.*, 1995) was sought since such treatment may account for the early basal activation levels observed in culture. Thus microglia were prepared according to Havenith *et al.*, 1998 with modifications. Brains were removed onto ice cold PBS (140 mM NaCl, 5 mM KCl, 25 mM Na_2HPO_4, 11 mM glucose, pH 7.4) and then gently homogenised with 10-15 strokes using a hand held homogeniser (Wheaton, USA). Next the suspension was centrifuged at 500 g for 10 min and the pellet from 4 neonate rat brains resuspended in 10 ml of 70% isotonic Percoll (a 100% stock Percoll is made from 9 parts Percoll plus 1 part 10X PBS). This was overlayered with 10 ml of 35 % isotonic Percoll and 10 ml PBS. The Percoll gradient was then centrifuged at 1250 g for 45 min at 20°C and the cells found at the 35/70 % interface removed, pooled and washed once in PBS at 500 g for 10 min. The cells were plated on coverslips at a density of 1 X 10^5 and maintained at 37°C with 5% constant CO_2. Cultures were used at 1-2 DIV.

Beside the preparation of neonate tissue, microglia were isolated from adult rat brains. Method 2 was used for this since low cell yields were obtained using enzyme digestion with little improvement even when the enzyme concentrations were increased ten fold. However, the number of brains was limited to 2 per gradient to ensure maximum

extraction of cells from the dense myelin/debris layer. Also human microglial preparations were provided by Dr D. Gveric, Institute of Neurology.

2.2.3 N9 microglial cell line

N9 microglial cells (Corradin *et al.*, 1993) were grown in tissue culture flasks and then using a cell scraper removed into Dulbecco's modified eagle medium (DMEM) supplemented with 4.4 mM $NaHCO_3$, 50 μM β-mercaptoethanol, 5% foetal calf serum and 50 U/ml penicillin plus 50 μg/ml streptomycin. Cells were pelleted by centrifugation at 200 g for 5 min, resuspended, counted using a haemocytometer and then either plated onto 13 mm coverslips at a density of 10 X 10^4/coverslip or 5 X 10^5 cells in a 35 mm tissue culture dish. Cells were maintained in the above culture medium at 37°C with 5% constant CO_2 and used one day after plating.

2.3 Neuronal cell culture

2.3.1 Rat cerebellar granule cell culture

Cerebellar granule neurones were isolated from 5-7 day old Wistar rat pups and prepared as previously described (Pocock *et al.*, 1995). Cerebella were removed into a filter sterilised solution A consisting of 100 mM phosphate buffered saline (PBS), 0.3% fatty acid free bovine serum albumin (FAF-BSA), 10 mM glucose and 0.38% $MgSO_4.7H_2O$. The meninges were removed using forceps before the tissue was minced with a McIlwain tissue chopper into 375 μm sections. The material was enzymatically digested with 4 mg/ml trypsin dissolved in solution A, for 20 min at 37°C and followed by the addition of solution B (solution A containing 500 U DNase I and 0.5 mg soybean trypsin inhibitor; SBTI). The latter solution prevents the suspension becoming gelatinous and inhibits any

further action of the trypsin thus allowing subsequent processing of the sample. Following centrifugation at 180 g for 1 min the loose pellet was resuspended in 2 ml of solution C, a 6X concentrated solution of the DNase/SBTI mixture. This was performed by trituration with glass pipettes of decreasing aperture until there were few clumps present and the solution was as homogenous as possible. The cell suspension was then transferred onto the top of a 4% BSA/Earles salts solution. This was centrifuged at 180 g for 5 min at room temperature to produce a pellet which was subsequently resuspended in a small volume of supplemented MEM as used in the microglial cultures.

The cell suspension was diluted 1 in 10 to allow a suitable density of cells to be counted using a haemocytometer. Cells were then plated at a density of 0.3 x 10^6/coverslip on 13 mm glass coverslips which had been coated with poly-D-lysine 24 h previously. Briefly, coverslips had been prepared by soaking them in ethanol for 2 days and then further sterilised by placing them at 220°C overnight. The next day the coverslips were incubated in sterile poly-D-lysine (15 mg/l) for 1 h at 37°C with frequent gentle inversion to ensure even coating of the coverslips and then allowed to dry overnight. Cells were maintained in supplemented MEM and after 24 h *in vitro* 10 μM cytosine arabinofuranoside was added to prevent proliferation of non neuronal cells such as astrocytes. The cultures were maintained at 37°C with constant 5% CO_2 and the medium changed on day 6. Cells were used at 10-12 DIV by which time the neurones had acquired an extensive network of neurites and resembled a differentiated phenotype.

2.3.2 HT22 hippocampal cell line

HT22 hippocampal cells (Davis and Maher, 1994) were grown in tissue culture flasks and then removed into culture medium using 0.25% trypsin. Following the removal of excess solution the cells were left at room temperature until they began to round up and detach from the flask. Culture medium made from Dulbecco's modified eagle medium (DMEM) with 10% FCS and 50 U/ml penicillin plus 50 μg/ml streptomycin was added to the cells which were then pelleted by centrifugation at 200 g for 1 min. Cells were resuspended, counted using a haemocytometer and then plated at a density of 10 X 10^4/coverslip or 2 X 10^5 cells in a 35 mm tissue culture dish. Cells were maintained in the above culture medium at 37°C with 5% constant CO_2 and used either 1 or 2 days following sub-culture.

2.4 Immunocytochemistry

To determine the purity of isolated microglia and the level of contaminating cells in neuronal cultures, cell specific antigens were analysed by immunolocalisation. This involved incubating the cells with primary antibody which could subsequently be detected using an avidin-biotin peroxidase technique (Hayes *et al.*, 1988). Such a method has the advantage that the primary antibody signal is amplified through the high affinity binding of avidin to biotin. Using diaminobenzidine tetrahydrochloride (DAB) as substrate results in the formation of a brown precipitate which can be viewed by light microscopy.

A number of primary antibodies were used; the microglia specific marker OX-42 (an anti-CR3 complement receptor antibody), ED1 (a marker for activated microglia which recognises a lysosomal protein), or anti-glial fibrillary acidic protein (GFAP) antibody, an astrocytic marker. Cells cultured on coverslips were fixed in either ice-cold ethanol (OX-

42), acetone (ED1), or 5:95 acetic acid:methanol (GFAP) for 10 min, washed twice in PBS and then blocked for 1 h in 1% BSA/PBS at room temperature. Excess blocking solution was removed from the edge of the coverslip by using absorbent tissue paper and then 25 µl of primary antibody diluted at 1:200 in blocking solution applied to the coverslips. Samples were then incubated for 1 h in a humid glass chamber to maximise antibody binding and minimise evaporation of the small volumes used. Controls were performed using 25 µl of blocking buffer instead of primary antibody. After four washes with PBS, the samples were replaced in the humid chamber and incubated with biotinylated anti-mouse IgG antibody (1:200 dilution) for 1 h followed by pre-formed avidin-biotin-horseradish peroxidase complex for 45 min. Following a brief wash with PBS, immunostaining was visualised with 0.5 mg/ml DAB in PBS with 0.03% H_2O_2. The samples were then washed extensively in H_2O and the coverslips mounted in an aqueous mountant before viewing under a light microscope.

2.5 Assessment of cell viability and apoptosis

Two different fluorescent dyes were used throughout the course of these studies fluorescein diacetate (FDA) to label live cells green and propidium iodide (PI) to label dead cells red (Bonfoco *et al.*, 1996). The assay works on the principle that staining is dependent upon the integrity of the plasma membrane. FDA is membrane permeable and on entry into the cell it is cleaved by endogenous esterases to a non-membrane permeable product which has a green fluorescence (530 nm) and therefore labels the cytoplasm of live cells. PI, can only bind directly with DNA when the plasma membrane has become compromised since it is non-membrane permeable. Thus PI complexes with DNA to label the nuclei of dead cells with a strong red fluorescence (>600 nm).

Microglial cell cultures were treated according to individual experimental protocols and then the number of live and dead cells assessed. When investigating the effects of microglial conditioned medium on neuronal viability the medium was either filtered using a 0.2 μm pore cellulose acetate filter or centrifuged at 13,500 g for 5 min to remove cellular material prior to addition to the neuronal cultures. Following treatments in culture medium the coverslips were briefly washed in basic medium (BM) consisting of 153 mM NaCl, 3.5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.4 mM KH_2PO_4 and 20 mM TES (N-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid) pH 7.4 (Pocock *et al.*, 1993) and then incubated with 15 μg/ml FDA and 5 μg/ml PI for 30 min at 37°C. Then the number of live (green) cells and dead (red) cells were observed under a fluorescence microscope using fluorescein and rhodamine interference filters respectively. Cells were scored in a minimum of 8 fields per coverslip on at least two coverslips per treatment and experiments performed using at least two different cell preparations ($n \geq 32$). This and all other data was analysed by Students *t*-test.

There are two distinct forms of cell death known as necrosis and apoptosis. Fast necrotic cell death is characterised by cell swelling and rupture of the plasma membrane and thus can be recorded using uptake of PI. This dye will also label late apoptotic cells when plasma membrane integrity can become compromised but not the early stages and thus the degree of apoptosis may be underestimated. Chromatin condensation and the appearance of pyknotic nuclei are two early changes characteristic of apoptosis. The fluorescent dye Hoechst 33342 (bisbenzimide trihydrochloride) labels the nucleus of 'living cells' and thus can be used to show nuclear aberrations (Yan *et al.*, 1994). Following treatment of cultures, medium was removed and cells washed in PBS and then fixed for 10 min in 4 % para-formaldehyde at 4°C. Following another wash in PBS the cells were stained with

Hoechst 33342 (5 μg/ml) for 10 min. Nuclear morphology was viewed using a fluorescent microscope with excitation at 365 nm, emission >490 nm. The number of condensed brightly stained nuclei indicative of apoptosis were counted and compared with control cultures. Cells were scored in a minimum of 8 fields per coverslip on at least two coverslips per treatment and experiments performed using at least two different cell preparations ($n \geq 32$).

2.6 DNA fragmentation analysis

Another method to distinguish between necrotic and apoptotic mortality is to use agarose gel electrophoresis. Necrotic cell death is characterised by the random digestion of DNA in contrast to apoptosis which gives rise to the non random fragmentation of DNA (Wyllie, 1980). DNA can be rapidly isolated and then analysed by gel electrophoresis to detect this characteristic 'laddering' pattern indicative of apoptosis. Thus DNA was isolated by a modification of the method of Laird *et al.*, 1991. Following experimental treatment of cultures, medium was removed, the cells washed once in PBS and then lysed into buffer consisting of 100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, proteinase K (100 μg/ml) pH 8.5 for 1 h at 55°C. Any 'floating' apoptotic cells were added to this mixture following centrifugation of the culture medium. After lysis, DNA was precipitated by the addition of one volume of isopropanol with continuous shaking for 1 h at room temperature. DNA was pelleted by centrifugation at 14,000 rpm for 15 min and resuspended in TE buffer consisting of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5. The DNA concentration was determined spectrophotometrically according to 1 OD at 260 nm = 50 μg/ml. A 1% agarose gel was prepared with 1 g agarose dissolved in 100 ml of TBE buffer (100 mM Tris-HCl, 100 mM borate, 0.1 mM EDTA pH 8.5) and 5 μg/ml ethidium bromide. 1 μg of total

DNA was resuspended in sample buffer (0.25% bromophenol blue and 40% (w/v) sucrose) and loaded onto the gel along with one lane of pre-stained ϕX174 DNA/Hae III standard base pair ladder (Promega). DNA was resolved for 2 h at 125V, the gel removed from the tank and destained in H_2O for 15 min. Fragments were visualised under UV illumination.

2.7 Nitrite and nitrate measurement

Determination of nitrite (NO_2^-) was carried out using Griess reagent (Ding *et al.*, 1988) to give an approximation of nitric oxide (NO) production. The product of nitric oxide synthase, NO, rapidly decays and is oxidised to nitrite a stable end product which can be easily assayed using this test. Another breakdown product is nitrate which can be converted to nitrite in the presence of nitrate reductase and thus taken together with a measurement of nitrite this method gives a more accurate measurement of NOS activity (Green *et al.*, 1982). 200 µl supernatants from control and treated cultures were combined with nitrate reductase (0.025 U/ml) and β-NADPH (100 µM) and incubated at 37°C for 15 min. The mixture was then combined with lactate dehydrogenase (100 U/ml) and sodium pyruvate (100 mM), vortexed gently and incubated at 37°C for 5 min. An equal volume of Griess reagent [0.1% (w/v) N-(1-naphthyl)-ethylenediamine plus 1% (w/v) sulphanilamide in 5% H_3PO_4 (v/v)] was then combined with the mixtures and incubated for 15 min in the dark at room temperature. The optical density of this solution was recorded spectrophotometrically at 540 nm and compared with a standard curve constructed with nitrite (0-100 µM) in cell culture medium. The efficiency of conversion of nitrate to nitrite was determined by comparing the optical density of treated nitrate standards with nitrite standards (Figure 2.7.1).

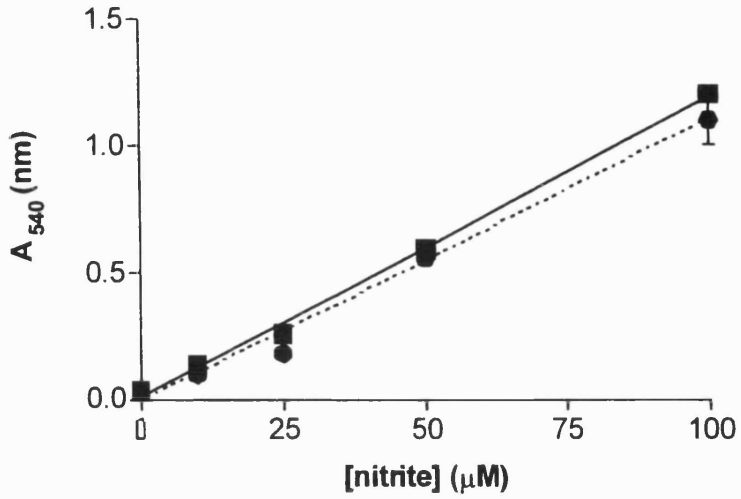


Figure 2.7.1 Typical linear standard curves for nitrite and nitrate following conversion to nitrite. Nitrite standards (squares) were incubated with an equal volume of Griess reagent for 15 min and the optical density recorded at 540 nm. Nitrate standards (circles) were first converted to nitrite with nitrate reductase and β-NADPH. The high efficiency of conversion is apparent as the two traces are virtually superimposable.

2.8 Determination of glutamate content in microglial supernatants

Cell culture supernatants were assessed for levels of glutamate by a colorimetric method using a commercially available kit (Boehringer Mannheim) according to previously published methods (Beutler, 1985; Piani and Fontana, 1994). The following two enzymatic reactions are coupled to generate a coloured end product (formazan):

(1) L-glutamate + NAD^+ + $H_2O \leftrightarrow$ 2-oxoglutarate + NADH + NH_4^+

(catalysed by glutamate dehydrogenase)

(2) NADH + INT + $H^+ \rightarrow NAD^+$ + formazan

(catalysed by diaphorase)

Briefly, in a 96 well plate, 60 μl of potassium phosphate/triethanolamine buffer, pH 8.6 was combined with 20 μl of NAD/diaphorase solution (0.03 U diaphorase and 0.2 mg

NAD) followed by the addition of 20 µl of cell culture media (diluted in 180 µl of H_2O) from experimental samples. Next 20 µl of INT was added, the mixture allowed to equilibrate and the reaction started with 3 µl of glutamate dehydrogenase (2.7 U). The optical density was recorded at 450 nm in a microplate reader until the reaction had reached an end point after 30 min. Since the culture media contained substances that can interfere with the reaction it was necessary to record the change in absorbance in media not exposed to cells. This blank was subtracted from test samples to give an accurate measurement of glutamate levels. Absolute values were obtained by constructing a standard curve using known concentrations of glutamate (1-10µg was within the linear range of the assay, see Figure 2.8.1) added to culture media. Experiments were performed on a minimum of two separate cultures.

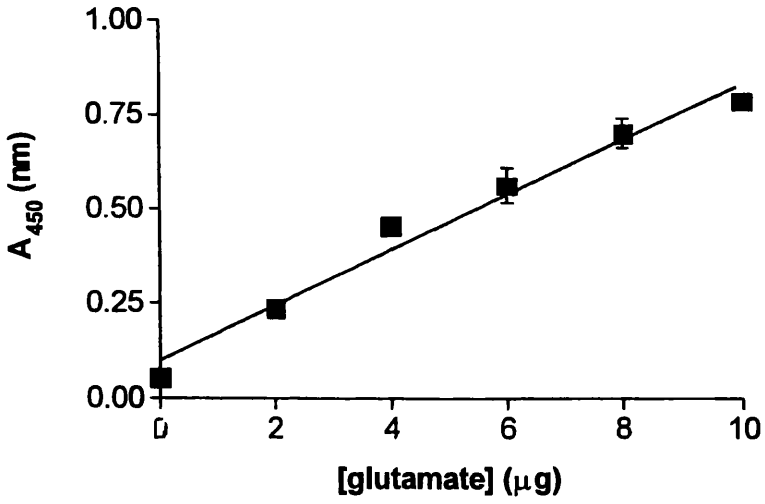


Figure 2.8.1 A typical linear standard curve for serial dilutions of a glutamate standard. Different quantities of glutamate standard were incubated with 20 µl INT solution, 20 µl NAD^+/diaphorase solution and 3µl of glutamate dehydrogenase. After 30 min the optical density at 450 nm was recorded in a microplate reader.

2.9 Measurement of lactate dehydrogenase (LDH) activity in culture supernatants

Cells release the cytoplasmic enzyme, lactate dehydrogenase, into the culture media when they are damaged. This can be assayed colorimetrically by following the conversion of iodonitrotetrazolium (INT) to a formazan in the presence of excess lactate (Klegeris and

McGeer, 1997). Briefly, using a 96 well plate, 100 µl of cell culture media was combined with 15 µl of a 50% lactate solution and 15 µl INT solution. The reaction was started by the addition of 15 µl NAD^+/diaphorase solution and was routinely complete within 20 min. The optical density of the mixture was recorded at 450 nm in a microplate reader and the levels of LDH determined from a standard curve (Figure 2.9.1).

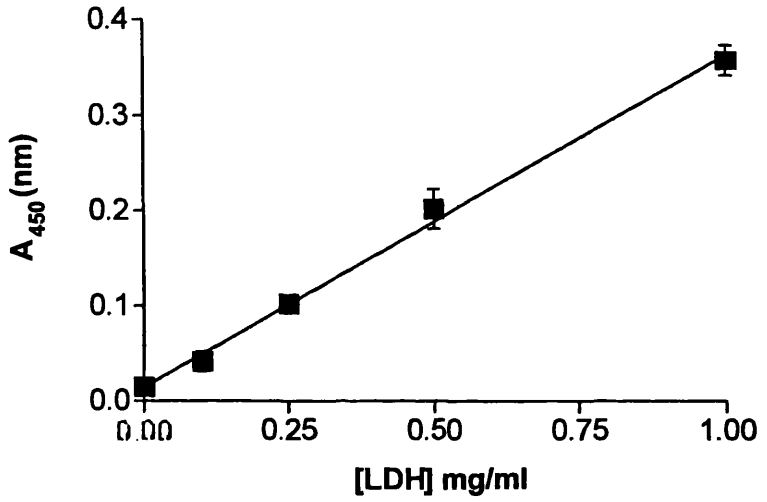


Figure 2.9.1 A typical linear standard curve for serial dilutions of lactate dehydrogenase. Increasing volumes of LDH standard were incubated with 15 µl INT solution and 15 µl NAD^+/diaphorase solution for 20 min and the optical density recorded at 450 nm in a microplate reader.

2.10 Measurement of cellular ATP/ADP levels

For cellular homeostasis to be maintained, high levels of free energy are required to drive such processes as macromolecular synthesis and the active transport of molecules and ions. ATP has two high energy phosphoanhydride bonds at the terminal phosphate group and thus serves as a suitable candidate as the carrier for this energy. The turnover rate of ATP is high with its continual regeneration from ADP. Thus a characteristic of cells under stress, particularly those undergoing necrosis, is a depletion of ATP levels. ATP can be assayed by measuring the bioluminescence generated during the following luciferase catalysed reaction:

$ATP + D\text{-luciferin} + O_2 \rightarrow oxyluciferin + PP_I + AMP + CO_2 + light$

using a luminometer. Any ADP in the reaction mixture can be converted to ATP in the presence of pyruvate kinase allowing for the calculations of final ATP:ADP ratios. Thus cells were treated experimentally in culture medium. This was then removed and the cells scraped into 100 µl ice cold acidic solution (10% perchloric acid and 50 mM EDTA). This was then vortexed briefly, centrifuged at 10000 g for 2 min and the supernatant combined with 42 µl of neutralising solution (3 M KOH and 1.5 M Tris base). The samples were then vortexed and centrifuged once more for 2 min. 5 µl of supernatant was combined with 185 µl of dilution buffer, 20 µl of pyruvate kinase (8 U), and 200 µl of luciferase reaction solution. The chemiluminescence was recorded in a Jade luminometer for 1 min (to give an indication of ATP levels) and then 20 µM phosphoenol pyruvate added to convert ADP to ATP. The concentrations of ATP and ADP were determined from standard curves (Figure 2.10.1) and expressed as µmoles/mg protein.

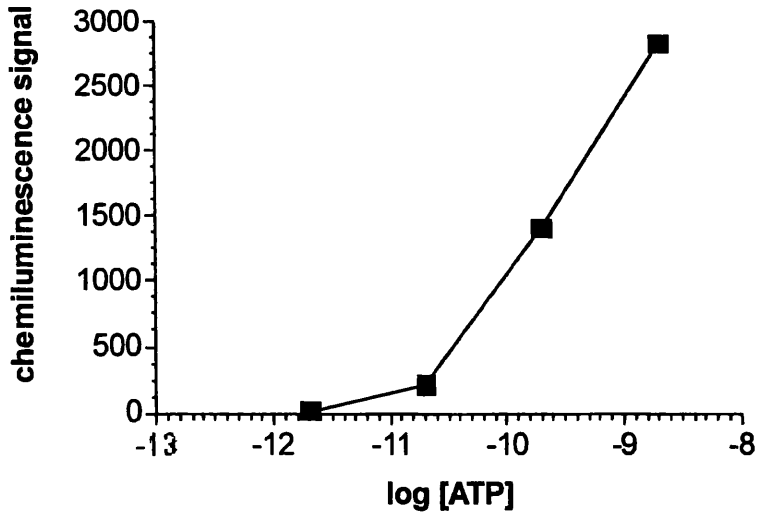


Figure 2.10.1 Typical standard curve for the chemiluminescence produced with serial dilutions of ATP. 5 µl of ATP standards were assayed in the presence of 185 µl of dilution buffer, 20 µl of pyruvate kinase and 200 µl of luciferase reaction solution. The chemiluminescence was recorded in a luminometer.

2.11 Measurement of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was assessed semi-quantitatively by using the fluorescent probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanine iodide (JC-1), which can selectively label mitochondria (Reers *et al.*, 1991). JC-1 monomers appear green when excited at 490 nm but if the mitochondrial membrane is polarised these can form aggregates and the colour of the dye changes to red or orange (Salvioli *et al.*, 1997). Mitochondria are not discernible above the green cytoplasmic fluorescence observed when JC-1 is in monomeric form but those labelled with aggregated JC-1 can be seen as discrete entities and morphological changes can be observed. Cells on coverslips were treated according to the experimental protocol, removed from culture medium and then briefly washed in BM. The cells were then loaded with 5 μM JC-1 dissolved in basic medium at 37°C for 10 min. After loading, the coverslips were washed extensively in basic medium to remove any non specific deposits of JC-1 and then mounted on the stage of an Olympus IX70 epi-fluorescence microscope. Using a dichroic filter set with excitation at 490 nm and emission >520 nm it was possible to view both cells which had discrete mitochondria fluorescing red and cells with depolarised mitochondria which appeared green. The absolute numbers labelled red and green were counted in 8 fields per coverslip and the degree of mitochondrial depolarisation expressed as the percentage green of total cells counted. A 550 nm emission filter (Omega Optical emission filter XF32 590DF35) was used to exclude the green fluorescence and allow the cells with red polarised mitochondria only to be counted. Mitochondrial membrane potential was also analysed by loading the cells with 0.5 μg/ml rhodamine 123 for 20 min at 37°C, or 200 nM tetramethyl rhodamine ester (TMRE) for 90 min at 37°C. Imaging with rhodamine 123 was carried

out at excitation 485 nm, emission >530 nm and TMRE fluorescence was recorded with excitation at 530 nm and emission >550 nm.

2.12 Cell lysis and protein preparation

Cells were grown on tissue culture dishes, treated experimentally and then whole cell lysates prepared for analysis by SDS-PAGE and Western blotting. Culture medium was removed and the dishes placed on ice. Cells were scraped into 100 µl of an ice cold lysis buffer containing detergent to solubilise proteins and a mixture of protease inhibitors to prevent protein breakdown (20 mM Tris-acetate, 1 mM EDTA, 1 mM EGTA, 10 mM Na β-glycerophosphate, 1 mM Na-orthovanadate, 1% Triton X-100, 0.27 M sucrose, 1 mM benzamidine, 4 µg/ml leupeptin and 0.1% mercaptoethanol, pH 7.4). The mixture was incubated on ice for 10 min, vortexed briefly and the nuclei pelleted by centrifugation at 13,200 g for 10 min. The supernatant contained the solubilised proteins which were subsequently assayed for protein.

For preparation of mitochondrial and cytosolic fractions microglial cells were grown in tissue culture plates, treated experimentally and then scraped into ice cold buffer consisting of 25 mM sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4. The cell suspension was then gently homogenised with 10-15 strokes until a smooth homogenate was obtained. Following centrifugation at 500 g at 4°C to pellet the nuclei the supernatant was removed and centrifuged at 10,000 g for 10 min at 4°C. The resultant supernatant was taken as the cytosolic fraction and the resuspended pellet as mitochondria and samples assayed for protein.

2.13 Bradford protein assay

The concentration of protein in the various preparations was determined by the technique of Bradford (Bradford, 1976). A standard curve of BSA (0.5-3 mg/ml protein) was constructed and 5 µl of sample cell lysate/preparation was added to tubes in duplicate. 1 ml of Bradford protein reagent was pipetted into the tubes and the solutions vortexed. After 5 min the absorbance at 595 nm was recorded against a blank of Bradford reagent and the protein concentration determined from the BSA standard curve. Using 5 µl of sample ensured that the protein concentration was within the linear range of the diluted BSA standard (Figure 2.13.1).

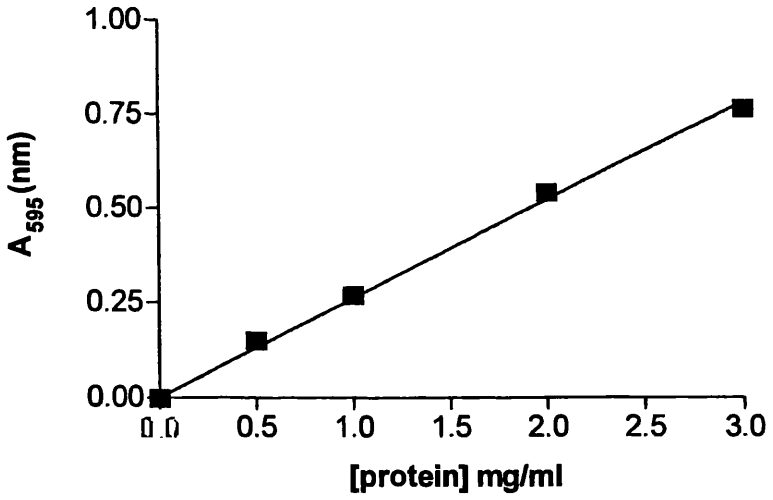


Figure 2.13.1 A typical linear protein standard curve for serial dilutions of BSA. 1 ml of Bradford reagent was added to varying concentrations of BSA and the absorbance recorded at 595 nm in a spectrophotometer.

2.14 SDS-polyacrylamide gel electrophoresis

Proteins in whole cell lysates and other preparations were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a BioRad Mini-Protean II electrophoresis cell. Gels were poured using the BioRad Casting Stand through two glass plates which had been thoroughly cleaned with ethanol. Depending upon the molecular

weight of the proteins to be separated the resolving gel comprised 8-15% acrylamide plus 0.2% bis-acrylamide 375 mM Tris/HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulphate and 0.05% TEMED. Above this a 4% stacking gel was cast made from 125 mM Tris/HCl, pH 6.8, 0.1% SDS, 4% acrylamide, 0.1% bis-acrylamide, 0.05% ammonium persulphate and 0.1% TEMED. 20-45 μg protein was dissolved in Laemmli sample buffer (Laemmli, 1970) (1% SDS, 5% glycerol, 1.25% mercaptoethanol, 62.5 mM Tris/HCl, pH 6.8 and 1% bromophenol blue) and loaded into the wells. 5 μl of pre-stained molecular weight standards (Sigma) were usually loaded in the end lane of the gel. The gel was electrophoresed in running buffer (125 mM Tris, pH 8.3, 1 M glycine and 0.01% SDS) at constant voltage of 180 V until the dye front had just begun to disperse. Typically this took 45-60 min. The glass plates were then disassembled and the gel removed and either Coomassie stained with 40% acetic acid, 20% methanol and 0.004% Coomassie brilliant blue for 1 h and then destained for 2 h with 40% acetic acid and 20% methanol or prepared for electrophoretic transfer.

2.15 Protein transfer and immunoblotting

Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membrane (0.45 μM pore size) using a BioRad Mini Trans-Blot electrophoretic transfer cell. The gel was first equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 0.01% SDS, 20% (v/v) methanol) for 20 min. Meanwhile, the PVDF membrane was activated in methanol for 1 min, washed in H_2O for 5 min and then incubated in transfer buffer for 15 min. A 'wet' transfer sandwich was constructed by laying a fibre pad on the inside of the gel cassette followed by two pieces of Whatmann 3MM paper and then the gel was laid on top of this. The activated PVDF membrane was then carefully lowered over the gel and a glass rod used to roll out any air bubbles. This was finally covered with a further two

pieces of Whatmann 3MM paper and another fibre pad and the cassette closed and slotted into the holder in the transfer tank. The tank was filled with transfer buffer and an ice pack inserted to preventing any overheating which might distort protein transfer. The samples were then electrophoretically transferred at either 80 V for 1 h or at 22 V overnight. The sandwich was then disassembled and the success of transfer indicated by the intensity of staining from the molecular weight markers. Alternatively Ponseau-S stain was used to highlight the proteins on the membrane. 0.5% Ponseau-S plus 1% acetic acid was added for 1 min and then excess washed off with H_2O until distinct bands could be resolved. The membrane was now ready for immunoblotting.

The membrane was first washed in Tween 20-Tris buffered saline (TTBS) consisting of 0.05 % Tween-20, 10 mM Tris/HCl pH 7.4, 150 mM NaCl to remove any residual transfer buffer. Next the membrane was incubated with blocking buffer (TTBS with 5 % (w/v) low fat skimmed milk powder) for 1h at room temperature with constant agitation to minimise any non specific antibody interactions. This was then replaced with primary antibody diluted in blocking buffer for at least 1 h followed by 3 x 10 min washes in TTBS. Then horse radish peroxidase (HRP)-conjugated secondary antibody at a suitable dilution in blocking buffer was added to the membrane for 1 h at room temperature and the blotting procedure was completed with a final 3 X 10 min washes in TTBS. Thus, via the interaction of primary and secondary antibodies any HRP coupled to the proteins of interest was detected with ECL substrate solution. Any excess TTBS was removed from the edges of the membrane with absorbent tissue paper and the membrane incubated with 2 ml of each ECL reagent. The membrane was dried by blotting between two pieces of Whatmann 3 MM paper, enclosed in a Saran wrap and affixed to an X-ray film cassette. The blot was then exposed to X-ray film for 1-30 min depending upon the intensity of the signal.

It was possible to probe a membrane with more than one antibody and each blot was typically screened with anti-β actin antibody to ensure that equal protein concentrations had been loaded throughout the wells. Any previously bound antibodies were removed from the membrane by incubation with 100 mM glycine, pH 2.9 for 30 min at room temperature with constant agitation and the membrane was ready to use again following several washes in TTBS. To quantify any differences in intensity of bands of interest the autoradiograms were scanned with a Molecular Dynamics densitometer and integrated using analysis software (Sunnyvale, USA).

2.16 Single cell $[Ca^{2+}]_i$ fluorescence imaging

The technique of calcium imaging in single cells has enabled the dissection of the spatial and temporal distributions of calcium signals that may control cellular function. It is possible to generate a picture of the cell and the concentration of Ca^{2+} ($[Ca^{2+}]_i$) at any particular region may be represented by the degree of brightness of the image. By collecting a continuous stream of these images it is possible to record real time changes in $[Ca^{2+}]_i$. A number of fluorescent indicators all based on structures developed by Tsien are used in the measurement of $[Ca^{2+}]_i$ and they include quin-2, indo-1, and Fura-2 (Cobbold and Rink, 1987). The later is most commonly used since it has a high affinity for calcium and can be used to measure concentrations in the range 50 nM to 2 μM. Fura-2 is taken up into the cell in the membrane permeable form of an acetoxymethyl ester (Fura-2 AM) where it is cleaved to its free acid form and retained within the cell. Fura-2 responds to changes in wavelength under different excitation conditions. At 361 nm there is a point where the fluorescence is independent of $[Ca^{2+}]_i$ but either side of this region binding of fura-2 to calcium results in fluorescence changes (an increase at 340 nm and a

corresponding decrease at 380 nm). Thus it is possible to calculate the ratio of the intensities at these two excitation wavelengths to record $[Ca^{2+}]_i$ and eliminate any artefacts which may be showing effects on both wavelengths. To determine exact $[Ca^{2+}]_i$ the ratio needs to be recorded in the absence of calcium (R_{min}) and the presence of saturating calcium (R_{max}) and these values inserted into the Grynkiewicz equation (Grynkiewicz *et al.*, 1985):

$[Ca^{2+}]_i = fK_D[(R-R_{min})/(R_{max}-R)]$ where K_D is the known dissociation constant for fura.

Thus single cell calcium responses were measured by fluorescence imaging as previously described with modifications (Pocock *et al.*, 1995; Harrold *et al.*, 1997). Briefly, cells plated on non-fluorescent 13 mm coverslips were washed with physiological bathing solution (BM). These cells were loaded with 5 μM of the Ca^{2+} indicator dye, Fura-2 AM in the same buffer with 1% BSA (to assist loading and prevent compartmentalisation of the dye) for 30 min at 37°C. Coverslips were then inserted into a thermostatted perfusion chamber (37°C) of an Olympus IX70 inverted epi-fluorescence microscope and 150 μl of BM added. Either compounds were added directly to the well or an aliquot of medium from microglia was applied to neurones and the Ca^{2+} responses within distinct cells acquired at 340 and 380 nm excitation wavelengths with emission at 510 nm using a Life Science Resources SpectraMASTER monochromator. Images were acquired by a 12 bit digital camera (Astrocam) and the output visualised with a Life Science Resources Merlin Imaging system, version 1.86. Data were analysed by calculating the 340/380 nm fluorescence ratios for each of the chosen cells. Experiments were always performed in duplicate from at least 2 independent cultures.

3. CGA MEDIATED MICROGLIAL ACTIVATION: THE ROLE OF NITRIC OXIDE AND GLUTAMATE IN CELL DEATH

3.1 Introduction

Microglia, the resident macrophages of the CNS exhibit a resting/ramified process bearing state in the normal brain but in response to various signals can undergo a morphological transformation to an active form that resembles the mature macrophage (Kreutzberg, 1996). This process is accompanied by changes in the expression of immunochemical markers. The CR3 complement receptor is expressed by microglia independent of activation status (Akiyama and McGeer, 1990) and thus can be used to distinguish microglia from other glial cell types. Only ameboid microglia will synthesise the macrophage specific lysosomal marker ED-1 (Graeber *et al.*, 1989b) and therefore by monitoring changes in the expression of this antigen it is possible to identify novel activating molecules. The nature of such activating signals *in vivo* is poorly understood but some advances have been made using cell culture systems.

Microglial cells have typically been prepared from mixed primary glial cultures under conditions where the influence of other cells over several weeks may lead to "activation" (Williams *et al.*, 1992). Instead, microglia can be freshly isolated using Percoll density gradients following enzymatic digestion of brain tissue (Sedgwick *et al.*, 1991) though this has been shown to alter the levels of cell surface molecules such as CD4 and CD25 (Ford *et al.*, 1996). To date the most suitable procedure to obtain cultures of resting microglia may be disruption of CNS tissue by homogenisation (Havenith *et al.*, 1998).

In an attempt to elucidate the signalling pathways occurring during activation of microglia, cultures have often been exposed to combinations of cytokines such as interleukin-1β and lipopolysaccharide (Chao *et al.*, 1995). Depending on the stimulus, a

number of factors released by activated microglia including nitric oxide (Kim and Tauber, 1996) and glutamate (Piani and Fontana, 1994) have been examined for their ability to influence neuronal survival. However, *in vitro* there is a progressive decline in the number of viable microglial cells which can prevented with antioxidants (Heppner *et al.*, 1997). Activation of microglia also results in loss of viability. Hypoxia treated microglia are protected from cell death with mannitol, a radical scavenger (Lyons and Kettenmann, 1998), and the J774 cell line demonstrates increased cell death following stimulation with LPS which can be attenuated with a NOS inhibitor (Deakin *et al.*, 1995). This suggests that the build up of damaging molecules following activation of microglia may be detrimental to their survival. The components of this toxic environment have not yet been elucidated.

Recently chromogranin A (CGA), a 48 kDa secretory protein, has been shown to be a novel activator of microglia (Taupenot *et al.*, 1996). Beside being a major component in secretory granules of chromaffin cells (Winkler and Fischer-Colbrie, 1992), CGA is found in both neurones and glia of the CNS (Munoz *et al.*, 1990; Mahata *et al.*, 1997). Secretion of CGA during synaptic activity can have a direct effect on neuronal signalling by controlling neurotransmitter release (Brudzynski and Munoz, 1994; El Majdoubi *et al.*, 1996). Indirect effects may mediated through the action of CGA on glial cells. Expression of CGA is upregulated in a number of neurodegenerative diseases and is particularly prominent in the senile plaques associated with Alzheimer's disease (Yasuhara *et al.*, 1994), a pathology associated with activated microglia (Larner, 1997). This chapter describes a initial characterisation of CGA induced activation of cultured microglial cells.

Summary of results

Primary microglial cells were isolated by Percoll density gradient centrifugation either following enzymatic disaggregation or homogenisation to disrupt brain tissue. Both methods resulted in highly enriched cultures of microglia which were positive for OX-42, the anti-CR3 antibody, and contained few GFAP positive astrocytes. The cultures were also sensitive to L-leucine methyl ester, a microglial cell toxin. When exposed to CGA the microglia underwent a morphological change to an activated state and upregulated expression of ED-1. Treatment of both primary cultures of microglia and the N9 microglial cell line with CGA resulted in the production of nitric oxide and the release of glutamate. Nitric oxide release occurred as a result of increased expression of iNOS, a pathway which involved tyrosine phosphorylation. Secretion of glutamate was reduced by the Na^+/cystine transporter inhibitor, aminoadipate, and also bafilomycin suggesting a portion may be released in a vesicular mode. The rise in nitric oxide and glutamate was detrimental to cell survival – both inhibitors of either NOS or glutamate release significantly attenuated microglial cell death. Furthermore, cell death was triggered by activation of a metabotropic glutamate receptor since it could be blocked by the type II/III mGluR antagonist MSPG. CGA activated microglia also released cathepsin B to the cell culture medium.

3.2 A morphological and immunocytochemical characterisation of unstimulated and CGA exposed primary rat brain microglia cultures

When microglia were prepared from 5-7 day old Wistar rat pups by using enzyme digestion to disaggregate the cells (method 1) the average yield was $0.42 \pm 0.08 \times 10^6$ cells/brain. Subsequently, it was found that by homogenising the brains and then separating the isolated cells through one Percoll gradient (method 2) the average yield was increased to $0.76 \pm 0.05 \times 10^6$ cells/brain. Occasionally adult rats (>30 days old) were used but these gave significantly lower yields per gram of tissue available ($0.50 \pm 0.05 \times 10^6$ cells/brain), even when using method 2. After plating onto coverslips, cells prepared from neonate rats, by either method, were initially small and rounded but after 24 h in culture increased in size and assumed a resting, ramified structure (Slepko and Levi, 1996) (Fig. 3.2.1A and Fig. 3.2.2A). The number of cells attached to the coverslips were counted after 24 h. The addition of 5 mM L-leucine methyl ester, a microglial toxin (Hewett *et al.*, 1999) reduced the mean number of cells per field from 101.15 ± 3.82 to 5.84 ± 0.63 (Fig. 3.2.1B) suggesting the cultures were enriched in microglia. The majority of the cells also stained positive for OX-42 suggesting a highly enriched culture of microglia (Table 3.2.1). Furthermore, the remaining cells showed no immunoreactivity toward GFAP (a marker of astrocytes) at 2 DIV and the number of GFAP positive cells remained low at 4 DIV (<2%) indicating minimal contamination of these cultures.

Previous reports have indicated that with time *in vitro* microglia will undergo a spontaneous morphological change from the process-bearing resting state to an active ameboid form (Slepko and Levi, 1996). Immunostaining with ED-1, a marker of activation status revealed this to be the case. At 2 DIV, cultures contained low levels of

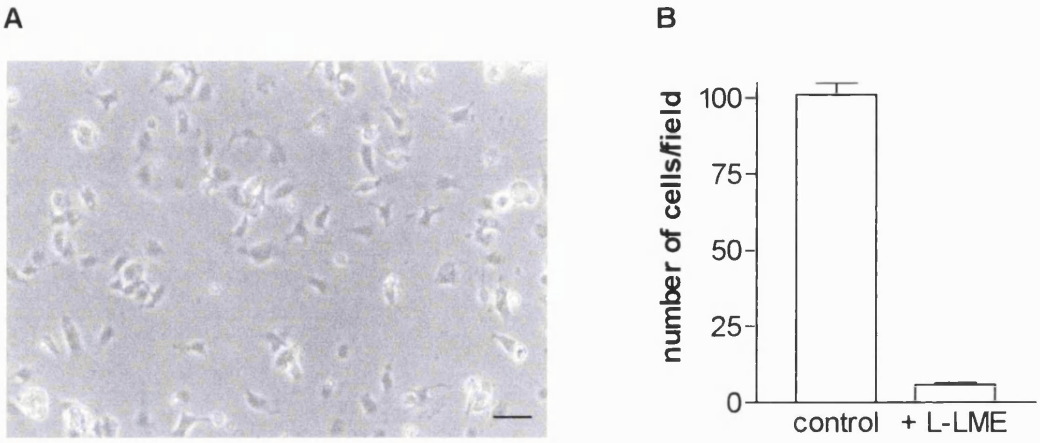


Fig. 3.2.1 Primary cultures of neonate rat brain microglia are sensitive to L-leucine methyl ester. (A) Following isolation using Percoll density centrifugation, cells were plated onto poly-D-lysine coated coverslips and cultured in MEM containing 10 % FCS. At 24 h the cells had developed many fine processes indicative of a resting state prior to activation. Scale bar = 30 μM. **(B)** The number of cells in control cultures and those treated with 5 mM L-leucine methyl ester (+ L-LME) were counted in 5 discrete fields per coverslip performed in duplicate from two independent preparations (n = 20).

	Method 1	Method 2
Cell yield (cells/brain)	$0.42 \pm 0.08 \times 10^6$	$0.76 \pm 0.05 \times 10^6$
OX-42 antibody		
Control 2 DIV	95.00 ± 3.50 %	92.00 ± 7.10 %
ED-1 antibody		
Control 2 DIV	18.77 ± 3.29 %	12.37 ± 2.96 %
Activated 2 DIV	92.78 ± 1.89 %	89.45 ± 0.85 %
Control 4 DIV	60.31 ± 2.53 %	61.56 ± 1.56 %
GFAP antibody		
Control 2 DIV	None detected	None detected
Control 4 DIV	1.65 ± 0.32 %	1.03 ± 0.30 %

Table 3.2.1 Cell yields and purity of different microglial culture technique. Microglia were prepared and then cell counts made using a haemocytometer. The mean number ± SEM from 10 different cultures is shown. Microglia were stained for immunoreactivity to OX-42, ED-1 and GFAP. Data are the mean ± SEM values expressed as the percentage of cell number in 10 discrete fields from 3 independent cultures that stained with the respective primary antibody following visualisation using avidin-biotin peroxidase.

ED-1 positive cells which increased 3 fold in the absence of any stimulating factor following another 2 days in vitro (Table 3.2.1). Thus, to discern any morphological or biochemical differences between basal levels and those in the presence of activating factor, it was necessary to treat the cells early in culture (at 1 DIV). Following exposure to 10 nM CGA for 12 h there was both a detectable change in morphology to an ameboid form (Fig. 3.2.2B) and an increase in the number of ED-1 positive cells (Table 3.2.1 and Fig. 3.2.2D).

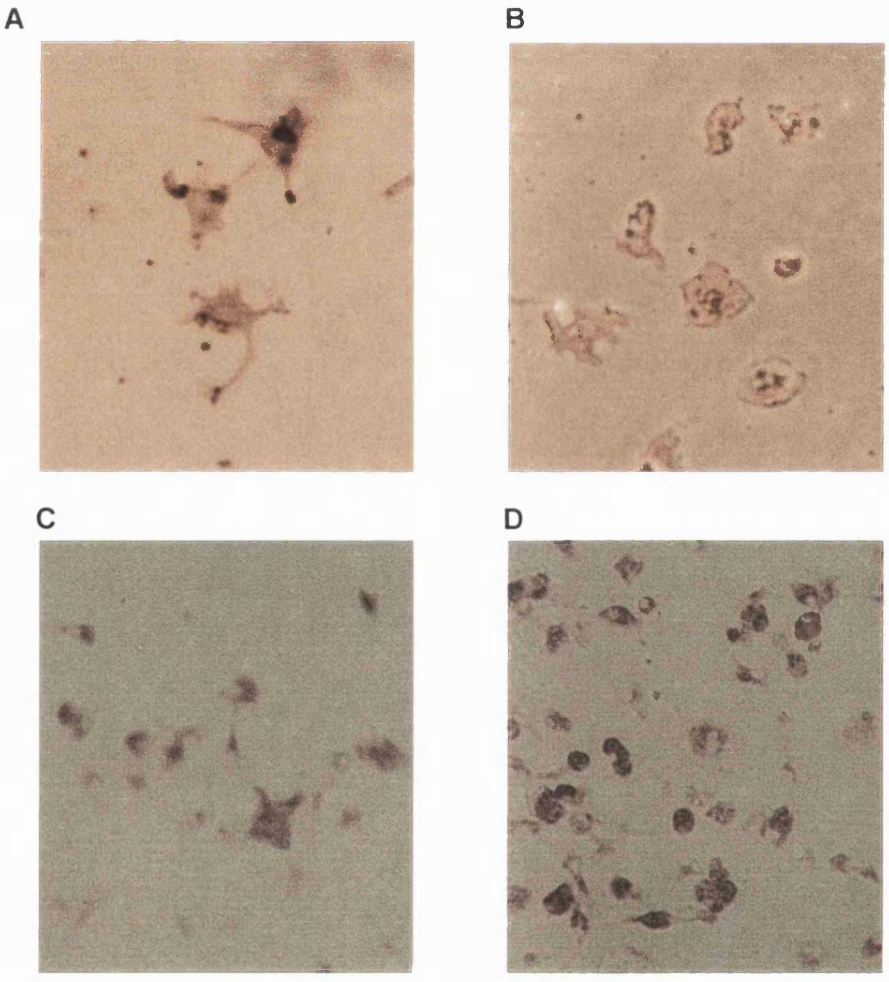


Fig. 3.2.2 Immunochemical staining of cultures with the macrophage/microglia specific markers, OX-42 (A/B) and ED-1 (C/D). One day after plating cells had extended fine processes **(A)** but after exposure to 10 nM CGA for 12 h there was an increase in the number of cells exhibiting an ameboid morphology **(B)**. Control cultures showed weak staining with ED-1 **(C)** but there was an increase in both the intensity and total number of ED-1 positive cells when treated with 10 nM CGA for 12 h **(D)**.

3.3 CGA activated microglia release nitric oxide and subsequently die

The retraction of processes and transition to an ameboid form as observed by immunostaining at 12 h was followed by a phase when the cells became more rounded and shrunken, a process accompanying certain forms of cell death (Wyllie *et al.*, 1980; Bortner and Cidlowski, 1999). This was investigated further using the fluorescent probes, fluorescein to detect live cells and propidium to label dead cells (Bonfoco *et al.*, 1996). Untreated cultures of neonate rat brain microglia contained a high percentage of viable cells (91.00 ± 1.60%) which fluoresced green with fluorescein and low levels of dead cells (9.00 ± 1.60%) stained red with propidium (Fig 3.3.1A,B and E). Treatment of microglia with 10 nM CGA for 24 h cell resulted in an increase in the number of propidium positive cells to 58.00 ± 0.90% (Fig. 3.3.1D and F). Whilst the number of live cells had decreased, the remaining cells that stained with fluorescein showed a more rounded morphology with few processes (Fig. 3.3.1C). The loss in cell viability was dependent on the concentration of CGA used to activate the microglia with maximal death observed using 10 nM CGA (Fig. 3.3.2A). Cultures were treated for 24 h with 1 μg/ml lipopolysaccharide (LPS), another known activator of microglia (Chao *et al.*, 1992) and similar counts for live and dead cells made. LPS resulted in a small but significant decrease in the number of live cells when compared with control cultures (Fig. 3.3.2A). A comparison between different cell preparations revealed that CGA caused a significant increase in the total number of dead cells in cultures of both adult rat brain microglia and the N9 cell line (Fig. 3.3.2B). Control cultures of primary adult rat brain microglia contained a significantly higher level of dead cells (26.50 ± 3.05%) compared with primary neonate preparations (9.00 ± 1.60%) but the number of dead cells following CGA treatment was similar (59.78 ± 1.34% in adult, 58.00 ± 0.90% in

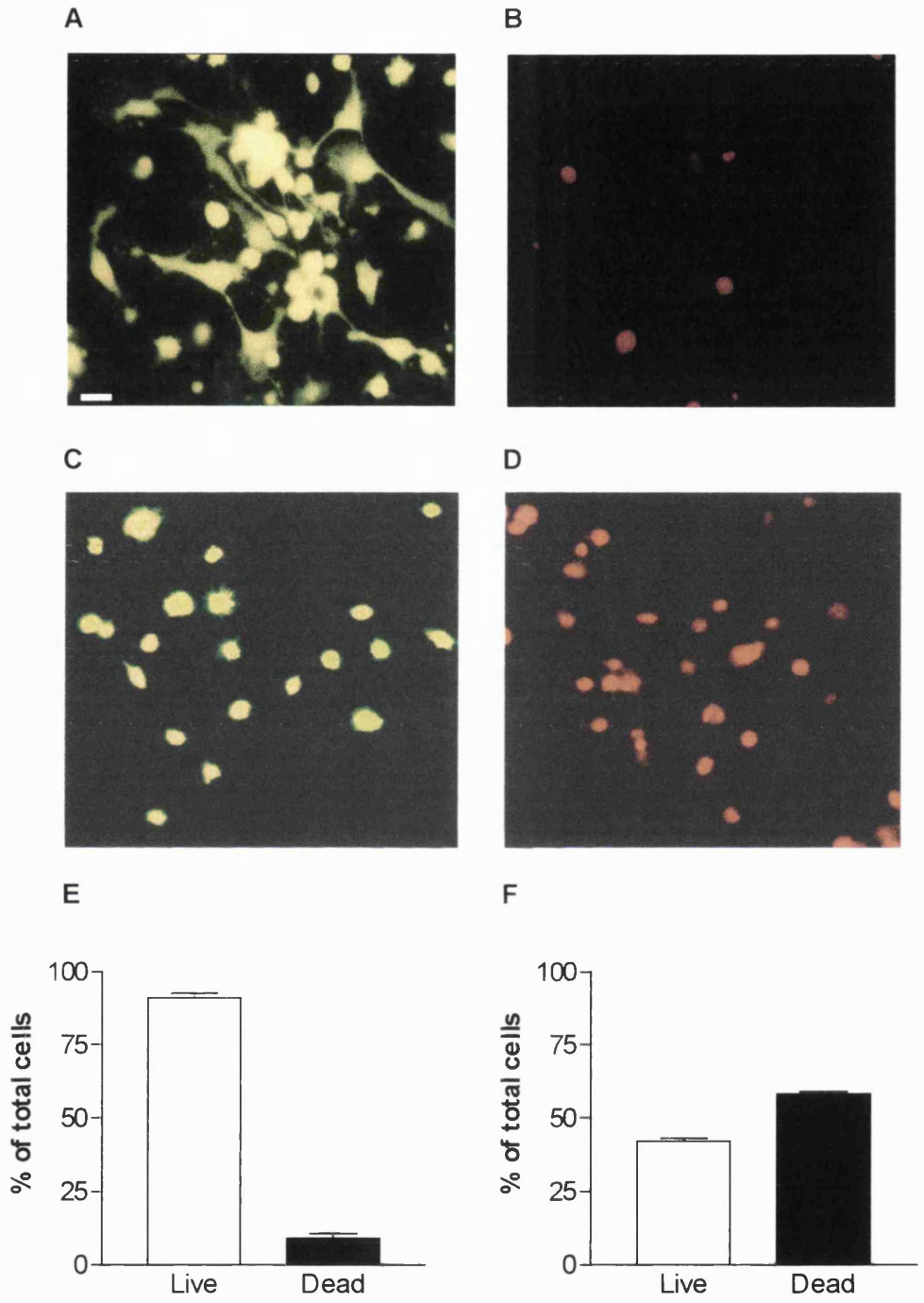


Fig. 3.3.1 CGA induces cell death in primary cultures of rat brain microglia. Control cultures stained with fluorescein **(A)** showed a resting morphology and there were few propidium positive cells **(B)**. Fluorescein stained cells exposed to 10 nM CGA for 24 h displayed a more rounded morphology **(C)** and the number of propidium positive cells had increased **(D)**. Absolute numbers of cells that stained positive for either fluorescein (Live) or propidium (Dead) were counted in 10 discrete regions from 2 coverslips per condition and 3 independent cell preparations (n = 60) and data expressed as mean number of cells ± SEM for control **(E)** or CGA treated cultures **(F)**.

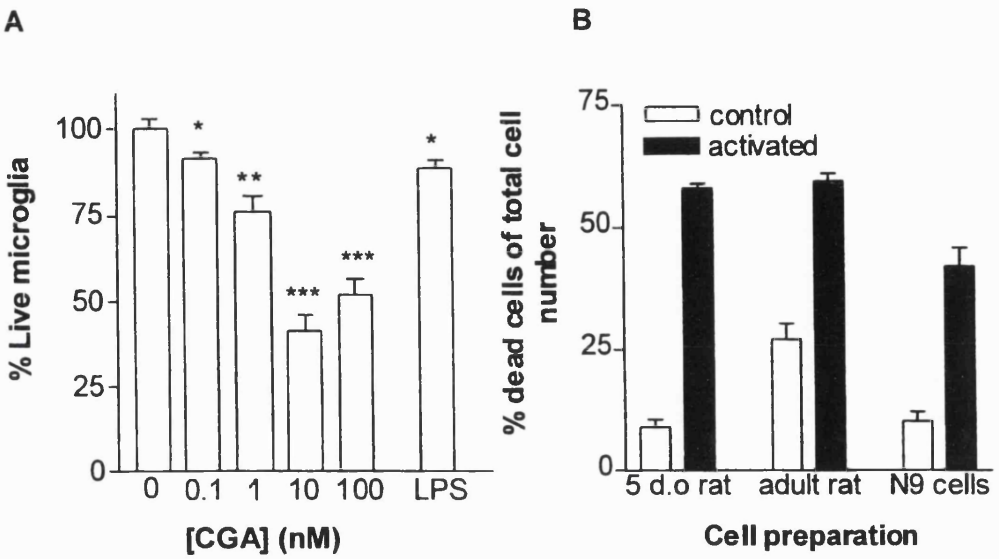


Fig. 3.3.2 Chromogranin A toxicity of microglial cells is dose dependent. After 24 h treatment with CGA, primary cultures of neonate rat brain microglia were stained with fluorescein and propidium. **(A)** Microglial viability decreased with increasing concentrations of CGA, plateauing at 50 % with 10 – 100 nM CGA. Thus in all subsequent experiments 10 nM CGA was used. Data are normalised to control cultures as 100 % live, and are the mean ± SEM values (n = 60). *p<0.05, **p<0.01, ***p<0.001 significantly different from control cultures. **(B)** The absolute number of dead cells stained with propidium was counted in control cultures and those exposed to 10 nM CGA for 24 h (activated) in preparations of adult rat brain microglia and the N9 cell line. These are plotted next to the data for cells cultured from neonates (5 d.o rat). Values are expressed as the % dead cells of total cell numbers counted, mean ± SEM (n = 60).

neonate cultures). The loss of viability in the N9 cell line was less marked than primary cultures but still significant (Fig. 3.3.2B). In accordance with previous reports (Taupenot *et al.*, 1996), primary cultures of neonate rat brain microglia exposed to CGA (0.1-100 nM) released nitric oxide (Fig. 3.3.3). Nitric oxide is generated by the enzyme nitric oxide synthase (NOS) and rapidly decays to the stable end products, nitrate and nitrite. Griess reagent can be used to assay total nitrite levels following conversion of any nitrate to nitrite with nitrate reductase and thus give an estimation of the levels of NO released (Green *et al.*, 1982; Ding *et al.*, 1988). The concentration of total nitrite in culture medium from cells exposed to CGA increased in a dose dependent manner from low basal levels (0.98 ± 0.01 μM) to a maximum level of 34.20 ± 1.05 μM with 10 nM CGA (Fig. 3.3.3A). These levels compared with 22.80 ± 1.90 μM total nitrite in the

presence of 1 μg/ml LPS. The concentration of nitrite observed in the absence of nitrate conversion was typically 30 % lower i.e. cultures exposed to 10 nM CGA showed levels of 24.02 ± 0.90 μM. Compared with neonate cells, microglia prepared from the brains of adult rats produced significantly higher levels of nitrite in unstimulated cultures (12.80 ± 2.50 μM) and also showed a smaller increase following stimulation with 10 nM CGA (Fig. 3.3.3B). There was also a significant increase in the level of nitrite in culture medium of N9 cells exposed to 10 nM CGA (Fig. 3.3.3B). In order to determine the kinetics of nitric oxide production, the levels of total nitrite were assayed in the culture medium of control and CGA exposed microglia at 8 h, 16 h, 24 h and 48 h. In primary cultures exposed to 10 nM CGA there was no detectable increase in levels of nitrite prior to 8 h but thereafter at 16 h the levels had increased significantly to 27.00 ± 0.50 μM and plateaued between 24 h and 48 h (35.05 ± 0.45 μM). Control unstimulated cultures showed no significant increase in nitrite over this 48 h period. A similar profile of nitrite production was observed using the N9 cell line (Fig. 3.3.3D). Microglia and macrophages express iNOS, the inducible form of NOS, responsible for the production of large quantities of NO over a long period of time (Lockhart *et al.*, 1998). To establish that iNOS was involved in CGA induced NO release, whole cell lysates were prepared and the proteins resolved by 12 % SDS-PAGE. Following transfer to PVDF membrane, the samples were immunoblotted using an anti-iNOS antibody (Fig. 3.3.4). There was no detectable iNOS protein in control cells but there were significant levels either at 16 h following treatment with 10 nM CGA or 24 h exposure to 1 μg/ml LPS. The presence of a specific inhibitor of iNOS, AMT-HCl, failed to prevent the increase in iNOS protein expression suggesting it acts at the catalytic step (Fig. 3.3.4).

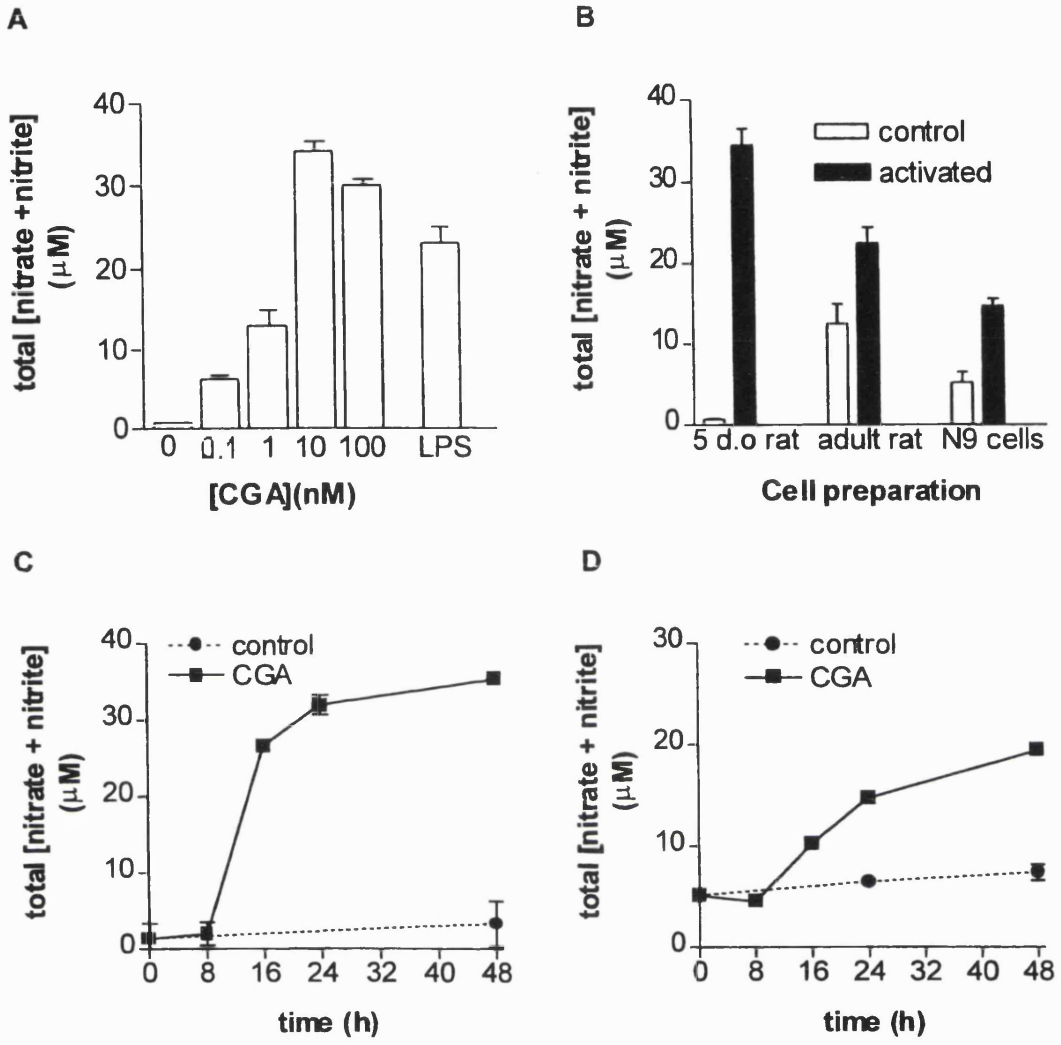


Fig. 3.3.3 Dose dependent and time course profile of nitric oxide generation by microglia activated with Chromogranin A. (A) Primary cultures of neonate rat brain microglia were exposed to CGA (0.1-100 nM) or 1 μg/ml LPS for 24 h and the total levels of nitrite following conversion of any nitrate to nitrite measured in the culture medium using Griess reagent. **(B)** Total nitrite measurements were made on culture medium from control cells and cells exposed to 10 nM CGA for 24 h in adult rat brain preparations of microglia and the N9 cell line. These are plotted next to the data for cells cultured from post natal day 5 rats (5 d.o rat). Total levels of nitrite were assayed at the time points indicated in control cultures and those exposed to 10 nM CGA in primary cultures of neonate rat brain microglia **(C)** and the N9 cell line **(D)**. All data are expressed as the mean ± SEM values of triplicate determinations of 3 coverslips per culture from a total of 3 independent preparations (n = 27).

Recent evidence suggests that microglial activators can induce tyrosine kinase dependent signalling pathways and these might be responsible for mediating the

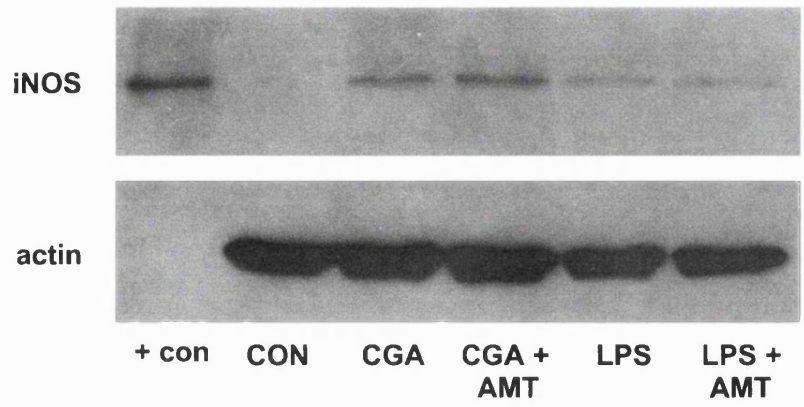


Fig. 3.3.4 Stimulation of the N9 microglial cell line with CGA or LPS results in the increased expression of iNOS protein. Cell lysates were prepared from control cultures (CON) or those exposed to 10 nM CGA for 16 h in the absence (CGA) or presence of the iNOS specific inhibitor, 150 nM AMT-HCl (CGA + AMT) or LPS (1 μg/ml for 24 h) treated cultures in the absence (LPS) or presence of 150 nM AMT-HCl (LPS + AMT). 30 μg of total cell protein was resolved by 12 % SDS-PAGE, transferred to PVDF membrane and then blotted with anti-macNOS antibody (1:1000). 1 μg of control cell lysate was used as a positive control (+ con). The blot was stripped and reprobed with anti-actin antibody (1:1000) to ensure equal loading of protein samples.

production of neurotoxic molecules such as superoxide (McDonald *et al.*, 1997; Combs *et al.*, 1999). CGA stimulates a tyrosine kinase activity in microglia and genistein, a tyrosine kinase inhibitor, can attenuate NO production (Taupenot *et al.*, 1996). However, the identity of the proteins phosphorylated and their importance in NO production remains unresolved. Thus, to determine whether tyrosine phosphorylation and NO production were involved in CGA induced microglial cell death, the effect of CGA on the profiles of phosphotyrosine proteins was examined by SDS-PAGE and Western blotting using an anti-phosphotyrosine antibody, PY54. With increasing incubation times a series of proteins with molecular weights between 20-60 kDa showed enhanced phosphorylation (Fig. 3.3.5A) and this was dependent on the concentration of CGA used (Fig. 3.3.5B). These increased levels of phosphotyrosine could be blocked with the tyrosine kinase inhibitor genistein (Fig. 3.3.5A). Genistein was also able to significantly reduce the levels of nitric oxide and cell death induced by

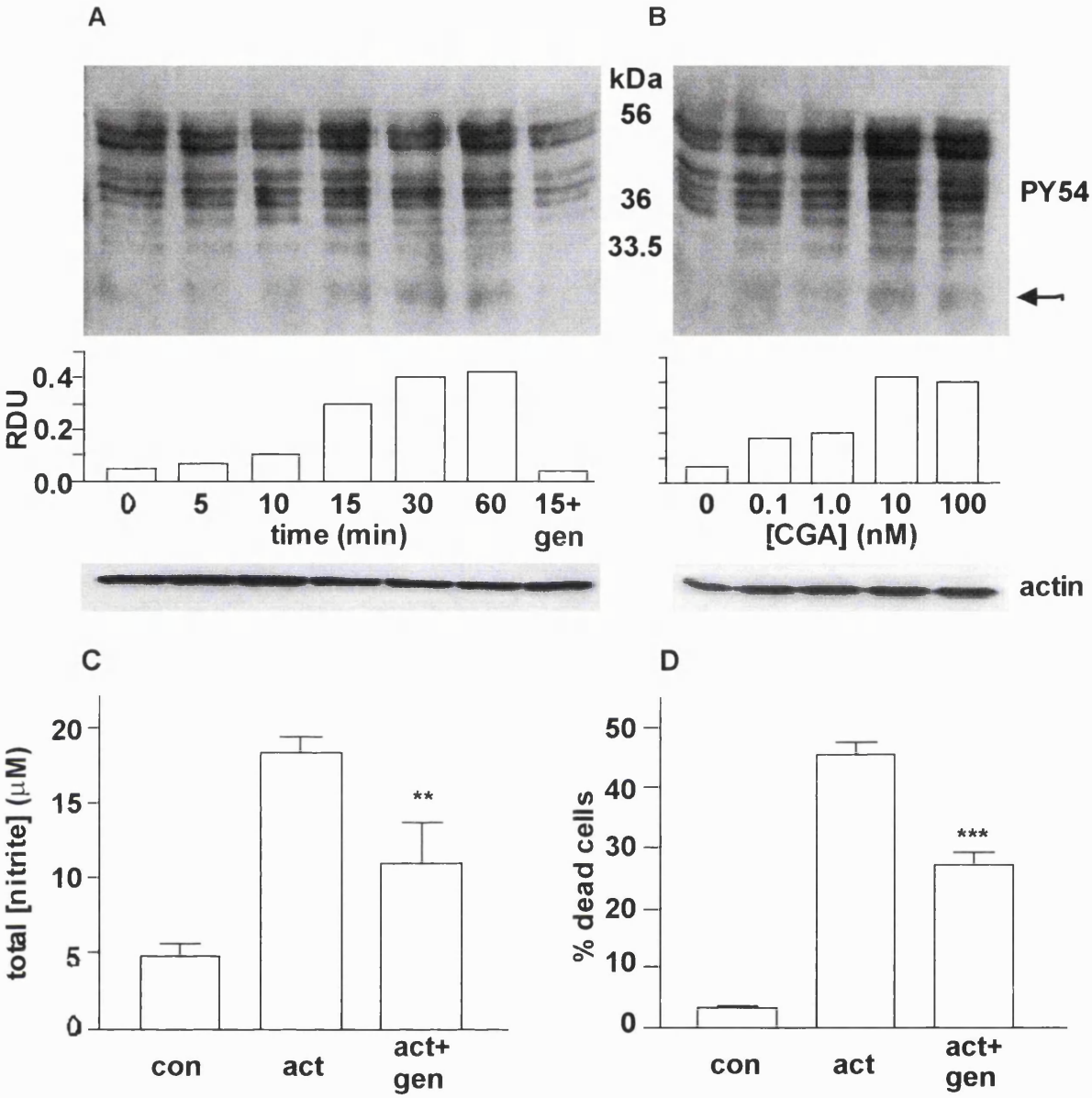


Fig. 3.3.5 Genistein inhibits CGA induced tyrosine phosphorylation, nitric oxide production and cell death in the N9 cell line. (A and B) Cell lysates were prepared from control cultures (0) and those either exposed to 10 nM CGA for 5, 10, 15, 30, or 60 min or 15 min in the presence of genistein, or different concentrations of CGA (0-100 nM) for 15 min. 30 μg of total cell protein was resolved by 12% SDS-PAGE, transferred to PVDF membrane and then blotted with anti-phosphotyrosine PY54 antibody (1:3000). The blot was stripped and reprobed with anti-actin antibody (1:1000) to ensure equal loading of the gel. The relative density units (RDU) of the lower band (arrow) are shown as representative of the overall increases in phosphorylation. **(C)** Griess reagent was used to assay total nitrite levels in the culture medium of control cells (con), cells activated with 10 nM CGA for 24 h in the absence (act) and presence of 200 μM genistein (act + gen). Data are the mean ± SEM values (n = 24); **p<0.01, significantly different to cultures treated with CGA alone. **(D)** The number of dead cells stained with propidium were counted in control cultures (con), cultures activated with 10 nM CGA for 24 h in the absence (act) and presence of 200 μM genistein (act + gen). Data are expressed as the mean % dead cells ±SEM (n = 32); ***p<0.001 significantly different to cultures treated with CGA alone.

CGA (Fig. 3.3.5C and D) suggesting that tyrosine phosphorylation may precede the production of NO and subsequent cell death. To confirm that nitric oxide played a role in the toxicity evoked by CGA, the broad NOS inhibitor, L-NNA, and the specific iNOS inhibitor, AMT-HCl, were used to inhibit NO production. Both of these compounds and the protein synthesis inhibitor, cycloheximide, significantly reduced the levels of nitrite in the culture medium of cells exposed to 10 nM CGA for 24 h (Fig. 3.3.6A). The increases in nitrite were also abolished in cultures treated with the microglial toxin, L-LME, suggesting that NO was produced by microglia and not any contaminating cells (Fig. 3.3.6A). Cell viability was improved from 43.75 ± 3.50% in cultures treated with CGA alone to either 100 ± 4.96%, 92.75 ± 3.25% or 81.25 ± 2.97% when pre-incubated

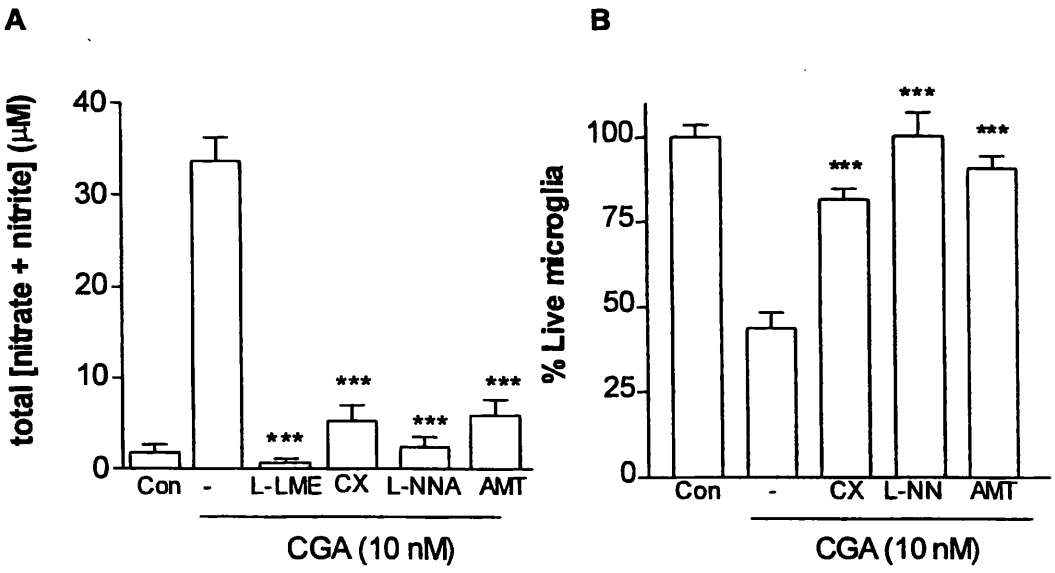


Fig. 3.3.6 CGA induced microglial cell death is prevented by NOS inhibitors. (A) Nitric oxide release over a period of 24 h was determined using Griess reagent to measure the total nitrite levels in the culture medium of control cells (con), those exposed to 10 nM CGA in the absence (-) or the presence of 5mM leucine methyl ester (L-LME), 1 μg/ml cycloheximide (CX), 1 mM L-NNA (L-NNA) or 150 nM AMT-HCl (AMT) each of which had been added 1 h prior to exposure to CGA. Data are expressed as the mean ± SEM values (n = 27). ***p<0.001; significantly different to cultures exposed to CGA alone. **(B)** The number of live cells stained with fluorescein was counted in control cultures (Con) and those exposed to 10 nM CGA in the absence (-) or the presence of 1 μg/ml cycloheximide (CX), 1 mM L-NNA (L-NNA) or 150 nM AMT-HCl (AMT) each of which had been added 1 h prior to exposure to CGA. Data are normalised to control cultures (100 % live) and are the mean % of live cells ± SEM (n = 60). ***p<0.001; significantly different to cultures exposed to CGA alone.

with 1 mM L-NNA, 150 nM AMT-HCl or 1 μg/ml cycloheximide respectively, prior to the addition of CGA (Fig. 3.3.6B). This protective effect of NOS inhibitors and the prevention of NOS protein expression suggests that NO may be involved in the CGA induced toxicity to microglia. To determine whether exogenously applied NO had the same affect as CGA, the ability of the nitric oxide releasing compound, S-nitrosopenicillamine (SNAP), to cause cell death in microglia was assessed. Increasing concentrations of the NO donor resulted in elevated levels of nitrite in the cell culture medium (Fig. 3.3.7A). However, even in the presence of double the concentration of NO generated by microglia following CGA treatment (released using 1mM SNAP) there was no significant reduction in cell viability (Fig. 3.3.7B). This suggests that only endogenously produced NO or other factors acting in concert with NO may be detrimental to microglial cells.

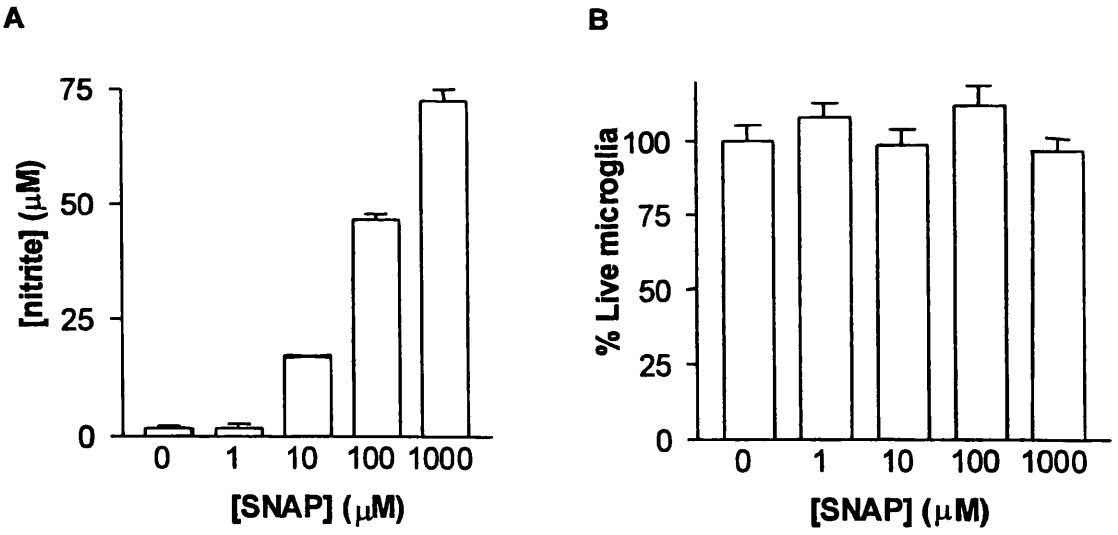


Fig 3.3.7 Effect of the nitric oxide donor, SNAP, on nitrite accumulation and cell death in primary cultures of rat brain microglia. (A) Following exposure to SNAP (1-1000 μM) for 24 h the levels of nitrite in the culture medium were measured using the Griess reagent. Data are the mean ± SEM from 3 independent cell preparations (n = 27). **(B)** SNAP (1-1000 μM) was added to cultures and cell viability assessed 24 h later using fluorescein and propidium staining to count the number of live and dead cells. Data are normalised to control cultures (100 % live) and the mean % of live cells ± SEM (n = 60).

3.4 CGA activated microglia release glutamate, attenuation of which prevents cell death

Previous studies have shown that microglia can release soluble factors such as glutamate into the culture medium (Piani and Fontana, 1994). Conditioned medium from primary microglia activated for 24 h with 0.1-100 nM CGA was measured for glutamate using the glutamate dehydrogenase diaphorase assay (Beutler, 1985). There was a significant increase in the amount of glutamate from a basal level of 37 ± 3.2 μg/mg protein in the absence of CGA to 139 ± 22.2 μg/mg protein in the presence of 10 nM CGA (Fig. 3.4.1A). Although basal and activated levels were lower, probably as a result of differences in plating density and culture media used (Patrizio and Levi, 1994), there was a similar significant increase in glutamate in the medium of N9 cells exposed to 10 nM CGA compared with control (Fig. 3.4.1B). Exposure of primary cells to LPS (1 μg/ml) resulted in lower levels of glutamate release than that observed with 10 nM CGA (Fig. 3.4.1A). Recent evidence suggests glutamate dependent toxicity in other glial cell types (Matute *et al.*, 1998) so the possibility that glutamate might be involved in CGA induced microglial death was first investigated by examining whether the nitric oxide inhibitor, L-NNA, had any effect on the glutamate release. CGA induced glutamate release in both primary cultures (Fig. 3.4.1A) and the N9 cell line (Fig. 3.4.1B) was significantly reduced in the presence of L-NNA. Further characterisation of the production of nitric oxide and glutamate release suggested that the increase in glutamate was dependent upon the induction of the nitric oxide synthase enzyme. NOS activity increased rapidly after 8 h (Fig. 3.4.1C) but between 8-16 h there was only a small increase in glutamate levels. Thus the production of glutamate showed a lag period following induction of NOS and then increased rapidly between 16 and 21 h (Fig. 3.4.1C). At this time point, there was no significant increase in the number of

microglial cells staining with propidium suggesting that the cells were not releasing glutamate as a result of cell lysis. This was confirmed by measuring the amount of lactate dehydrogenase (LDH) in the culture medium. This cytosolic enzyme is released when cells are damaged and have leaky plasma membranes. The levels observed did not change significantly at early time points but by 24 h there was a small but significantly elevated concentation (Fig. 3.4.1C). Thus, the accumulation of high levels of glutamate in the culture medium may be responsible for the reduction in microglial cell viability (Fig. 3.4.1C).

Further characterisation involved activating microglia for 24 h with 10 nM CGA in the presence of a variety of pharmacological agents and measurements of glutamate content and cell viability performed. The iNOS specific inhibitor, AMT-HCl attenuated glutamate release to a similar degree observed with the broad NOS inhibitor, L-NNA, suggesting that the nitric oxide produced comes from the iNOS enzyme. Aminoadipate, an inhibitor of the Na^+/cystine transporter which is known to be required for glutamate release (Piani and Fontana, 1994) significantly blocked release as did propentofylline (Fig. 3.4.2A). The later compound suppresses microglial activation and subsequent generation of free radicals (Schubert, 1994) and may thus exert its effect through this function. 1 μM bafilomycin, a H^+/ATPase inhibitor, which can prevent vesicular glutamate uptake in neuronal systems (Bowman *et al.*, 1988), had a smaller effect (Fig. 3.4.2A). One possible reason for this is that the compound could only be applied over a short period of time as it was toxic to the cells when used for the full 24 h. Therefore, it was added at approximately 16 h which was prior to any significant increase in glutamate.

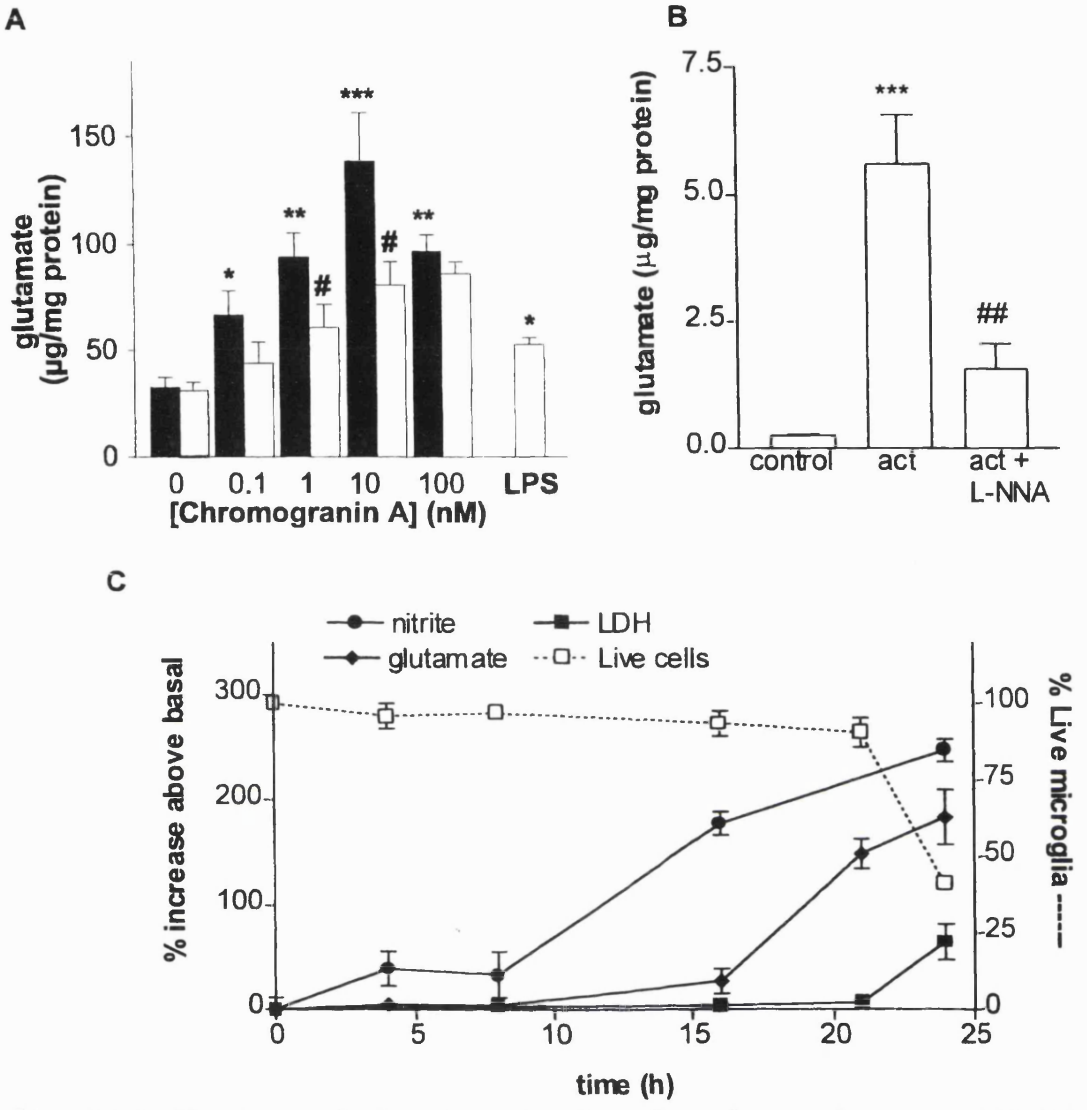


Fig. 3.4.1 Chromogranin-A activated microglia release glutamate. (A) Primary cultures of neonate rat brain microglia were either exposed to increasing concentrations of CGA (0.1-100 nM) or 1 µg/ml LPS in the absence (solid bars) or presence of 1 mM L-NNA (open bars) for 24 h and then the culture medium analysed for glutamate using the glutamate dehydrogenase diaphorase assay. Data are expressed as the mean concentration of glutamate released per mg of protein ± SEM from three independent cell preparations (n = 12). *p<0.05, **p<0.01, ***p<0.001 significantly different concentration of glutamate from control cultures and #p<0.05 significantly different from cells exposed to the given concentration of CGA alone. **(B)** Similar experiments were performed using the N9 cell line and glutamate levels determined in control untreated cultures (control), those exposed to 10 nM CGA for 24 h (act) or those treated with 10 nM CGA in the presence of 1 mM L-NNA (act + L-NNA). Data are expressed as the mean concentration of glutamate released per mg of protein ± SEM from three independent cell preparations (n = 12). ***p<0.001 significantly different to control cultures and ##p<0.01 significantly different to cultures exposed to 10 nM CGA alone. **(C)** Primary cultures of neonate rat brain microglia were exposed to 10 nM CGA for a total of 24 h and the cell viability measured using fluorescein/propidium (open squares) or the supernatants taken and assayed for total nitrite using Griess reagent (circles), glutamate by the glutamate dehydrogenase diaphorase assay (diamonds) or lactate dehydrogenase (LDH, closed squares) at the time points indicated.

The effect of glutamate receptor antagonists was also investigated. Neither the ionotropic receptor antagonists, (+)-5-methyl-10,11,-dihydro- 5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK801) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or the type II/III metabotropic glutamate receptor antagonist MSPG could prevent glutamate release induced by CGA (Fig. 3.4.2A). Cells treated with aminoadipate or propentofylline significantly reduced CGA induced microglial death (Fig. 3.4.2B) as did bafilomycin to a smaller extent. Neither of the ionotropic glutamate receptor antagonists afforded any significant protection but the metabotropic glutamate receptor antagonist, MSPG, was protective (73 ± 1.37%). Thus,

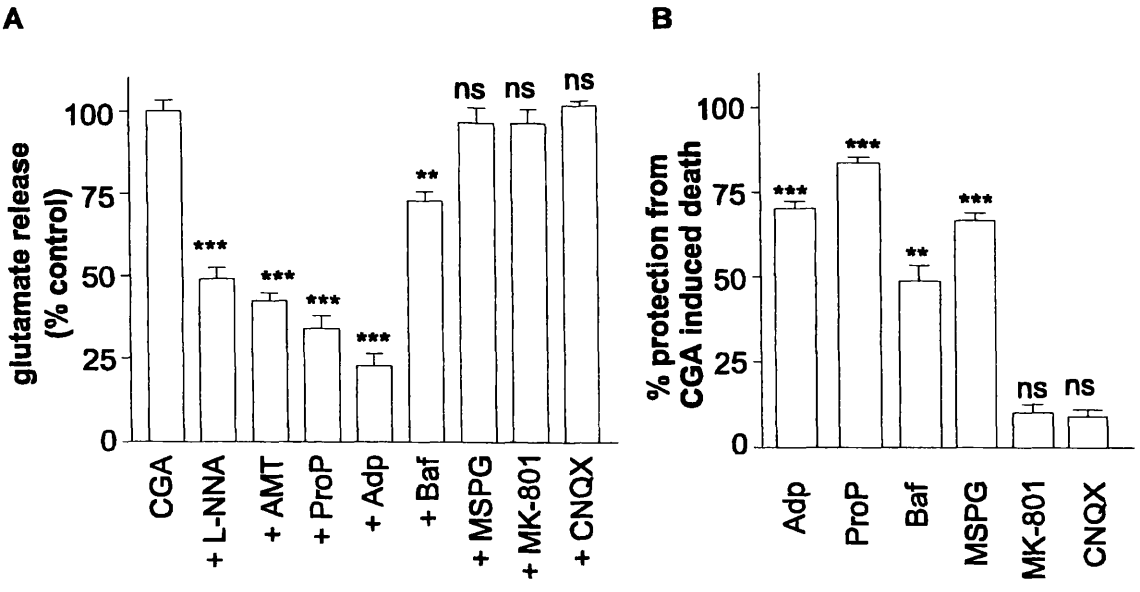


Fig. 3.4.2 Blockade of glutamate release attenuates microglial cell death. A number of drugs were assessed for their ability to influence glutamate release and subsequent cell death. **(A)** The levels of glutamate in the culture medium of cells exposed to CGA alone (CGA) or CGA in the presence of 1 mM L-NNA, 150 nM AMT, 50 μM propentofylline (ProP), 2.5 mM aminoadipate (Adp), 1 μM bafilomycin (Baf), 200 μM MSPG, 10 μM MK-801, or 20 μM CNQX was assayed using the glutamate dehydrogenase diaphorase assay. Data are mean ± SEM values (n = 18) expressed as a percentage of the glutamate released from cultures exposed to CGA alone where release = 100%. **p<0.01, ***p<0.001 significantly different to glutamate released in cultures exposed to CGA alone, ns not significantly different. **(B)** Microglial viability using fluorescein/propidium staining was determined following treatment with 10 nM CGA for 24 h in the presence of 2.5 mM aminoadipate (Adp), 50 μM propentofylline (ProP), 1 μM bafilomycin (Baf), 200 μM MSPG, 10 μM MK-801, or 20 μM CNQX. Data is expressed the mean ± SEM % protection from death induced by CGA alone (n = 60). **p<0.01, ***p<0.001 significant protection, ns not significantly different.

the presence of compounds that reduced the levels of glutamate in the media had a beneficial effect on cell survival suggesting that it may be glutamate or another unidentified glutamate receptor agonist that is responsible for cell death. Furthermore, the fact that MSPG could significantly protect the cells rather than MK801 or CNQX suggests that it may be acting via the mGluR group II/III receptors.

3.5 CGA activated microglia secrete cathepsin B to the culture media

Microglia are known to upregulate a multitude of cytokines and secreted proteins and CGA itself can induce TNF-α production in microglia (Ciesielski-Treska *et al.*, 1998). The release of proteins from CGA activated microglia was investigated by resolving

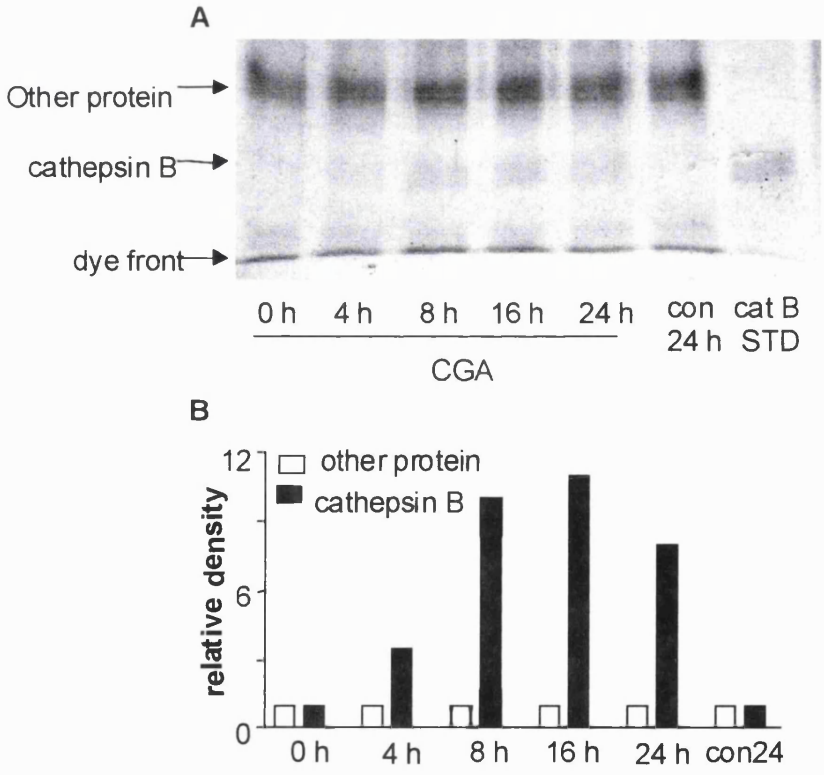


Fig. 3.5.1 Stimulation of microglia with CGA results in the extracellular release of cathepsin B. (A) 20 μl samples of cell culture medium from control cells at 0 h and 24 h (con 24 h) and those treated with 10 nM CGA for 4, 8, 16 and 24 h were combined with an equal volume of sample buffer and the proteins resolved with 10% SDS-PAGE. The gel was then Coomassie stained to reveal the separated proteins. **(B)** The gel was scanned and densitometric analysis performed on two protein bands. The values are displayed as relative density with respect to the individual bands at time 0 h.

equal volumes of cell culture medium from cells activated for various period of time using 10% SDS-PAGE. The gel was Coomassie stained and revealed elevated levels of a protein doublet with a molecular weight of approximately 25 kDa which corresponded to a cathepsin B standard (Fig. 3.5.1; Ryan *et al.*, 1995). This effect was not due to differences in overall protein loading since other protein levels remained stable over 24 h (Fig. 3.5.1). The consequences of this observation are discussed in Chapter 5.

3.6 Discussion

The results presented here indicate that microglia treated with Chromogranin A exhibit the characteristic signs of an activation cascade progressing from a resting morphology to an active ameboid state and as a consequence show a subsequent loss in cell viability. CGA results in the production of nitric oxide and the release of glutamate from both primary cultures of rat brain microglia and the N9 cell line. Inhibition of NOS and blockade of glutamate release both significantly attenuate cell death. Furthermore, microglial cell death is triggered via the activation of a metabotropic glutamate receptor. CGA activated microglia also secrete the cysteine protease, cathepsin B.

3.6.1 Microglia and NO

Microglial activation and death are often observed in cultures especially when microglia are grown in the presence of serum. Studies investigating factors that may influence the morphology of microglial cells have suggested that ramification is induced by influencing the levels of free radicals either via co-culture with astrocytes which act as scavengers (Sievers *et al.*, 1994) or with the antioxidant, vitamin-E (Heppner *et al.*, 1997). Even when microglia are cultured in chemically defined serum free media they

may become activated since many necessary constituents such as insulin suppress ramification (Fujita *et al.*, 1996). Microglia were isolated from Percoll gradients and initially put out processes suggesting a resting phenotype. However, over time they became progressively activated as shown by both a morphological change and increased expression of ED-1. As has previously been reported (Taupenot *et al.*, 1996) when microglia were exposed to CGA (0.1-100 nM) there was an increase in NOS activity as measured by the levels of nitrite in the culture medium assayed using Griess reagent. This was due to the iNOS form since the effects of L-NNA, a broad specificity inhibitor of NOS, on both nitrite levels and microglial function could be mimicked by the selective iNOS inhibitor, AMT-HCl, and the increase in NO could also be blocked by incubating the cells with cycloheximide suggesting the necessity for new protein synthesis. Western blots showed that there was an increase in the expression of iNOS protein. Furthermore, the increase in nitrite levels follows a similar kinetic profile to that studied in macrophages (Stueher and Marletta, 1987) where there is a lag period of between 6 – 8 h prior to a dramatic increase in NOS activity which remains at high levels for approximately 24 h. This lag period is likely to involve tyrosine phosphorylation. Genistein, a tyrosine kinase inhibitor, reduces both NO production and prevents an enhanced tyrosine phosphorylation of a number of proteins following CGA stimulation of microglia. Other studies have shown the importance of such phosphorylation events in mediating gene transcription (Bhat *et al.*, 1998).

Since CGA treated cultures were generating high levels of free radicals the involvement of these in microglial cell death was investigated. Cultures exposed to CGA showed a decrease in viability with increasing dose to 10 nM CGA and furthermore this could be prevented by incubating the cells with NOS inhibitors suggesting the cell death was due to the increase in NO levels. Using a combination of IFN-γ and LPS to activate cells, it

has been shown that the life span of peritoneal macrophages correlates inversely with the activity of NO synthase (Albina *et al.*, 1993). However, there are few reports on the direct effect of nitric oxide on microglial cells and most studies have involved the use of exogenously produced NO via NO donors. The role of nitric oxide in the pathology of primary glial cells has been studied previously by exposing oligodendrocytes, astrocytes and microglia to S-nitroso,N-acetyl-DL-penicillamine (SNAP), a nitric oxide releasing chemical (Mitrovic *et al.*, 1994). Such studies showed that metabolic function and cellular viability were most compromised in oligodendrocytes (supporting the hypothesis that nitric oxide production by other cells such as microglia is responsible for the cell loss in multiple sclerosis) whereas microglia appeared to be unaffected. Those using different culture systems such as the RAW 264.7 macrophage (Boggs *et al.*, 1998; Von Knethen and Brune, 1997) have observed the induction of apoptosis by exogenously supplied nitric oxide. The fate of a cell is often determined by its redox state and many of the enzymes necessary for cell death pathways are highly sensitive to oxidation. Selection for macrophages that were resistant to programmed cell death indicated that these cells had lower levels of glutathione (GSH), a thiol that controls the redox state of the cell (Boggs *et al.*, 1998). Thus, it may be that microglia exposed to the influences of other cells, when prepared from mixed glial cultures, acquire enhanced levels of GSH activity.

These conflicting reports lead to the investigation of whether SNAP would have a detrimental effect on the microglial cells isolated directly from a Percoll gradient. There was no significant loss in viability even at high concentrations (1mM) suggesting that the toxic effects observed with CGA were not due to the direct action of nitric oxide. These results suggested that by inhibiting the induction of NOS either there was an increase in the release of a protective compound or a decrease in the levels of a toxic

substance which is produced in a nitric oxide dependent manner. Recent evidence suggests that besides the induction of iNOS, cyclooxygenase-2 (COX-2) is also upregulated when microglia are activated with LPS (Minghetti *et al.*, 1996). This enzyme is responsible for the production of prostanoids which have diverse protective and pathological roles in the CNS. Inhibition of NOS results in an enhancement of LPS induced COX-2 and prostanoid synthesis (Minghetti *et al.*, 1996). It is conceivable that the increase in prostanoids may protect neighbouring microglia from a mixture of toxic mediators released by activating the cells with CGA. Experiments using J774 macrophages resulted in a reduced viability with LPS/IFNγ treatment which could be prevented by L-NIO, a NOS inhibitor (Deakin *et al.*, 1995). Furthermore, the effect of this compound was to reduce the levels of prostanoid, PGE2 and also TNF-α whilst increasing the levels of IL-6.

3.6.2 Microglia and glutamate

Besides secreting cytokines, microglia have also been reported to release glutamate. Piani and Fontana, 1994 showed that when cells were treated with LPS for a short period of time (6 h) there was no noticeable increase in glutamate levels in the media but when left for 48 h there was a 350% increase above control. This suggests that there could be a need for the induction of NOS prior to glutamate release. Thus this was investigated in cells exposed to CGA. There was a CGA dose dependent increase in glutamate release up to 10 nM when the supernatants were assayed 24 h after activation. Furthermore, when a time course was performed, the glutamate release followed iNOS induction and corresponded with an increase in cell death in treated cells. Recently, studies have shown that β-amyloid enhances macrophage production of glutamate (Klegeris and McGeer, 1997) and propose that this may contribute to the

pathogenesis seen in Alzheimer's disease. However, the peritoneal macrophages released a significant amount of glutamate in the absence of stimulus which was not the case in the results presented here. This difference may be due to the highly active state in which peritoneal macrophages appear following preparation. Overall, the percentage increase in glutamate secreted by treatment with β-amyloid (Klegeris and McGeer, 1997) was low in comparison to the levels recorded when cells were exposed to CGA. A comparison of CGA and LPS mediated glutamate release revealed the later increases to be of a similar magnitude to those elicited by β-amyloid (Klegeris and McGeer, 1997). This suggests CGA may be a particularly relevant molecule during activation of microglia in Alzheimer pathology. Furthermore, the control levels of glutamate in the peritoneal system were very similar to the maximal levels observed upon stimulation with 10 nM CGA which may account for the greater increase above control in microglial cells. Culture technique could also account for the differences as it is known that glutamate release is dependent upon cell density, media composition (levels of cystine and glutamine) and the serum concentration (Patrizio and Levi, 1994, Piani and Fontana, 1994). Glutamate can leak out of cells during cell lysis so it was necessary to ensure that this was not the cause of the increases observed in these experiments. This was not the case since there was no noticeable increase in LDH levels prior to or at the same time as increases in glutamate.

The influence of various pharmacological agents on the release were studied. Aminoadipate almost completely blocked the CGA induced release of glutamate indicating that it was dependent upon the Na^+-independent x_c^- transport system (Piani and Fontana, 1994). Interestingly, in fibroblasts it has been shown that the uptake of cystine, which is necessary for glutamate release, is enhanced by the levels of oxygen

free radicals (Bannai and Ishii, 1988) suggesting that the induction of NOS may contribute to the increased levels of glutamate secretion. Propentofylline, an adenosine reuptake and cAMP phosphodiesterase inhibitor reduces both the rate of microglial proliferation and also acts as a general de-activator of these cells inhibiting their transformation into mature macrophages (Rudolphi and Schubert, 1997). It has also been shown that the levels of free radicals produced during the respiratory burst activity of treated microglia is significantly reduced (Banati *et al.*, 1994). When CGA activated microglia were pre-incubated with propentofylline, there was a significant reduction in glutamate release probably as a result of the reduction in free radical formation and the activation status of the microglia. Finally, bafilomycin, an H^+/ATPase inhibitor which prevents vesicular glutamate uptake in neurones (Bowman *et al.*, 1988) was investigated. There was a small but significant decrease in the levels of glutamate released in the presence of this compound suggesting glutamate may be released in a vesicular manner. It has been reported that H^+/ATPase inhibitors can inhibit the respiratory burst in macrophages stimulated with PMA or zymosan (Murphy and Forman, 1993) so this may contribute to the effects observed. Furthermore, recent evidence suggests that microglia possess an homologue of the SNAP-25 protein, an important component of neuronal exocytosis (Hepp *et al.*, 1999). Since the time course of glutamate release and cell death suggested that glutamate may be toxic to the cells the influence of these compounds on cell viability was also measured. All afforded a high degree of protection when the cells were assessed 24 h after activation with CGA.

To determine whether a specific glutamate receptor was involved in microglial cell death, a number of glutamate receptor antagonists were used. Neuronal excitotoxicity occurs via the ionotropic NMDA and AMPA/Kainate glutamate receptors, but using

antagonists of these showed no significant protection from CGA induced death. Microglia have recently been shown to possess metabotropic glutamate receptors (Biber *et al.*, 1999). Using the mGluR group II/III receptor antagonist, MSPG, there was a significant protection from CGA induced loss in viability. Other glial cells possess metabotropic glutamate receptors. Astrocytes become swollen in response to glutamate, an effect mediated by metabotropic receptors (Yuan and Wang., 1996). Rather than a direct action of glutamate it may be that glutamate receptor activation triggers other receptor responses within the glial cells. Stimulation of mGluR's in the rat hippocampus can lead to activation of adenosine receptors via the release of cAMP (Winder *et al.*, 1996) and also adenosine itself can act as co-activator of mGluR's (Ogata *et al.*, 1994). The importance of adenosine in microglial function is well documented. For instance, microglia exposed to the non-selective adenosine receptor agonist, 2-chloro-adenosine for 12 h undergo programmed cell death (Ogata and Schubert, 1996). Propentofylline, is proposed to reinforce the actions of endogenous adenosine and cAMP and thereby control the number of activated microglia. However, instead of increasing the level of CGA induced microglial death as might be expected, there was an improvement in viability and this probably arises as a result of the inhibition of free radical formation and glutamate release. Finally the use of mGluR antagonist can only increase viability of the cells to 80-85% of control suggesting that other toxic mediators may be present. These might conceivably be protein molecules secreted by activated microglia. For instance, TGF-β, an immunosuppressive cytokine is known to cause apoptosis in microglia (Xiao *et al.*, 1997). Cathepsin B, a cysteine proteinase, was found to be secreted by CGA activated microglia and the consequences of its release are discussed in Chapter 5.

3.6.3 Conclusions

In summary, these *in vitro* studies suggest that nitric oxide and the release of glutamate from CGA activated microglia are detrimental to cell survival. The role of these two mediators in neuronal damage during pathological conditions such as Alzheimer's disease is well documented but their role in controlling microglial function has not been noted. This could have consequences for shifting the balance of microglial function between promoting neuronal repair or damage and intervention at this stage may provide new therapeutic strategies to be developed for the treatment of Alzheimer's disease. The mechanisms of cell death are characterised in Chapter 4.

4. MICROGLIAL APOPTOSIS: THE ROLE OF MITOCHONDRIA AND CASPASES

4.1 Introduction

During development of the CNS microglia play an important role in synaptic re-organisation by phagocytosing redundant neurones (Moore and Thanos, 1996). Activation of microglia can also result in the production of neurotoxic mediators that might be involved in diseases like Alzheimer's disease (Gonzalez-Scarano and Baltuch, 1999). In both these situations a regulatory mechanism to control the intensity of the microglial reaction would appear necessary. One possible mechanism by which this might occur is through the induction of a programmed cell death pathway (apoptosis) that might be mediated by the factors released during microglial activation (Jones *et al.*, 1997). *In vivo* studies using the rat model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), suggest that microglia are particularly sensitive to apoptosis (White *et al.*, 1998). This could be potentially mediated by the cytokines TNF-α and IFN-γ (Spanaus *et al.*, 1998) both of which are up-regulated during the acute phase of the disease (Renno *et al.*, 1995; Merrill *et al.*, 1992). Post mortem analysis of diseased tissue from Alzheimer brain (Lassmann *et al.*, 1995) also suggests extensive DNA fragmentation associated with activated microglia. Furthermore, in culture, microglial apoptosis can be induced by ATP (Ferrari *et al.*, 1997), adenosine (Ogata and Schubert, 1996), and transforming growth factor-β (TGF-β) (Xiao *et al.*, 1997) all factors that are released from microglia following inflammatory stimuli suggesting the operation of a controlling feedback mechanism. To date there is very limited knowledge on the nature of the intracellular pathways activated during microglial apoptosis.

A number of characteristic morphological and biochemical features are indicative of apoptosis including nuclear condensation and fragmentation (Wyllie *et al.*, 1980).

These processes are effected through the activation of a cascade of intracellular proteases, known as caspases (Martin and Green, 1995). Ced-3, a cysteine protease, was long known to be involved in apoptosis studies of *C. elegans* (Ellis and Horvitz, 1986). The subsequent discovery of interleukin 1β converting enzyme (ICE) as the first mammalian homologue of this protein (Yuan *et al.*, 1993) lead to the identification of a whole family of these enzymes which are synthesised as inactive proenzymes and then activated following cleavage at specific aspartate residues (thus the name, cysteinyl aspartic acid protease). Caspase cascades can occur following caspase induced caspase activation (Slee *et al.*, 1999) but there may be a requirement for initial cytosolic triggering factors (Liu *et al.*, 1996).

Recently, it has been suggested that as well as caspases, mitochondria may play a role in the pathways to programmed cell death (Mignotte and Vayssiere, 1998; Kroemer, *et al.*, 1998). Prior to DNA fragmentation changes in mitochondrial function can commit a cell to apoptosis. The first alteration to occur is a fall in membrane potential ($\Delta\psi_m$) which may lead to an uncoupling of oxidative phosphorylation (Vayssiere *et al.*, 1994). Agents which can prevent this, such as cyclosporin A, may block apoptosis but once a cell has reached a low $\Delta\psi_m$ there is a point of no return (Zamzami *et al.*, 1995). Cyclosporin A has been proposed to inhibit the phenomenon known as permeability transition (PT) (Zoratti and Szabo, 1995) which involves the opening of a large pore complex in the inner mitochondrial membrane. Once in the open state mitochondrial function is compromised and $\Delta\psi_m$ collapses along with uncoupling of the respiratory chain (Zoratti and Szabo, 1995). During PT apoptosis may proceed via the release of certain mitochondrial proteins such as apoptosis inducing factor (AIF) (Susin *et al.*, 1996; 1999) or cytochrome c (Liu *et al.*, 1996), a carrier of electrons to the cytochrome

oxidase complex in the respiratory chain. Following accumulation of cytochrome c outside the mitochondria it can interact with other cytosolic factors such as apoptosis protease activating factor 1 (Apaf-1) (Zhou *et al.*, 1997) to trigger the activation of caspases and cause nuclear modifications such as DNA fragmentation (Thompson, 1998).

Summary of results

Microglial cell death induced by CGA shows the characteristics of apoptosis. Treatment of both primary cultures and the N9 microglial cell line with CGA causes nuclear condensation and fragmentation and the activation of caspase-1. Cell death is blocked by the caspase-1 inhibitor, YVAD-CHO but neither the caspase-3 inhibitor, DEVD-fmk or the caspase-6 inhibitor, VEID-fmk. CGA induced microglial apoptosis occurs via mitochondrial membrane depolarisation which can be blocked with the iNOS inhibitor, AMT-HCl. Mitochondria in cells exposed to CGA become swollen in comparison with the rod-like structures observed in control cells. Cyclosporin A prevents both mitochondrial depolarisation and apoptosis implicating a role for the PT. Caspase activation occurs downstream of nitric oxide production and mitochondrial depolarisation. Cytochrome c is not released from the mitochondria to the cytosol in CGA induced apoptosis but occurs following treatment of microglia with staurosporine. Staurosporine mediated apoptosis is dependent on caspase-3 activity suggesting distinct pathways exist.

4.2.1 CGA induced microglial apoptosis involves caspase-1 activation

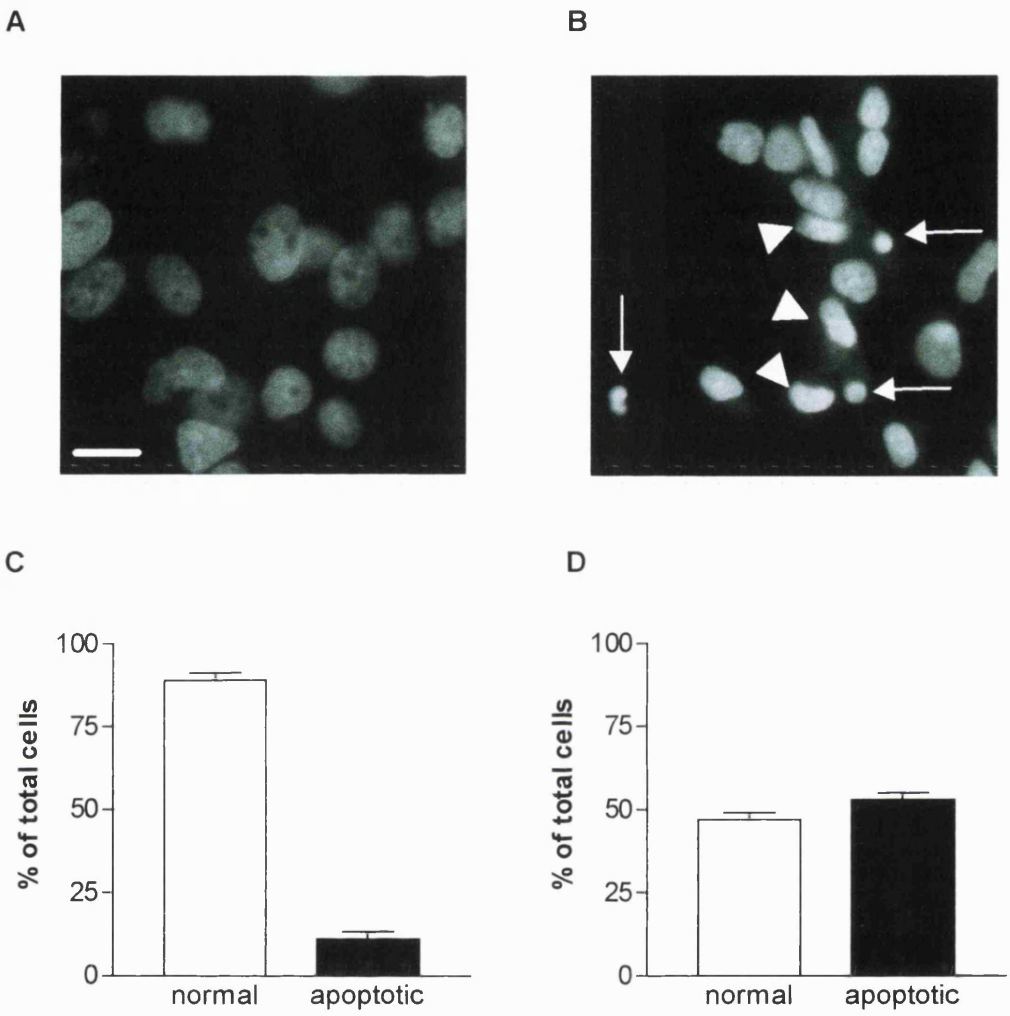


Fig. 4.2.1 Chromogranin A induces apoptosis in microglia. Control untreated microglia **(A and C)** or those exposed to 10 nM CGA for 24 hours **(B and D)** were stained with Hoechst 33342 and the number of apoptotic cells showing pyknotic nuclei (arrows represent late stage apoptotic nuclei and arrowheads early stage) counted using a fluorescence microscope with excitation at 365nm, emission >510nm. Values are the mean % ± SEM of the total nuclei counted in 10 distinct regions per coverslip performed in duplicate from 3 independent cultures (n = 60). Bar = 20 μM.

The results from chapter 3 suggested that CGA evoked microglial cell death was mediated by nitric oxide dependent pathways. Previous studies have suggested that nitric oxide can cause apoptotic cell death in macrophages (Meβmer *et al.*, 1996); thus the mode of microglial cell death was investigated following activation with CGA.

Primary cultures of rat brain microglia were either left untreated or stimulated with 10 nM CGA and then stained with Hoescht 33342 to identify cells undergoing nuclear condensation, one of the typical signs of apoptosis (Yan *et al.*, 1994). Control cells contained low levels of apoptotic nuclei, approximately 10%, (Fig. 4.2.1A and C) whereas cells treated for 24 h with CGA contained approximately 50% cells with condensed nuclei (Fig. 4.2.1B and C). A time course to identify the point at which nuclear condensation begins revealed that following stimulation of primary microglial cultures with CGA there was no noticeable nuclear modification prior to 16 h but there was significant loss of viability at 24 h by which time the number of apoptotic microglia had increased to 48.90 ± 3.75% and by 48 h this number had increased further to 69.00 ± 3.60% (Fig. 4.2.2A). Similarly there were significant increases in apoptosis by 24 h in the N9 microglial cell line (Fig. 4.2.2B). When CGA stimulated microglia were incubated with AMT-HCl, an iNOS specific inhibitor, for the full 24 h prior to assessment of apoptosis there was a significant increase in the cell viability from 46.30 ± 8.54% to 90.11 ± 3.10%, which is similar to control levels (92.53 ± 3.05%) (Fig. 4.2.2C). Furthermore, if AMT-HCl was applied either 2, 4, or 8 h following CGA treatment there was also a significant reduction in the number of apoptotic cells. However, if AMT-HCl was added following the induction of NOS i.e. at 12 h it failed to significantly reduce cell death. Thus CGA evoked microglial apoptosis is mediated by NO.

Cellular caspases may be activated during apoptosis (Lazebnik *et al.*, 1991). Thus, microglia were incubated with specific peptide inhibitors of either caspase-1, caspase 3 or caspase 6 to determine whether these caspases were involved in CGA induced

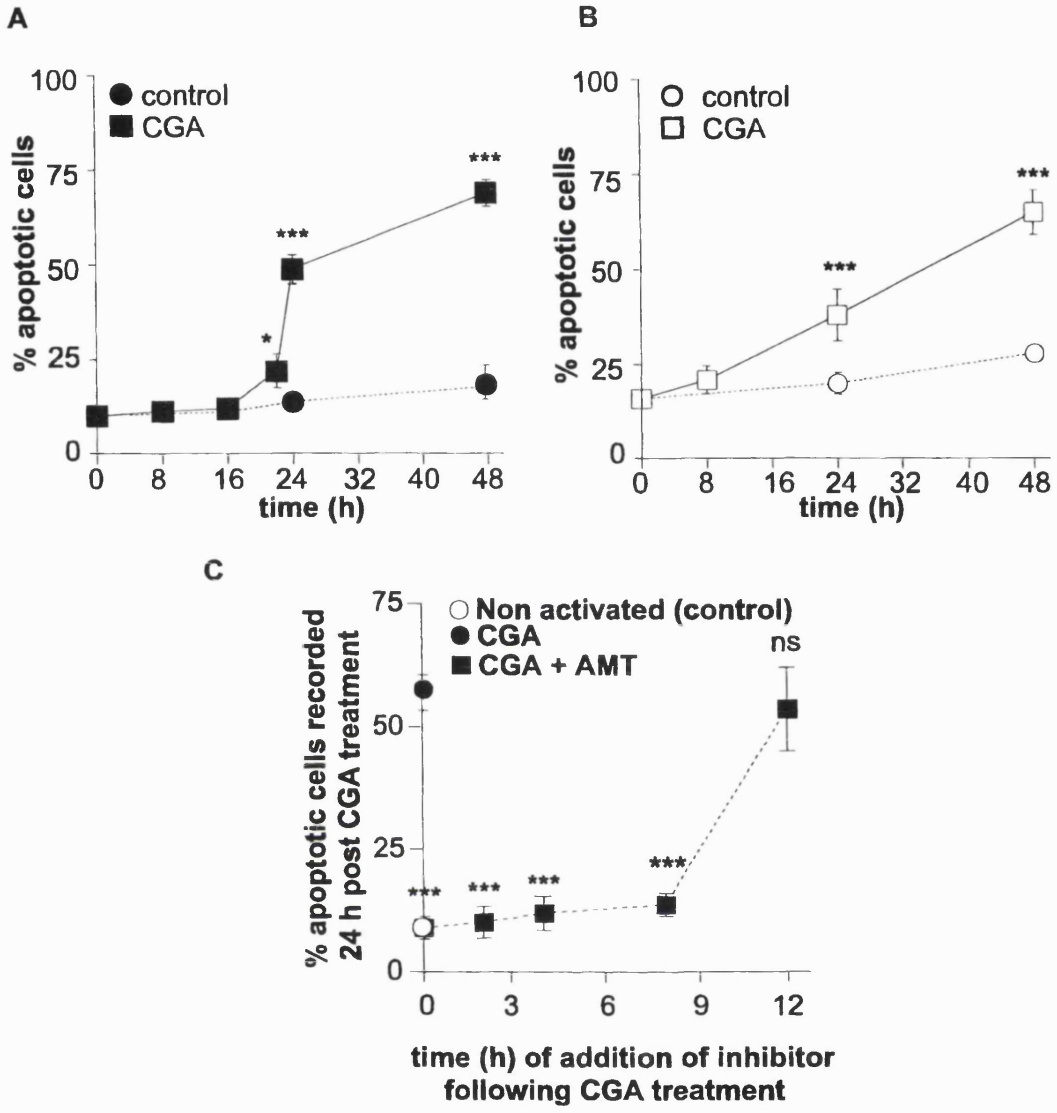


Fig. 4.2.2 Time course of CGA induced microglial apoptosis. Primary microglia **(A)** and the N9 cell line **(B)** were plated onto coverslips and then stimulated with 10 nM CGA at 1 DIV. Cultures were stained with Hoescht 33342 and the number of apoptotic cells showing pyknotic nuclei in control (circles) and CGA treated (squares) cultures were counted using a fluorescence microscope with excitation at 365nm, emission >510nm. Values are the mean % ± SEM of the total nuclei counted in 8 distinct regions per coverslip performed in duplicate from 3 independent cultures (n = 48). ***p<0.001, *p<0.05 significantly different compared with the number of apoptotic cells at time 0 h. **(C)** Microglia were treated with AMT-HCl (100 nM) at various time points following stimulation with 10 nM CGA and the degree of apoptosis assessed 24 h later. Data are the mean % apoptotic cells in control (open circles), CGA treated (squares) or CGA treated in the presence of AMT-HCl (closed circles). ***p<0.001 significantly different to the level of apoptosis in the presence of CGA alone, ns not significantly different.

microglial apoptosis. Preincubation of microglia with 100 μM YVAD-CHO or 10 μM WEHD-fmk before stimulation with 10 nM CGA significantly prevented apoptosis (Fig. 4.2.3A) suggesting that cell death was dependent on caspase-1 activation (Hibi *et al.*,

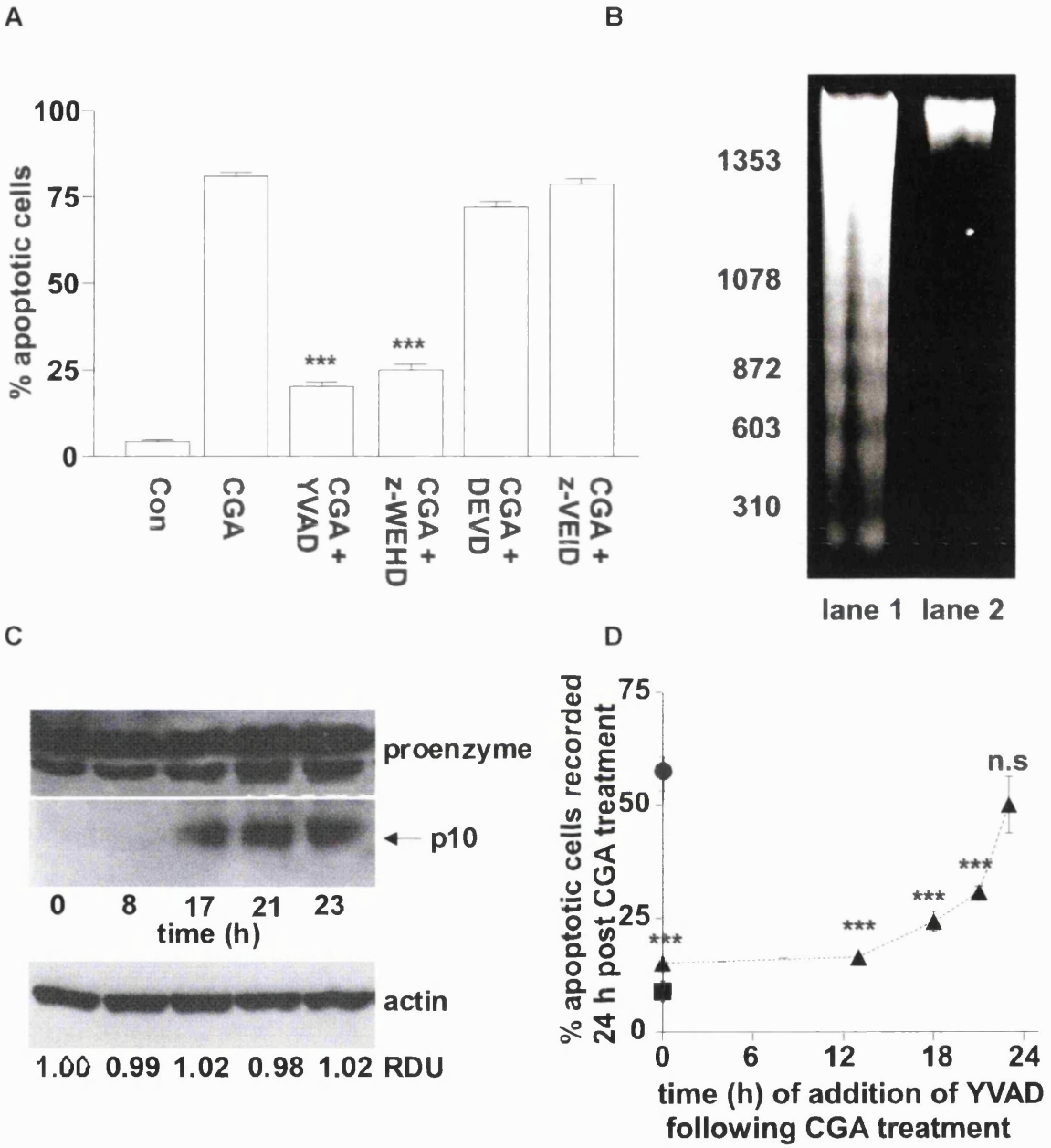


Fig. 4.2.3 Microglial apoptosis involves caspase 1 activation. **(A)** Microglia were preincubated with 100 μM YVAD-CHO, 10 μM WEHD-fmk, 10 μM VEID-fmk or 50 μM z-DEVD-fmk for 1 h before exposure to 10 nM CGA for 48 h. The number of apoptotic cells were counted in a minimum of 20 discrete areas from two independent experiments. Data are the mean ± SEM values. ***p<0.001. **(B)** DNA was extracted from cells exposed to 10 nM CGA for 24 h in the absence (lane 1) or presence of the caspase 1 inhibitor, YVAD-CHO (lane 2) and fragments separated on a 1% agarose gel for 2 h at 125V and viewed with UV illumination. **(C)** Cell lysates were prepared from control N9 microglia (0) and those exposed to 10 nM CGA for 8, 17, 21 or 23 h, resolved by SDS-PAGE and immunoblotted with anti-ICE p10 (c20) antibody. The blot was reprobed with actin and the relative density (RDU) is shown with respect to levels at time 0 h. **(D)** Microglia were treated with YVAD-CHO (10 μM) at various time points following stimulation with 10 nM CGA and the degree of apoptosis assessed 24 h later. Data are the mean % apoptotic cells in control (squares), CGA treated (circles) or CGA treated in the presence of YVAD-CHO (triangles). ***p<0.001 significantly different to the level of apoptosis in the presence of CGA alone.

1997; Bermudez *et al.*, 1999). Neither 50 μM DEVD-fmk (caspase-3 inhibitor) nor 10 μM VEID-fmk (caspase-6 inhibitor) were able to significantly reduce apoptosis (Fig. 4.2.3A). Also when cells were incubated with IL-1β neutralising antibody (1:100) there was no significant protection from CGA induced apoptosis (activated cells, 48.35 ± 2.14% apoptotic versus activated cells plus antibody, 53 ± 1.97% apoptotic after 24 h stimulation) suggesting that the product of caspase-1 activation, IL-1β, is not directly involved. Morphological changes to the nucleus and caspase activation are often accompanied by the degradation of chromatin to low molecular weight fragments (Wyllie, 1980) which give a characteristic DNA ladder when subjected to gel electrophoresis. Exposure of microglia to 10 nM CGA resulted in the appearance of such DNA laddering which could be prevented by incubation with YVAD-CHO (Fig. 4.2.3B). Cell lysates of the N9 microglial cell line were prepared from cells treated with 10 nM CGA for varying lengths of time and then immunoblotted for cleavage of the ICE (caspase-1) proenzyme (Gu *et al.* 1999). p10 fragments were first detectable at 17 h indicating the activation of this caspase (Fig. 4.2.3C). Finally the time course of caspase activation in relation to apoptosis was investigated by adding YVAD-CHO at different time points after stimulation of cells with CGA. The addition of YVAD-CHO 13 h (prior to the appearance of p10 fragment) into CGA exposure gave the same degree of protection as if added at the same time as CGA (Fig. 4.2.3D). If the inhibitor was added at 18 h or 21 h which is even after the appearance of active fragment there was still a significant decrease in the number of apoptotic cells but the protective effect was lost if YVAD-CHO was added any later (Fig. 4.2.3D) suggesting there may be a certain threshold activation required to elicit nuclear changes. Changes in cellular ATP levels may control whether a cell becomes necrotic or apoptotic (Nicotera *et al.*, 1998, 1999). Thus to investigate the significance of this phenomenon in CGA induced microglial cell

death, cellular ATP levels were determined. Following exposure to 10 nM CGA the levels of ATP and ADP were determined in primary microglia (Fig. 4.2.4A and B) and the N9 microglial cell line (Fig. 4.2.4C and D). Following 24 h treatment with CGA there was a small drop in ATP levels and over time a gradual decline in the ATP/ADP ratio which appears to occur following the induction of apoptosis. This was not the case for sodium cyanide treatment, which rapidly depletes ATP levels (Pocock and Nicholls, 1998). In this instance, the ATP levels showed a significant drop within 15 min (Fig. 4.2.4A and C).

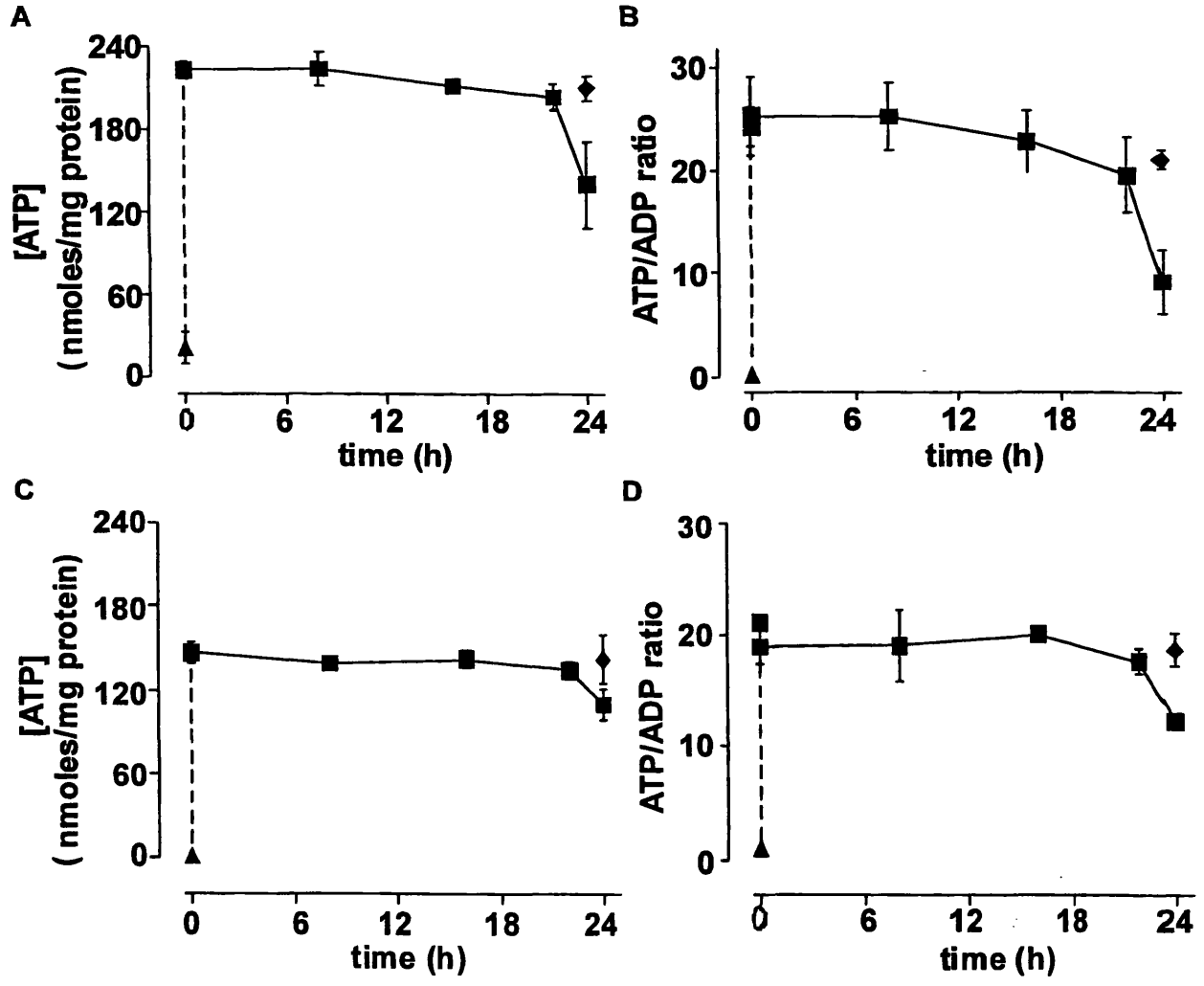


Fig. 4.2.4 ATP levels remain high until late time points during CGA induced microglial apoptosis. Primary microglia (A and B) or the N9 cell line (C and D) were either left untreated (◆) or exposed to 10 nM CGA (■) for the times indicated and then samples prepared for ATP determination. A 15 min exposure to 5 mM cyanide was used as a postive control for rapid depletion of ATP levels (▲).

4.3 CGA induces mitochondrial depolarisation in microglia

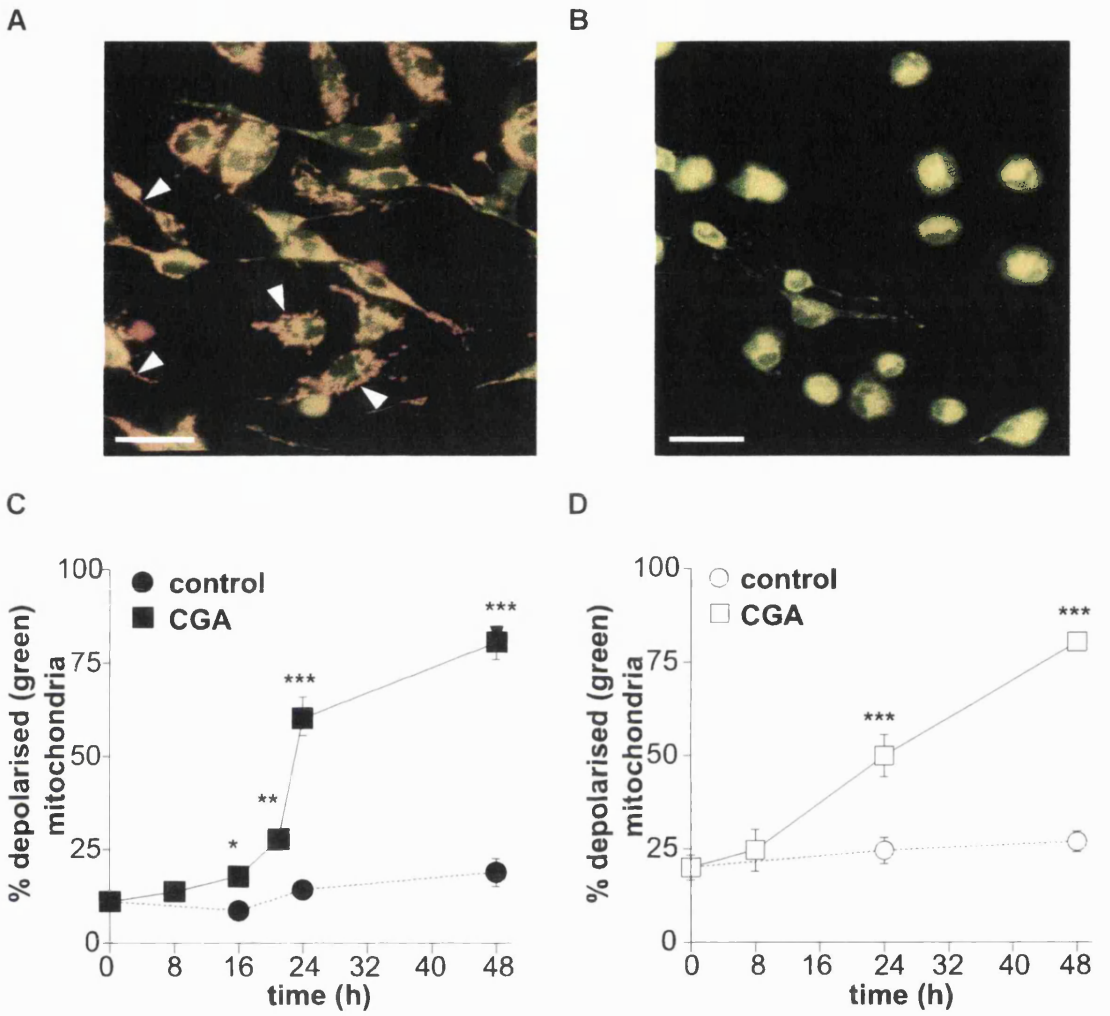


Fig. 4.3.1 CGA induces mitochondrial depolarisation in microglia. Cells were loaded with the mitochondrial membrane potential sensitive probe JC-1 as described in Chapter 2. Discrete mitochondria which fluoresced red/orange were visible in control untreated microglial cultures **(A)** but following exposure to 10 nM CGA the cells displayed only a green diffuse fluorescence and no punctate staining mitochondria. **(B)** The number of cells exhibiting a red/orange or green fluorescence were counted in either primary microglial cultures **(C)** or the N9 cell line **(D)** at time points following addition of 10 nM CGA (squares) or their corresponding controls (circles). *p<0.05, **p<0.01, ***p<0.001 signficantly different to controls at time equal 0 h.

Previous studies have shown that prior to DNA fragmentation cells destined to undergo apoptosis exhibit a fall in mitochondrial membrane potential ($\Delta\psi_m$) and thus an increase in the number of depolarised mitochondria. JC-1, a mitochondrial specific dye, can be used to measure such changes. It passes into the mitochondrial matrix where individual monomers can form J aggregates which fluoresce red when $\Delta\psi_m$ is high. At depolarised

mitochondrial membrane potentials JC-1 will remain as a monomer and fluoresce green. Thus microglial cells were loaded with JC-1 at various time points following treatment with 10 nM CGA and the number of cells exhibiting a red or green fluorescence recorded. In control untreated primary cultures, cells mainly fluoresced red (Fig. 4.3.1A) and the number with depolarised mitochondria (green) accounted for 11.15 ±1.3% of the total cell population (Fig. 4.3.1C). This number did not increase significantly during the course of the experiments and after 24 h was only 14.28 ± 1.98% (Fig. 4.3.1C). However, cells stimulated with CGA showed a small but significant increase in the number of cells with depolarised mitochondria at 16 h (17.87 ± 2.48%) and a more marked increase to 60.19 ± 5.79% by 24 h and 80.30 ± 4.5% by 48 h (Fig. 4.3.1C). A similar trend was observed for the N9 microglial cell line (Fig. 4.3.1D). To confirm that the cells fluorescing green contained mitochondria that were completely depolarised, cells stimulated with CGA for 24 h were loaded with JC-1 and the mitochondrial uncoupler, carbonyl cyanide p-(tri-fluoromethoxyl)phenylhydrazone (FCCP) added to the cells and sequential images captured. In control microglia, FCCP addition resulted in a decrease in fluorescence (Fig. 4.3.2A), indicating that the mitochondria were previously polarised and thus capable of depolarisation by FCCP. In contrast microglia which had been exposed to 10 nM CGA for 24 h and contained depolarised microglia failed to respond to FCCP indicating that the mitochondria were fully depolarised (Fig. 4.3.2B). In order to confirm that this was due to a net decrease in red fluorescence, the experiments were repeated with an Omega Optical XF32 emission filter which only allows passage of fluorescence above 550nm therefore excluding green fluorescence. Again the fluorescence levels were reduced in control cultures upon

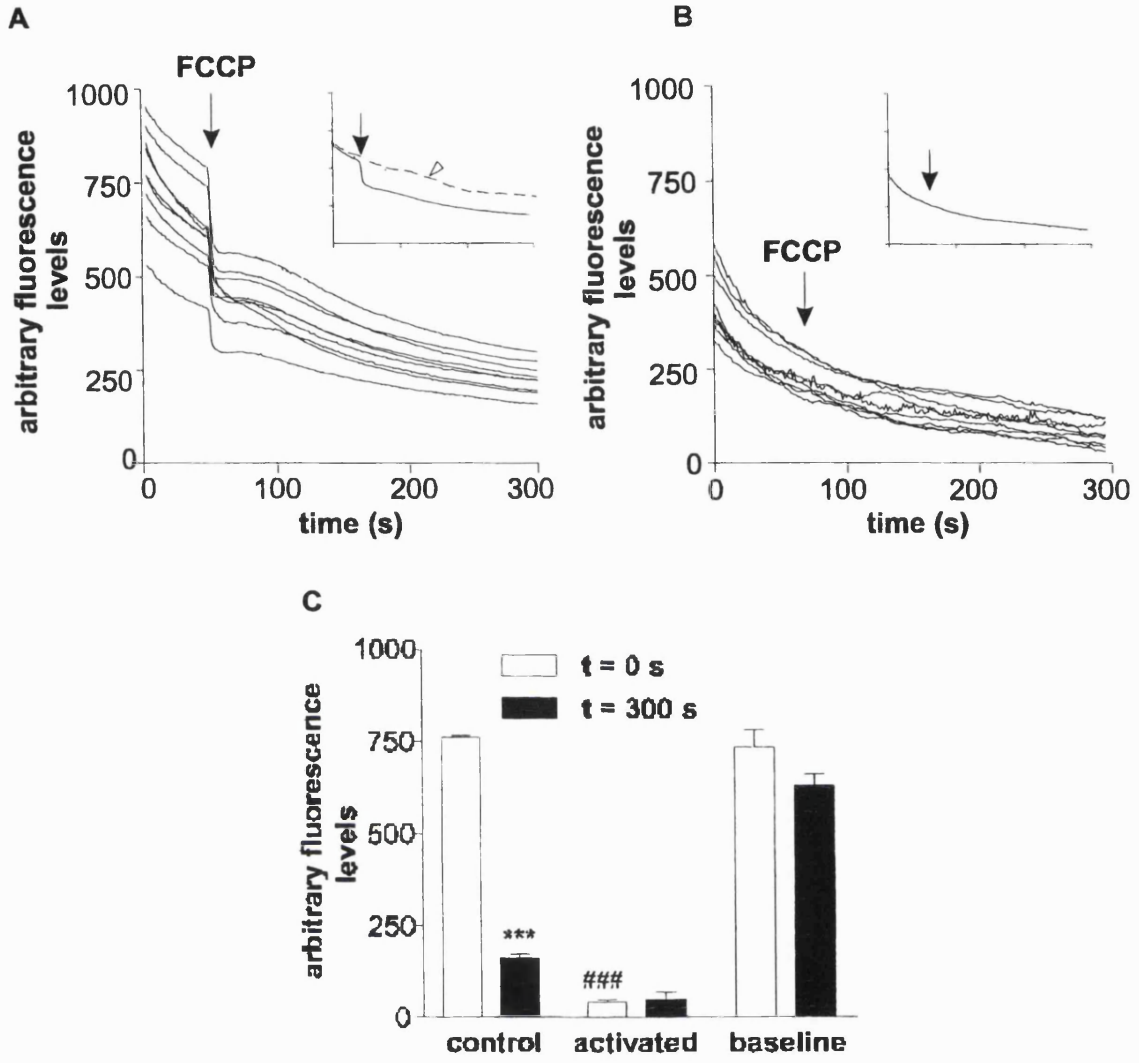


Fig. 4.3.2 Complete mitochondrial depolarisation following CGA activation. Microglia were loaded with JC-1 and single cell fluorescence imaging performed. The addition of 200 μM FCCP (solid arrow) resulted in decreased fluorescence (indicative of mitochondrial membrane depolarisation) in control untreated cultures **(A)** but did not affect the fluorescence levels in CGA stimulated cells **(B)**. Each line represents the response from a single cell and the insets are the respective means of 20 cells (hatched line indicates background drift due to fluorescence quenching). **(C)** Using a filter to show only polarised mitochondria (red/orange) the mean fluorescence levels of microglia were recorded prior to (t = 0 s) and following (t = 300 s) the addition of 200 μM FCCP in untreated cultures(control) and those exposed to 10 nM CGA for 24 h (activated). The change in fluorescence without addition of FCCP was recorded in control cells (baseline). ***$p<0.001$ indicates there is a significantly decreased fluoresence following FCCP treatment. ###$p<0.001$ indicates there is a significantly decreased fluorescence in CGA activated cells compared with control cells.

exposure to FCCP but significantly there was already a diminished fluorescence level in activated microglia which failed to change with FCCP (Fig. 4.3.2.C). These findings were mirrored (Fig. 4.3.3A-D) when similar experiments were conducted using

tetramethyl rhodamine ethyl ester (TMRE) as a mitochondrial probe (Bindokas *et al.*, 1998). This is another dye that is both readily and specifically taken up into the mitochondrial matrix and as such presents very few artifacts (Cossarizza *et al.*, 1993). Finally, rhodamine 123 was also used to confirm mitochondrial depolarisation

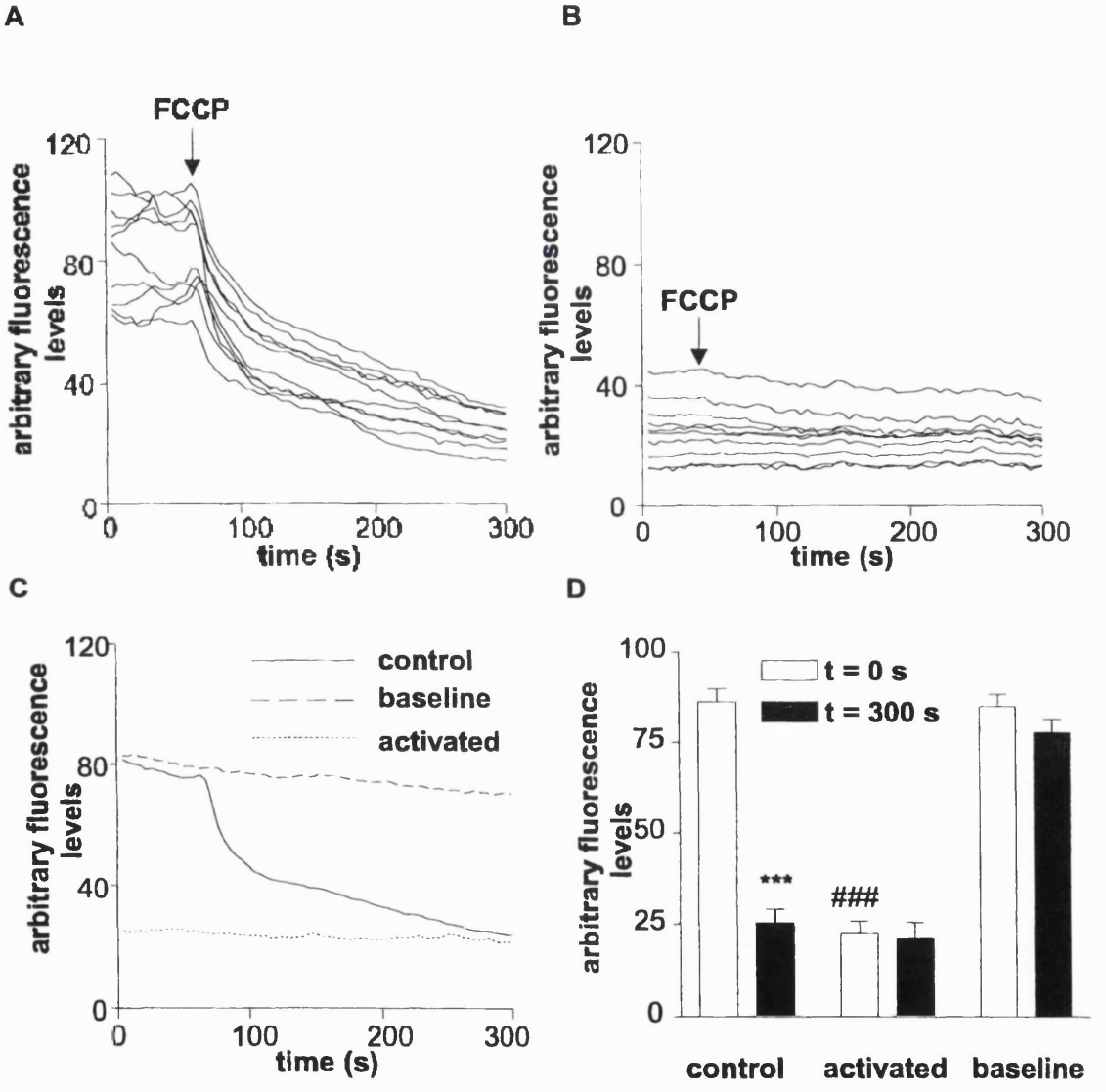


Fig. 4.3.3 CGA causes reduced mitochondrial membrane potential as measured with TMRE. **(A)** Addition of 200 μM FCCP (arrow) to untreated N9 cells loaded with TMRE (200 nM) results in a decrease in fluorescence indicative of mitochondrial depolarisation whereas cells which had been stimulated with 10 nM CGA failed to respond **(B)**. The mean responses of 20 cells treated in experiments as described above **(C)**. The average fluorescence levels in 80 N9 cells were recorded before (t = 0 s) or after FCCP addition (t = 300 s) in untreated cultures (control) or those stimulated with 10 nM CGA (activated) and cells without addition of FCCP (baseline) **(D)**. ***$p<0.001$ significantly different to fluorescence levels prior to FCCP addition in control cultures. ###$p<0.001$ significantly lower fluorescence levels following CGA treatment compared with control microglia (prior to FCCP addition).

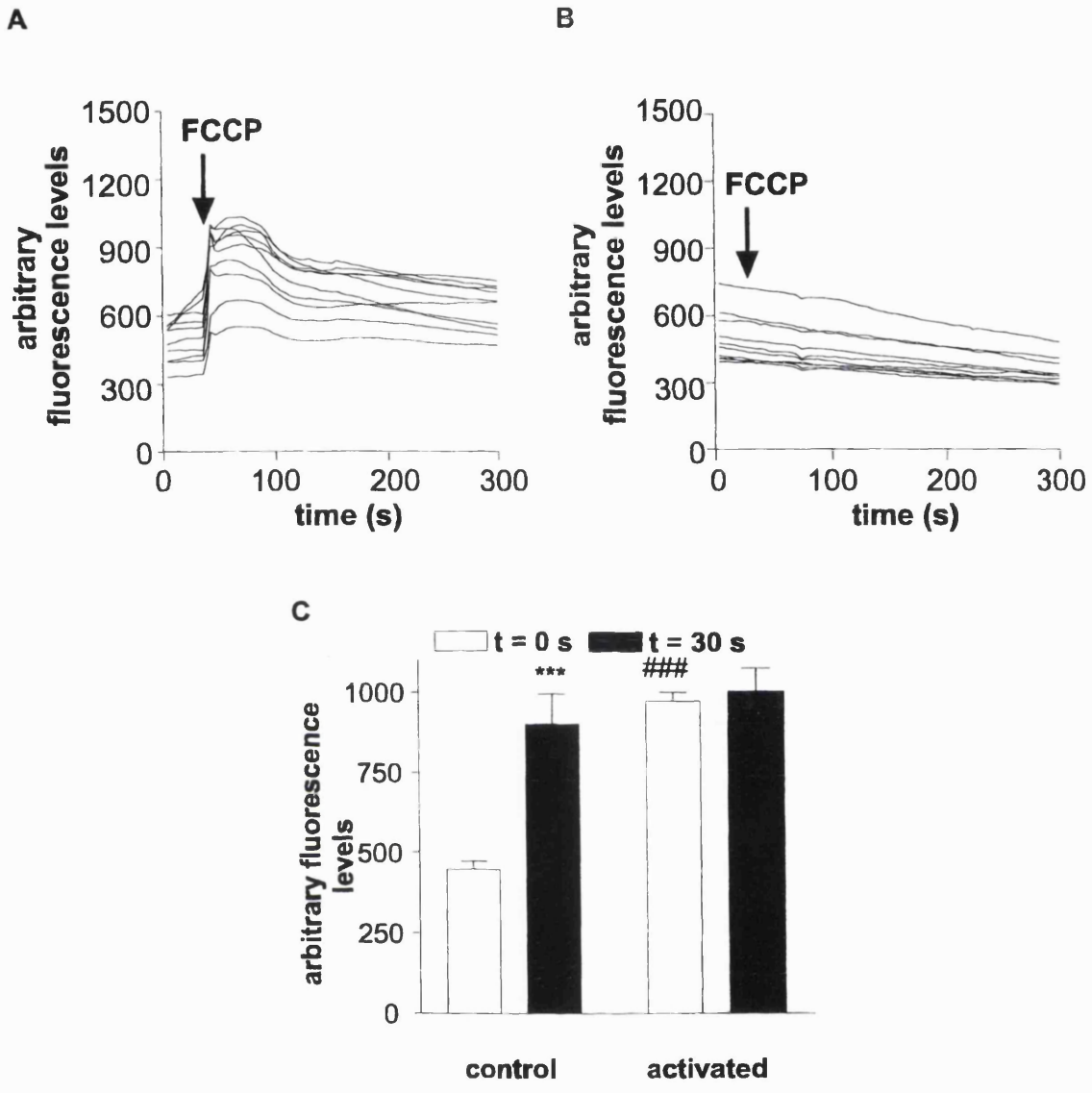


Fig. 4.3.4 CGA activation of microglia causes reduced mitochondrial membrane polarisation as measured with rhodamine 123. A) Addition of 200 μM FCCP (arrow) to untreated N9 cells loaded with rhodamine 123 (0.5 μg/ml) results in an increase in fluorescence indicative of mitochondrial depolarisation whereas cells which had been stimulated with 10 nM CGA failed to respond **(B)**. **(C)** The average fluorescence levels of rhodamine 123 loaded cells prior to FCCP challenge (t = 0 s) and immediately following FCCP addition (t = 30 s). Data are the mean ± SEM (n = 80 discrete cells). ***p<0.001 significantly higher fluorescence levels following FCCP addition, ###p<0.001 significantly higher fluorescence levels compared with control untreated cells prior to FCCP addition.

(Duchen, 1992). This mitochondrial probe is more difficult to work with since it aggregates easily. Therefore, rather than measuring mitochondrial membrane potential, rhodamine 123 is most suitable as a sub-cellular marker for mitochondria where it exhibits a yellow/green fluorescence. Nevertheless, exposure of microglia to CGA for

24 h resulted in a higher mean fluorescence level than control cultures (Fig. 4.3.4C) and whereas untreated cells responded to FCCP with an increase in fluorescence, CGA stimulated cells failed to (Fig. 4.3.4A and B).

4.4 Mitochondrial permeability transition, NO and microglial apoptosis

Beside the increase in the number of cells with depolarised mitochondria it was clear that there was a change in mitochondrial morphology following treatment with CGA. In control cultures, the mitochondria generally exhibited a rod like appearance and were widely distributed throughout the cell soma and also found in high abundance in the fine processes of the resting cell (Fig. 4.4.1A). While as previously discussed there was a small increase in the number of cells with depolarised mitochondria following 16 h incubation with 10 nM CGA the majority of the cells still had discrete red fluorescing mitochondria. However, the mitochondria had become more rounded at this stage (Fig. 4.4.1B). Confocal microscopy would have allowed the investigation of this in greater detail but these observations at least suggested that the permeability transition (PT) phenomenon might be occurring. PT involves the opening of a pore complex in the inner membrane of the mitochondrion allowing movement of molecules between matrix and cytosol thus leading to the swelling effect (Zoratti and Szabo, 1995). Thus the effect of cyclosporin A (CsA), a known inhibitor of the PT, on CGA induced mitochondrial depolarisation and apoptosis in microglia was investigated. CsA (1 μM) was added 13 h following the application of CGA (prior to any significant increase in mitochondrial depolarisation) and the cells stained with JC-1 and Hoescht 33342 at 24 h (Fig. 4.4.2). The number of apoptotic cells in primary cultures following CGA stimulation for 24 h was reduced from 52.10 ± 3.75% of the total to 22.90 ± 3.95% of the total in the presence of CsA (Fig. 4.4.2). Preincubation with CsA also resulted in a

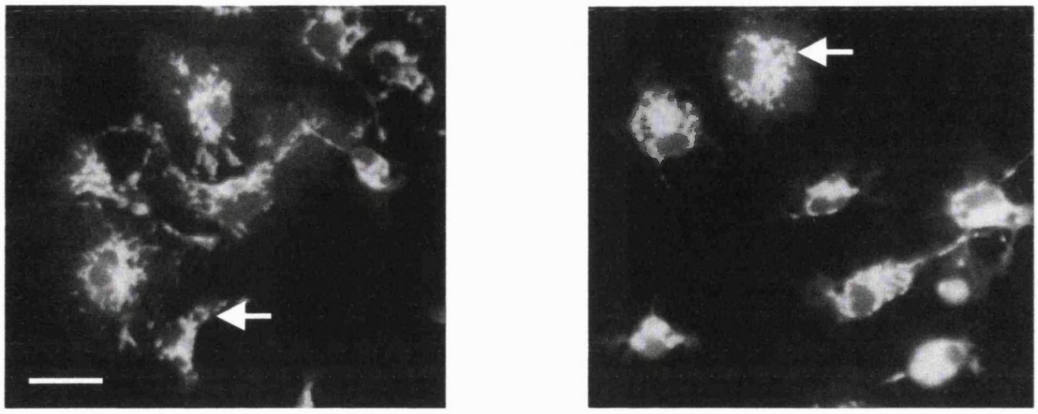


Fig. 4.4.1 Changes in mitochondrial morphology following CGA activation. Microglial cells were loaded with JC-1 and then mounted on the stage of an inverted fluorescence microscope. Images of JC-1 staining are shown for control microglia (A) and microglia exposed to 10 nM CGA for 16 h (B). Arrows indicate the discrete mitochondrial labelling of typically rod like structures in control microglia but a more rounded swollen morphology in activated cells.

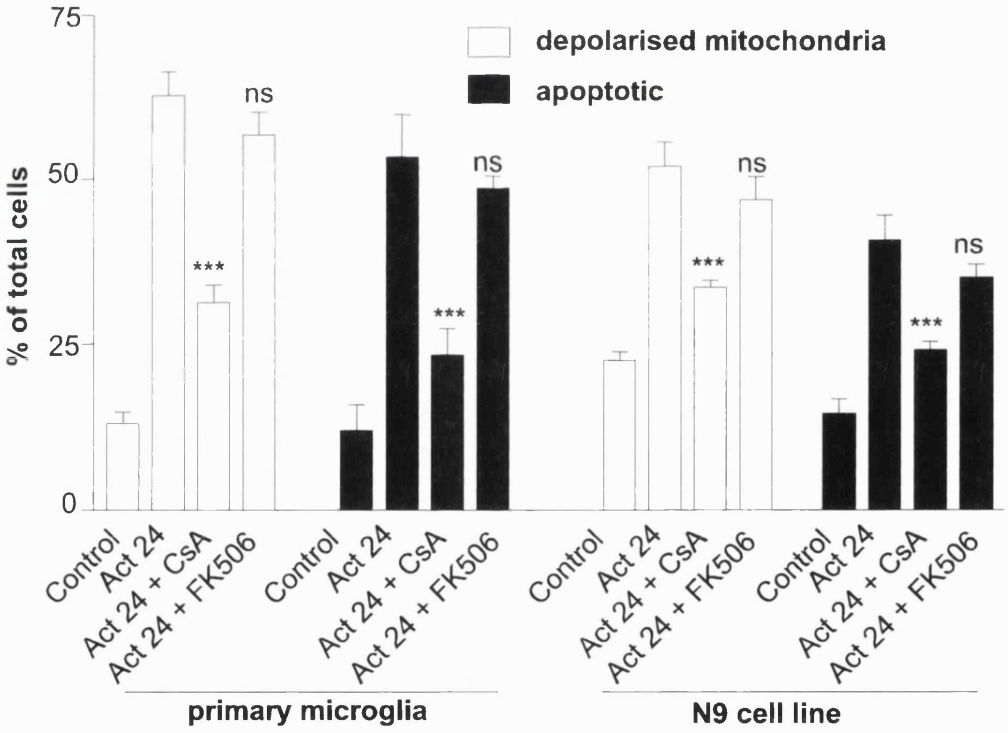


Fig. 4.4.2 Microglia activated with CGA undergo the mitochondrial permeability transition. Primary microglia or the N9 cell line were exposed to CGA for 24 h in the absence (act 24) or presence of 1 μM cyclosporin A (act 24 + CsA) or 1 μM FK506 (act 24 + FK506) and these and control cultures subsequently stained with JC-1 or Hoechst 33342. The number of cells with depolarised mitochondria (open bars) or those exhibiting apoptotic nuclei (closed bars) were counted in 8 distinct regions per coverslip with 2 coverslips per preparation and a total of 3 independent preparations and the values expressed as the mean percentage of total cell number ± SEM (n = 48). ***$p<0.001$ significantly different compared with control cultures.

reduction in the number of microglia with depolarised mitochondria from 60.19 ± 5.80% in cultures treated with CGA alone to 30.00 ± 2.60% in cells stimulated with CGA in the presence of CsA (Fig. 4.4.2). Since CsA also has effects on calcineurin (Dawson *et al.*, 1993; Ankarcrona *et al.*, 1996) the specific calcineurin inhibitor FK506 was tested for effects on mitochondrial depolarisation and apoptosis. This compound did not prevent the CGA mediated effects (Fig. 4.4.2). This data was mirrored in the N9 microglial cell line (Fig. 4.4.2). CsA also had no effect on NO production as indicated by total nitrite in the cell medium (CGA activated microglia 20.00 ± 1.97 μM, activated + CsA 20.00 ± 2.03μM). The role of NO in eliciting mitochondrial depolarisation was investigated (Fig. 4.4.3).

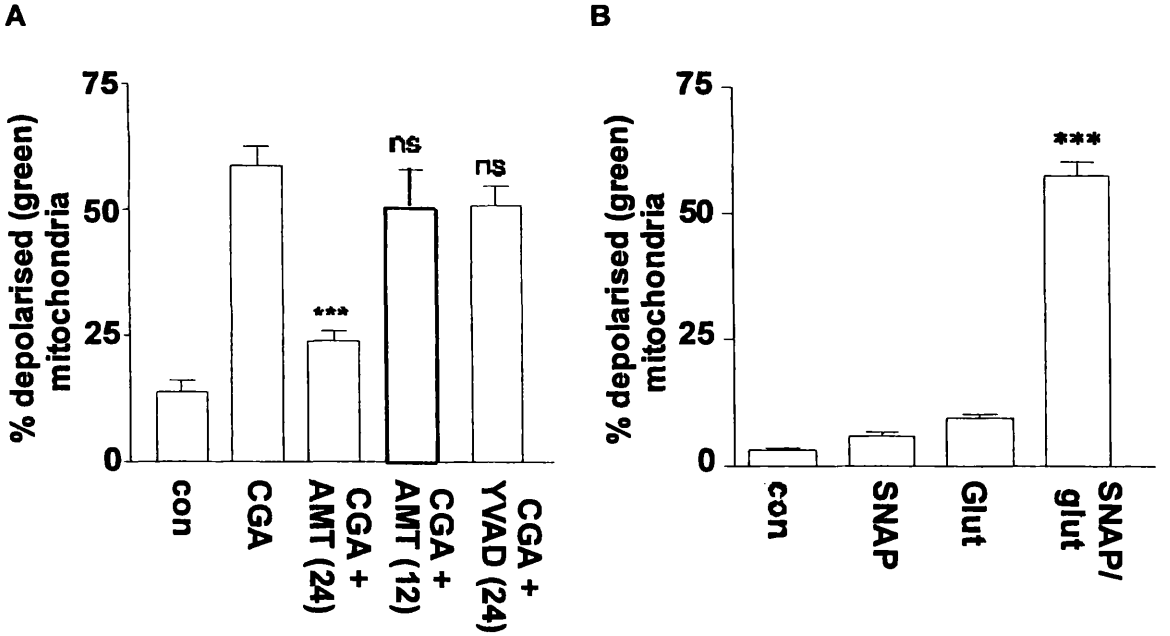


Fig. 4.4.3 Nitric oxide dependent mitochondriai depolarisation. (A) Cells were loaded with JC-1 and the level of mitochondrial depolarisation determined in unstimulated cultures (con), those exposed to CGA alone (CGA) or those exposed to CGA when AMT-HCl was added for the full 24 h incubation (CGA + AMT (24)) or for the last 12 h (CGA + AMT (12)) or cells exposed to CGA in the presence of YVAD-CHO for the full 24 incubation (CGA + YVAD-CHO (24)). Data are expressed as the percentage of cells displaying a green fluorescence (n = ≥ 32). ***p<0.001 significantly lower level of mitochondrial depolarisation compared with cells treated with CGA alone, ns not statistically different. **(B)** Microglia were either left untreated (con) or exposed to 100 μM nitric oxide donor alone (SNAP), 5 mM glutamate (glut) or NO donor with glutamate (SNAP/glut) for 24 h and then stained with JC-1. Data are expressed as the percentage of cells displaying a green fluorescence. ***p<0.001 significantly different from control cultures (n = ≥ 40).

Incubation of CGA stimulated cells with AMT, the iNOS inhibitor, prevented the increase in the number of cells with depolarised mitochondria (Fig. 4.4.3A). Thus suggesting nitric oxide as an upstream initiator of mitochondrial depolarisation. This was further tested by using the NO donor, SNAP. Application of SNAP to microglia did not result in mitochondrial depolarisation (Fig. 4.4.3B). Microglial apoptosis might be mediated by glutamate, however glutamate alone failed to evoke mitochondrial depolarisation (Fig. 4.4.3B). This might suggest a reason for the lack of apoptosis observed in the presence of SNAP or glutamate/mGluR agonist alone (Fig. 4.4.4A and B). However, treatment of cells with NO donor and glutamate together resulted in both significant mitochondrial depolarisation (4.4.3B) and apoptosis (Fig. 4.4.4A and B) suggesting that nitric oxide might sensitise microglia to the effects of mGluR agonists.

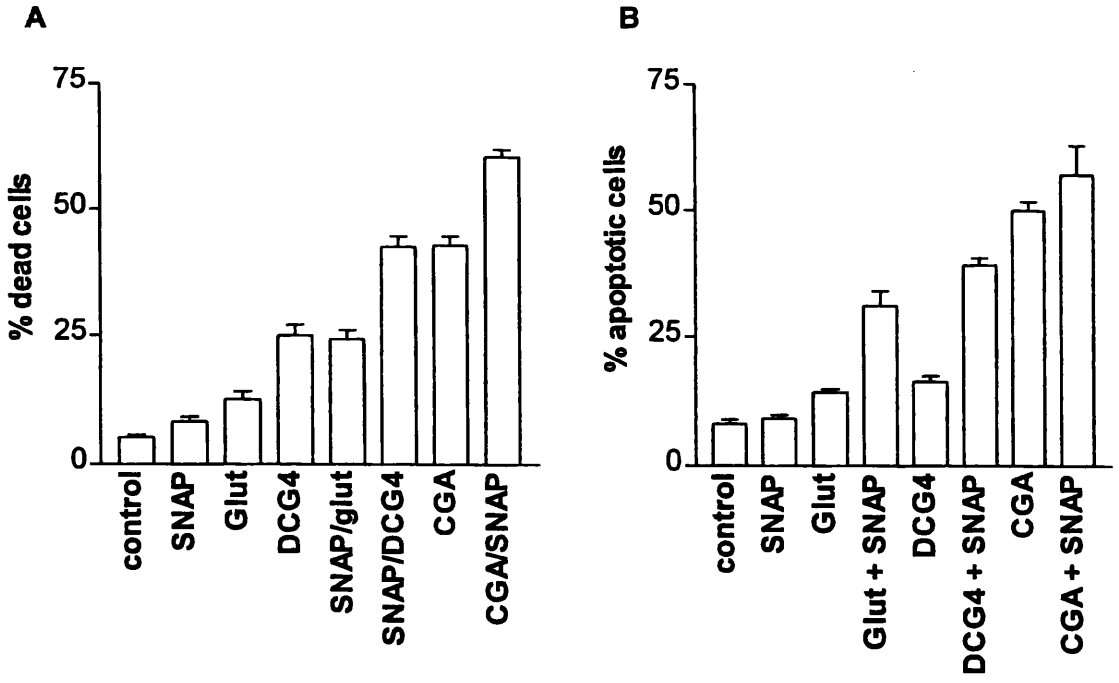


Fig. 4.4.4 The nitric oxide donor, SNAP, sensitizes microglia to the toxic effects of glutamate. N9 microglia were either left untreated (control) or exposd for 24 h to either 100 μM nitric oxide donor alone (SNAP), 5 mM glutamate (glut), 500 nM metabotropic glutamate receptor agonist (DGC4) or NO donor and glutamate (SNAP/glut), NO donor and mGluR angonist (SNAP/DCG4), 10 nM CGA (CGA) or CGA and NO donor (CGA/SNAP). Cells were then either stained with propodium iodide **(A)** or Hoechst 33342 to detect apoptotic nuclei **(B)**. Values are the mean % ± SEM of the total counts in 10 distinct regions per coverslip performed in duplicate from at least 2 independent cultures ($n \geq 40$).

4.5 Cytochrome c release is dependent on the nature of apoptotic stimulus

Microglial apoptosis could be prevented with cyclosporin A suggesting activation of the permeability transition, a phenomenon which may involve the release of cytochrome c from the mitochondria to the cytosol. Thus, mitochondrial and cytosolic fractions were prepared by homogenisation, under conditions that minimised mitochondrial damage, and cytochrome c protein levels were assayed by immunoblotting. Cytosol from CGA stimulated cells was free of any detectable cytochrome c as were the untreated samples (Fig. 4.5.1A). An extensive time course from 0-28 hours revealed that whilst cytosolic cytochrome c did not increase there were significant elevations in mitochondrial cytochrome c (15 kDa species) at 16 h and 24 h (Fig. 4.5.1B). Densitometry revealed these increases to be between two and three fold. Furthermore, analysis of cytochrome c content in whole cell lysates also revealed a similar increase in cytochrome c, indicating the results are not due to uncontrolled losses and redistributions between different fractions during the isolation procedure (Fig. 4.5.2A and B). Preincubation with 0.1 μg/ml cycloheximide reduced this increase suggesting that new protein synthesis was a requirement (Fig. 4.5.2A and B). Staurosporine, a known inducer of apoptosis and cytochrome c release in many cell types (Tang *et al.*, 1998) did promote the translocation of cytochrome c to the cytosol (Fig. 4.5.3A) and there were no increase in the levels in the mitochondrial fractions. Densitometry revealed that the levels of cytochrome c in the cytosol peaked with 2 h stimulation of staurosporine (1 μM) increasing ten fold above control samples (Fig. 4.5.3B). There was a corresponding loss of cytochrome c from the mitochondria. Both CGA and staurosporine blots were stripped and reprobed with cytochrome oxidase to reveal enrichment only in the mitochondrial fractions indicating that there was no contamination of cytosolic extracts.

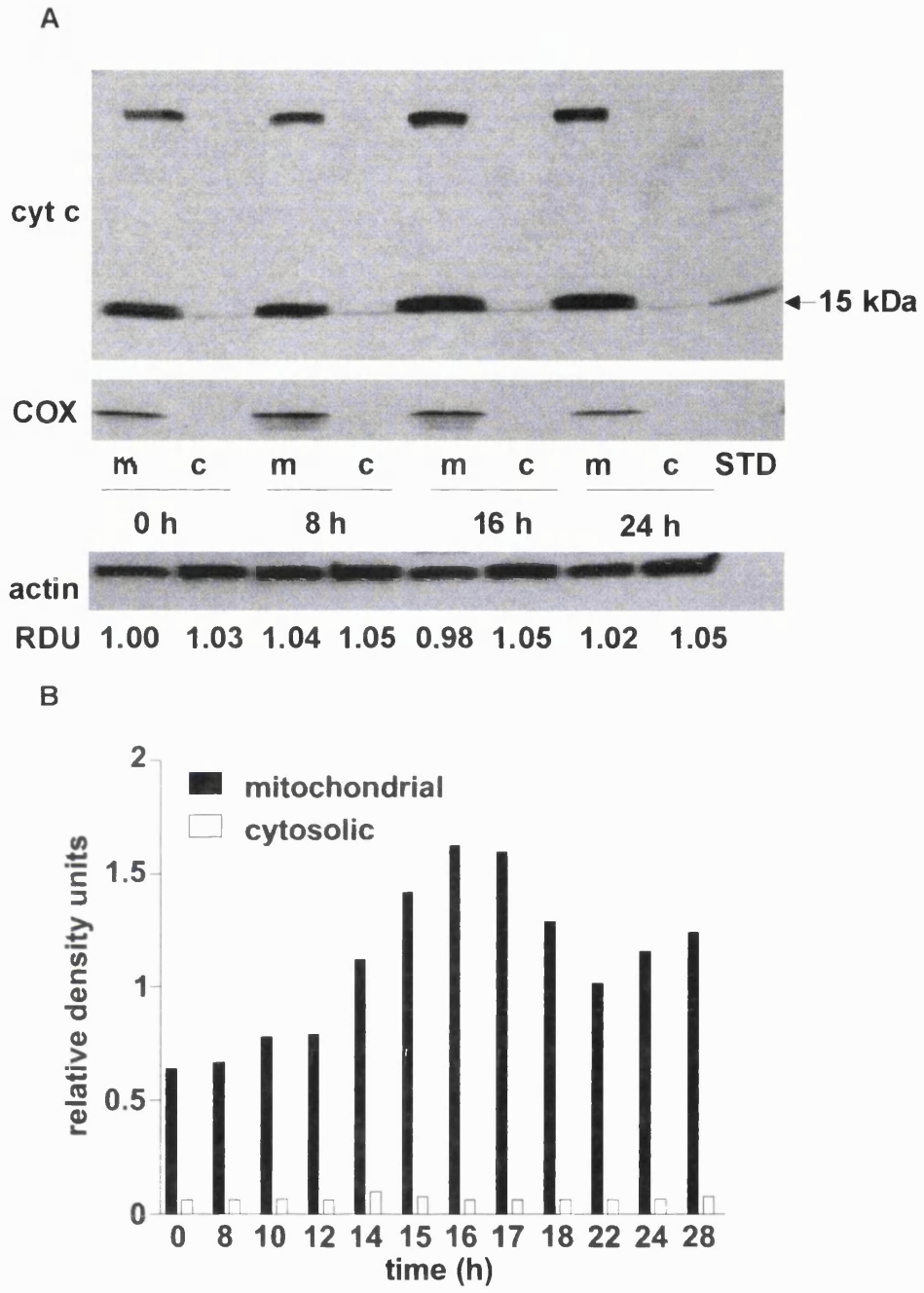


Fig. 4.5.1 CGA activation of microglia causes increased mitochondrial cytochrome c expression rather than translocation to the cytosol. (A) N9 cells were incubated with 10 nM CGA for 0, 8, 16, or 24 h and then fractionated into mitochondrial (m) or cytosolic (c) fractions. 30 μg of protein was resolved by 15% SDS-PAGE and immunoblotted with anti-cytochrome c (1:500). 0.05 μg of cytochrome c was used as a positive control (STD). Blots were stripped and reprobed with anti-cytochrome oxidase-subunit V (COX) to ensure purity of preparation and anti-actin to ensure equal protein loading. The relative density units (RDU) of the actin bands are shown in relation to that at time 0. **(B)** Densitometric analysis over a period of 0-28 h taken from various gels was performed on the 15 kDa cytochrome c band and correlated with actin protein. Values were expressed relative to the levels of cytochrome c in control untreated cells (0 h).

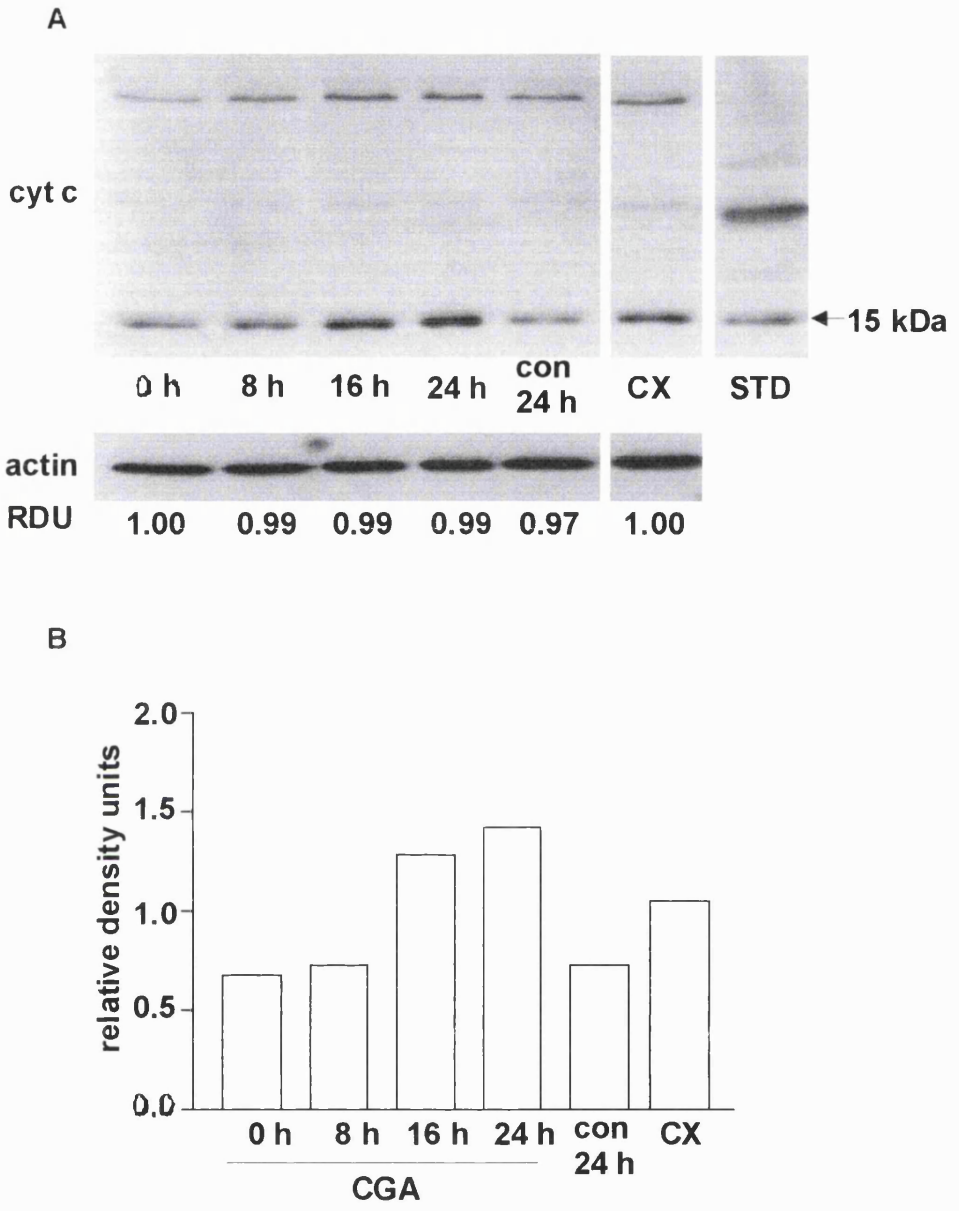


Fig. 4.5.2 Whole cell lysates of CGA treated microglia reveal increased levels of cytochrome c. (A) N9 microglia were either left untreated (0 h and con 24 h) or treated for 8, 16, or 24 h in the absence (8 h, 16 h, 24 h) or presence of 0.1 μg/ml cycloheximide for 24 h (CX) with 10 nM CGA and whole cell lysates prepared. 30 μg of protein was resolved by 12% SDS-PAGE and the samples immunoblotted with anti-cytochrome c antibody (1:500). The blot was stripped and reprobed with anti-actin antibody (1:1000) to ensure equal protein loading and the levels of protein (RDU) are shown relative to the band at time 0 h. **(B)** Densitometric analysis of the 15 kDa band was performed and correlated with actin protein. Values were expressed relative to the levels of cytochrome c in control untreated cells (0 h).

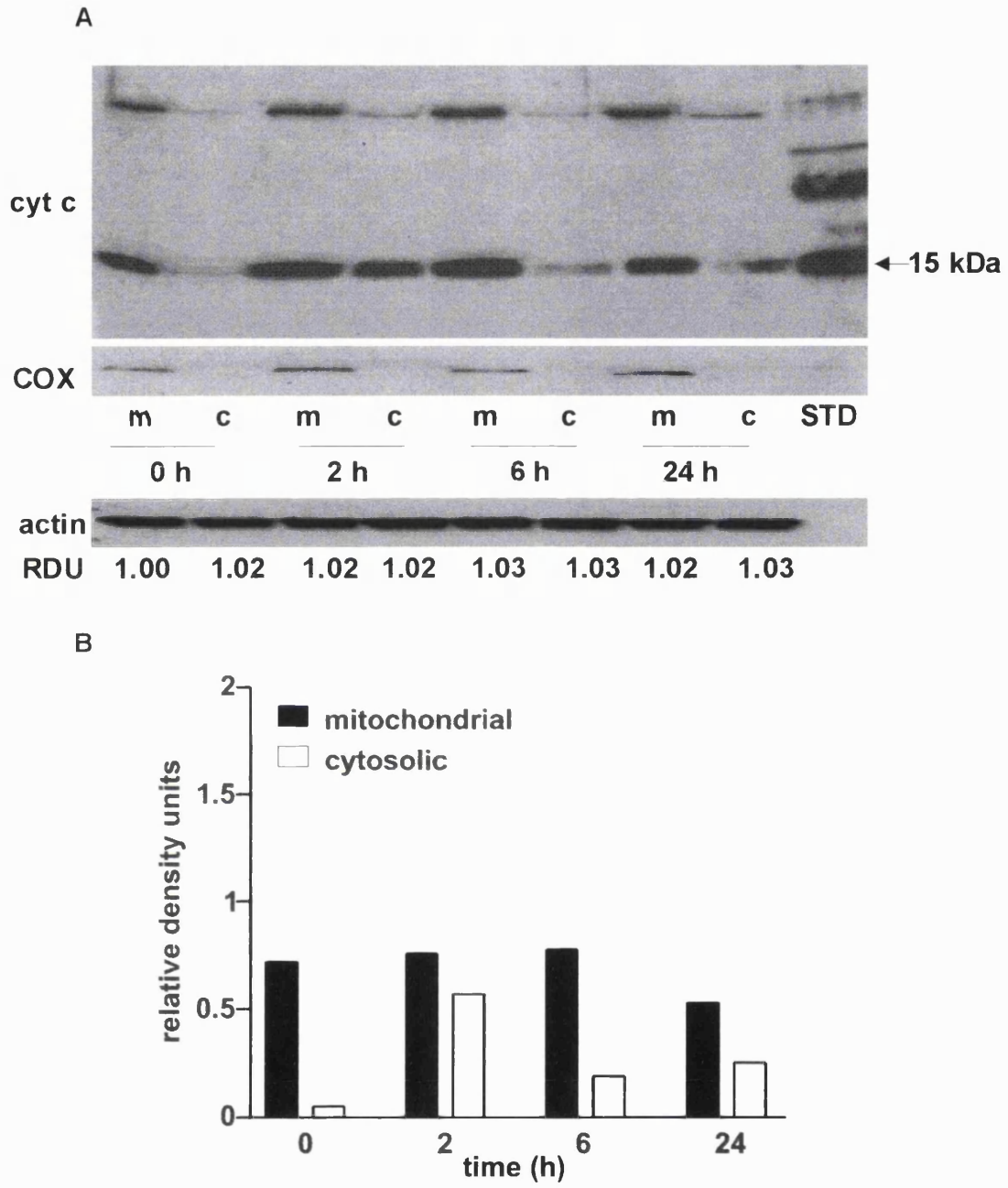


Fig. 4.5.3 Staurosporine causes the release of cytochrome c to the cytosol. (A) N9 cells were incubated with 1 μM staurosporine for 0, 2, 6, or 24 h and then fractionated into mitochondrial (m) or cytosolic (c) fractions. 30 μg of protein was resolved by 15 % SDS-PAGE and immunoblotted with anti-cytochrome c. 0.05 μg of cytochrome c was used as a positive control (STD). Blots were stripped and reprobed with anti-cytochrome oxidase-subunit V (COX) to ensure purity of preparation and anti-actin to ensure equal protein loading. The relative density units (RDU) of the actin bands are shown in relation to that at time 0. **(B)** Densitometric analysis was performed on the 15 kDa cytochrome c band and correlated with actin protein. Values were expressed relative to the levels of cytochrome c in control untreated cells.

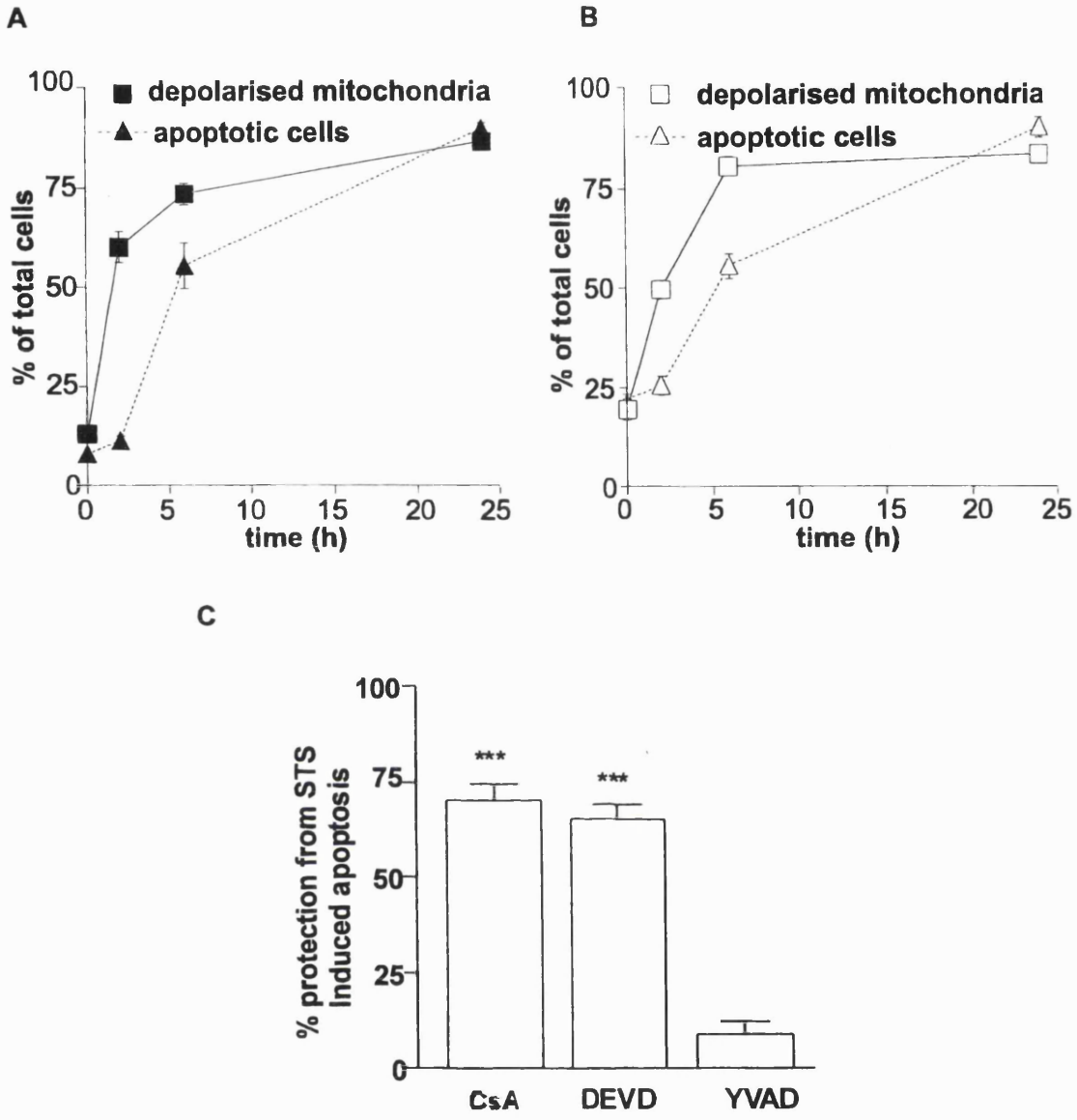


Fig. 4.5.4 Staurosporine induced microglial apoptosis involves mitochondrial depolarisation. Primary microglia **(A)** or the N9 cell line **(B)** were exposed to 1 μM staurosporine for 0 to 24 h and then loaded with the mitochondrial membrane potential sensitive dye, JC-1 (squares) or Hoescht 33342 to detect apoptotic cells (triangles). The number of cells displaying green fluorescence with JC-1 and the number of cells with bright pyknotic nuclei were determined 8 discrete regions on two coverslips per preparation from at least two independent cultures (n = ≥32). Data are the mean ± SEM. **(C)** The number of apoptotic cells in cultures of primary microglia was determined at 24 h in those exposed to 1 μM staurosporine and those in the presence of staurosporine and 1 μM cyclosporin A (CsA) or staurosporine and 50 μM DEVD-fmk (DEVD), and staurosporine plus 10 μM YVAD-CHO (YVAD). Cells were counted in 8 discrete regions on two coverslips per preparation from at least two independent cultures (n = ≥32). Data are the mean ± SEM. ***p<0.001 significantly less apoptosis compared with cultures treated with staurosporine alone.

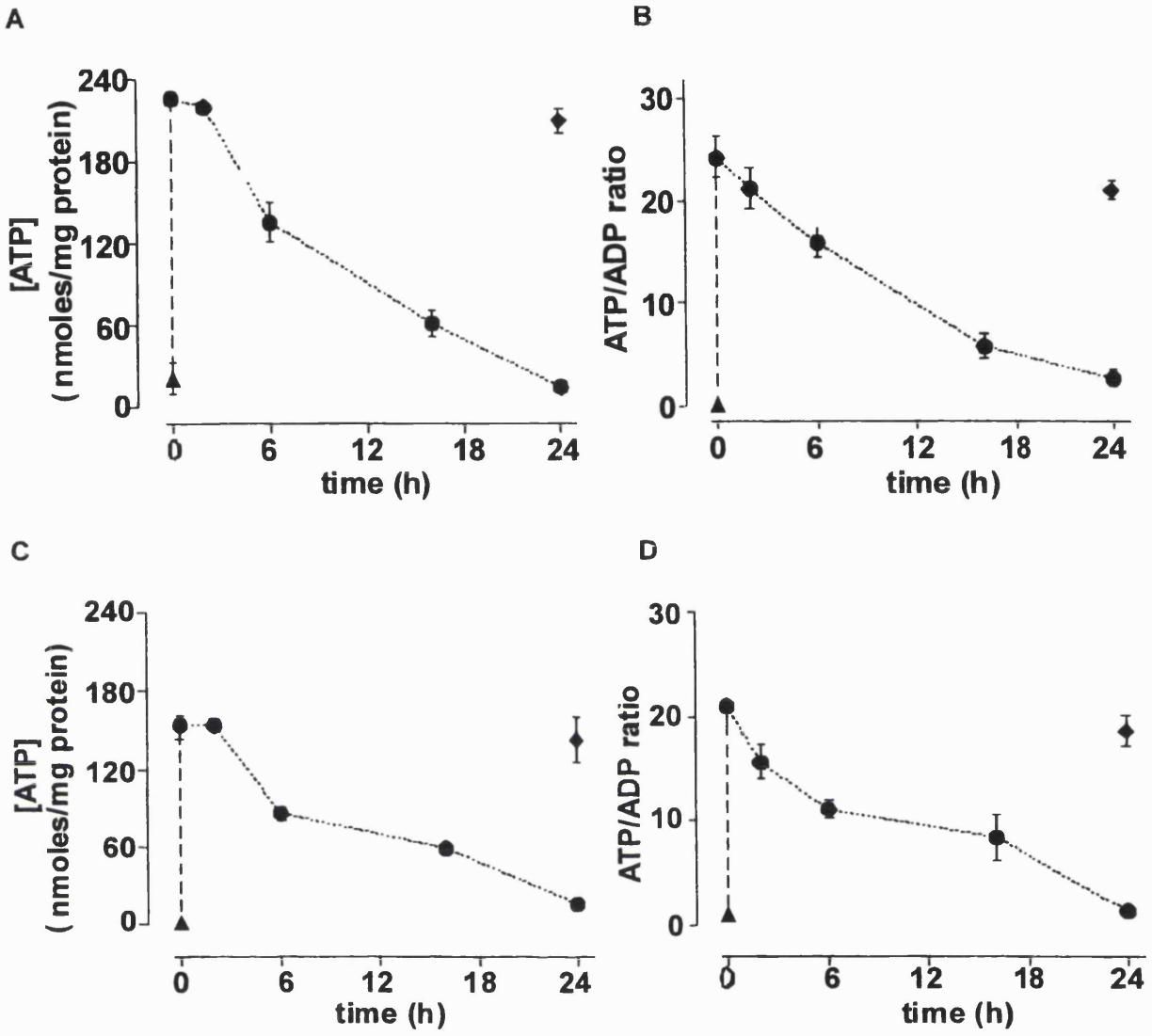


Fig. 4.5.5 ATP levels fall following the induction of microglial apoptosis by staurosporine. Primary microglia **(A and B)** or the N9 cell line **(C and D)** were either left untreated (◆) or exposed to 1 μM staurosporine (●) for the times indicated and then samples prepared for ATP determination as described in Chapter 2. A 15 min exposure to 5 mM cyanide was used as a postive control for rapid depletion of ATP levels (▲).

There was also no significant reduction in actin levels. The staurosporine induced release of cytochrome c correlated with the early onset of mitochondrial depolarisation and apoptosis observed in both primary microglia (Fig. 4.5.4A) and the N9 cell line (Fig. 4.5.4B). Furthermore, staurosporine evoked microglial apoptosis could be prevented by the caspase-3 inhibitor, DEVD-fmk and also CsA (Fig. 4.5.4C). However, YVAD-CHO was ineffective. Finally, the levels of ATP were assayed. A decrease in

ATP levels and ATP/ADP ratios occurred in both primary microglia (Fig. 4.5.5A and B) and the N9 cell line (Fig. 4.5.5C and D) at 6 h which is post mitochondrial depolarisation and the induction of apoptosis. Thus the differences in caspases activated and the involvement of cytochrome c release suggest that distinct signalling pathways are activated following treatment with CGA or staurosporine.

4.6. DISCUSSION

The data here shows that microglia stimulated with CGA undergo apoptosis as a consequence of disruption of mitochondrial function. Following the induction of NOS by CGA there is a fall in $\Delta\psi_m$ which precedes the activation of a downstream caspase-1 dependent pathway and the resultant morphological signs of apoptosis (pyknosis and DNA fragmentation). Both mitochondrial membrane depolarisation and apoptosis can be blocked by cyclosporin A, implicating the permeability transition phenomenon in these processes. Cytochrome c release from the mitochondria to the cytosol is not involved.

4.6.1 Microglial apoptosis involves NO and caspase activation

CGA induced microglial apoptosis involves the induction of NOS. Cholesterol oxides generated in the CNS may cause tissue damage during oxidative stress (Vatassery *et al.*, 1997) and have recently been shown to induce apoptosis in the N9 microglial cell line (Chang *et al.*, 1998). Whilst these substances cannot directly activate iNOS they do potentiate NO production in microglia stimulated with LPS and the cytotoxicity is

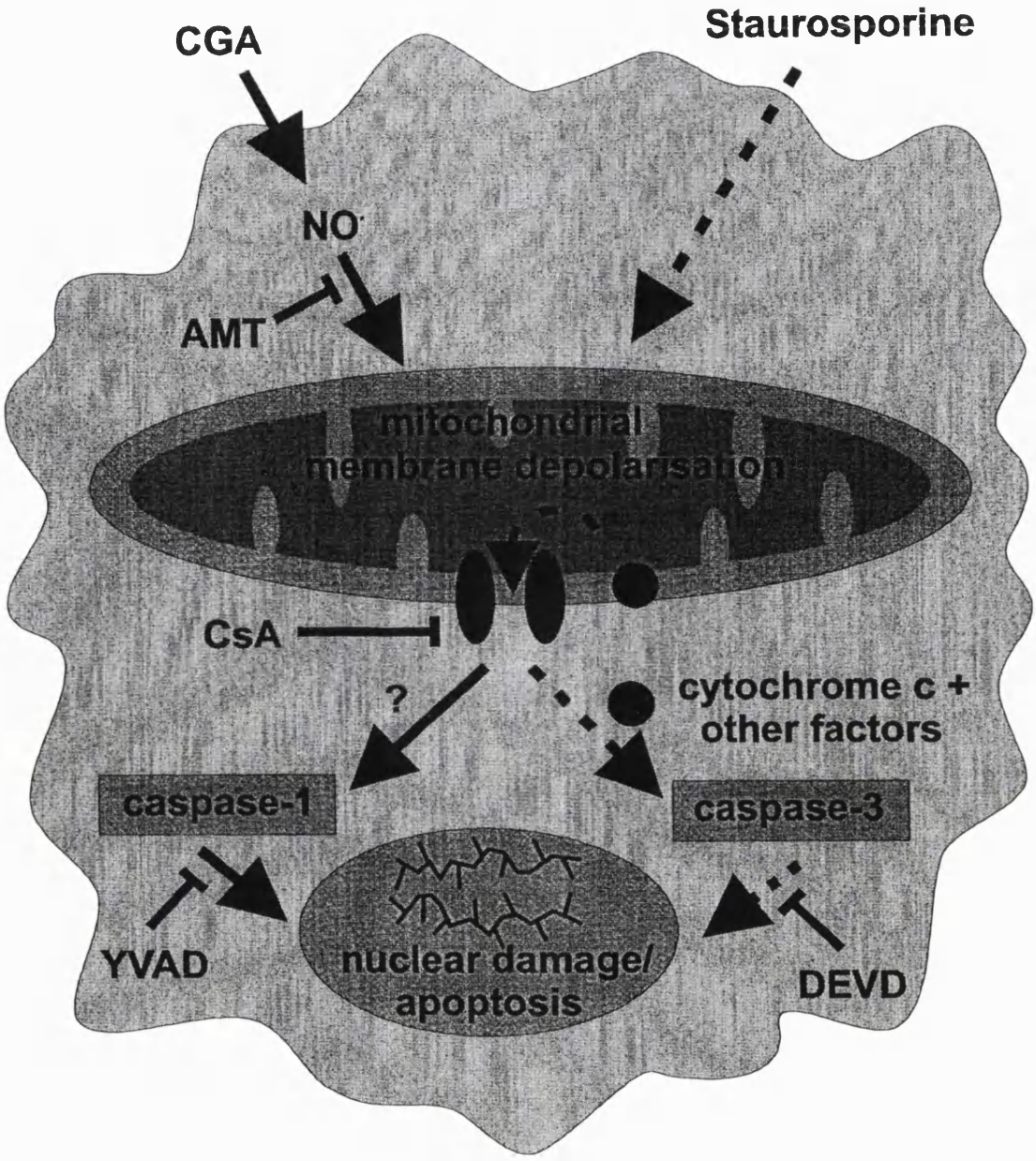


Fig. 4.6.1 Distinct apoptotic pathways evoked by CGA and staurosporine. CGA causes an increased production of nitric oxide via the induction of iNOS. This in turn is responsible for mitochondrial membrane depolarisation which can be blocked with NOS inhibitor, AMT-HCl. A fall in $\Delta\psi_m$ results in mitochondrial swelling without cytochrome c release and subsequent microglial apoptosis which can block be blocked by CsA implying the phenomenon of the permeability transition. Downstream of mitochondrial effects, caspase-1 is activated and leads to nuclear condensation which can be blocked by the peptide inhibitor, YVAD-CHO. Staurosporine causes a fast fall in $\Delta\psi_m$ and involves the release of cytochrome c from the mitochondria. Once in the cytosol, cytochrome c may interact with other factors to activate the downstream caspase-3. Apoptosis can thus be blocked by DEVD-fmk.

directly related to levels of NO. The involvement of reactive oxygen intermediates (ROI) has also been shown in Fas-L induced microglial apoptosis of cytokine stimulated cells and hypoxia exposed microglia where free radical scavengers are protective (Spanaus *et al.*, 1998; Lyons and Kettenmann, 1998). The intracellular signalling pathways have not been well investigated and to date there is only one report of the involvement of caspases in microglial apoptosis (Takai *et al.*, 1998). The authors observed 6-hydroxydopamine, a derivative of dopamine found in patients with Parkinsons disease (Glinka *et al.*, 1997), induced microglial apoptosis which could be prevented by inhibitors of both caspase-1 (YVAD-CHO) and caspase-3 (DEVD-CHO). CGA induced apoptosis was found to be dependent on caspase-1 activation since it could also be protected by YVAD-CHO and WEHD-fmk whereas inhibitors of caspase-3 and 6 had no effect. Caspase-1 or IL-1β-converting enzyme (ICE) has been extensively investigated for its role in inflammatory cell activation when pro-IL-1β is processed to yield active IL-1β (Black *et al.*, 1989). However, this was also the first protein identified to have extensive homology to the pro-apoptotic *Caenorhabditis elegans* gene product, ced-3 (Yuan *et al.*, 1993) and thus exhibits the properties of the other caspases in that it can degrade key cellular proteins. Thus, the role that caspase-1 plays in cell survival may be dependent on the substrate processed. A number of bacteria can induce macrophage apoptosis. *Shigella flexneri* the causative agent of bacillary dysentery can up-regulate caspase-1 activity during cellular apoptosis (Hilbi *et al.*, 1997). Similarly, *M. Avium* infected macrophages can be protected from cytokine mediated apoptosis by the caspase-1 inhibitor, WEHD-fmk (Bermudez *et al.*, 1999). A role for endogenous and exogenously supplied NO in promoting macrophage apoptosis has been reported previously (Meβmer *et al.*, 1996). NO induced apoptosis of the RAW 264.7 and U937 macrophage cell lines involves caspase activation (Meβmer *et al.*,

1998). Morphine induced macrophage apoptosis involves the up-regulation of iNOS and can be prevented by NOS inhibitors and caspase-1 inhibitors (Singhal *et al.*, 1998). Microglia exposed to CGA did not show a significant fall in cellular ATP levels until after the induction of apoptosis which is consistent with the notion that apoptosis requires significant levels of ATP (Leist *et al.*, 1997; Eguchi *et al.*, 1997). A fall in ATP is proposed to prevent the activation of multiple steps involved in the execution phase of apoptosis (Nicotera and Leist, 1997).

4.6.2 Mitochondrial depolarisation and the permeability transition

There is increasing evidence that changes in mitochondrial function may play a critical role in determining the fate of the cell. Thus to investigate this the mitochondrial specific dye, JC-1 was used to evaluate mitochondrial membrane potential. This has an advantage over conventional dyes such as rhodamine-123 as it is possible to easily visualise both cells with depolarised and normal $\Delta\psi_m$. Rhodamine binds with a high affinity to both mitochondria with high and low potentials and thus any differences in fluorescence intensity are hard to quantify (Johnson *et al.*, 1981). Following stimulation of microglia with 10 nM CGA there was a time dependent increase in the number of cells that displayed depolarised mitochondria. Cells that were characterised as depolarised did not respond any further to the addition of the mitochondrial uncoupler, FCCP. Control TMRE loaded cells showed a decline in fluorescence when treated with FCCP indicative of mitochondrial depolarisation. CGA treated cells failed to respond to FCCP. The use of rhodamine-123 further confirmed that effects observed with JC-1 were not artefactual. Cells exposed to CGA exhibited a higher mean fluorescence intensity which would be expected of a stimulus that depolarised the mitochondria (Duchen, 1990; Petit *et al.*, 1990).

Temporally the mitochondrial depolarisation followed the generation of nitric oxide but preceded the appearance of pyknotic nuclei suggesting that these changes might be responsible for mediating the apoptotic pathway. This contrasts with work which suggests that mitochondrial dysfunction occurs as a consequence of the activation of apoptotic pathways (Barbieri *et al.*, 1998; Bossy-Wetzl *et al.*, 1998). There are also situations where there is no detectable change in $\Delta\psi_m$ and apoptosis still occurs (Krohn *et al.*, 1999). However, the effect of CGA on microglia mimics reports with other systems describing a decrease in $\Delta\psi_m$ as an early step to apoptosis (Zamzami *et al.*, 1995). While differences may be due to the cell types and nature of the apoptotic stimuli used it could be that the phenomenon of the permeability transition (PT) does not operate similarly in all apoptotic processes.

Following the opening of the proposed mitochondrial megachannel, the collapse of $\Delta\psi_m$ proceeds and mitochondrial dysfunction occurs (Zoratti and Szabo, 1995; Bernardi and Petronilli, 1996). The channel opening can be blocked by cyclosporin A (CsA) and thus in theory the resulting apoptotic pathways inhibited. Consequently, CsA was used to try and block both the CGA induced fall in $\Delta\psi_m$ and subsequent apoptosis. The results indicate that the PT is required for CGA induced microglial apoptosis. Prior to any JC-1 fluorescence changes the mitochondria in cells exposed to CGA exhibited a more rounded morphology similar to observations previously reported in astrocytes (Kristal and Dubinsky, 1997) and hippocampal neurones (Dubinsky and Levi, 1998) following addition of ionophore to induce the PT. This swelling effect is consistent with the theory of the PT (Zoratti and Szabo, 1995). However, changes in the mitochondrial matrix volume may not necessarily indicate that a cell is to undergo apoptosis. Treatment of a human microglial cell line, CHME 5, and the monoblastoid, U 937 cell,

with acetyl-ceramide, a possible messenger in the signal transduction pathway mediating apoptosis, resulted in mitochondrial swelling but only induced apoptosis in the later cell type (de Gannes *et al.*, 1998). Similarly, CsA sensitive mitochondrial swelling induced by CCCP is a reversible effect and there is no sign of apoptosis following a lengthy period of mitochondrial depolarisation (Minamikawa *et al.*, 1999). However, CsA prevented both the fall in $\Delta\psi_m$ and cell death following microglial exposure to CGA which correlates with studies of PT in isolated mitochondria (Zamzami *et al.*, 1996). The possibility of any non specific effects of CsA on calcineurin rather the PT (Dawson *et al.*, 1993; Ankarcrona *et al.*, 1996) was eliminated by the lack of attenuation of the CGA induced fall in $\Delta\psi_m$ or apoptosis by the calcineurin inhibitor, FK506. This contrasts to a report suggesting that both CsA and FK506 protect LPS stimulated macrophages from apoptosis (Hortelano *et al.*, 1999). In this instance the authors report that such stimulation barely affects caspase-1 activity but involves caspase-3 thus different pathways activated in LPS and CGA mediated apoptosis may explain the anomaly.

4.6.3 NO and the permeability transition

The involvement of nitric oxide in triggering the PT is suggested from the studies which show that the NOS inhibitor, AMT-HCl prevents both the fall in $\Delta\psi_m$ and subsequent apoptosis. Nitric oxide may act directly on mitochondria to result in cellular apoptosis. NO can induce apoptosis in thymocytes via the induction of the permeability transition or through the formation of peroxynitrite in the mitochondria during neuronal apoptosis (Hortelano *et al.*, 1997; Kruman *et al.*, 1999). Alternatively, NO itself might not be directly responsible for downstream effects of the PT but act by making the mitochondria more sensitive to other inducers of the PT. For instance, in rat liver

mitochondria, NO accelerates both the onset of cyclosporin A sensitive swelling through the inhibition of the respiratory chain but can also modulate the response of the mitochondria to $[Ca^{2+}]_i$ induced PT (Balakirev *et al.*, 1997). This would fit with the data in this chapter showing the inability of the nitric oxide donor, S-nitrosopenicillamine (SNAP) to induce either mitochondrial depolarisation or apoptosis in primary cultures of microglia. Rather other molecules released during the CGA activation cascade such as glutamate could be responsible. In hippocampal neurones, glutamate can cause changes in mitochondrial morphology which are sensitive to cyclosporin A (Dubinsky and Levi, 1998). Glutamate also induces mitochondrial depolarisation directly in cultured neurones, an effect which can be prevented by NOS inhibitors (Almeida *et al.*, 1999). Other factors released by microglia such as TNF-α (Ciesielski-Treska *et al.*, 1998) have also been implicated in the PT (Bradham *et al.*, 1998; Pastorino *et al.*, 1996). This cytokine has not been investigated further in the current work but it is unlikely that TNF-α is responsible since blocking protein synthesis with cycloheximide potentiates apoptosis in the presence of the cytokine (Cossarizza *et al.*, 1995) which is the opposite of the effects observed with CGA (see chapter 3).

4.6.4 Microglial apoptosis and cytochrome c

During the PT, a number of proteins including cytochrome c are released from the mitochondrial intermembrane space that may be necessary for apoptosis to proceed (Scarlett and Murphy, 1997; Liu *et al.*, 1996). Nitric oxide has been reported to induce cytochrome c release from mitochondria during neuronal apoptosis (Uehara *et al.*, 1999). Furthermore, Bcl-2 which is known to prevent nitric oxide mediated apoptosis can also prevent the release of an apoptogenic protease (Susin *et al.*, 1996) and

cytochrome c from mitochondria (Bossy-Wetzel *et al.*, 1998). The localisation of cytochrome c in CGA treated microglia was performed by Western blot analysis. There was no significant release from the mitochondria to the cytosol whilst cytochrome c remaining within the mitochondria actually increased during apoptosis. One possible explanation for this is that there is an increase to compensate for inactivation of the cytochrome c as has been reported during Fas mediated apoptosis in Jurkat cells (Krippner *et al.*, 1996). Microglia were also exposed to staurosporine and subsequently underwent apoptosis over a faster time scale (0-6 h) and released cytochrome c suggesting that both the method of detection was sensitive enough and that distinct pathways exist. Despite the general belief that cytochrome c release is an essential feature of apoptosis evidence does exist for the lack of release during such cell death (Vier *et al.*, 1999, Tang *et al.*, 1998; Adachi *et al.*, 1998). It was suggested that cytochrome c independent apoptosis might be a feature of apoptosis in the absence of mitochondrial involvement (Tang *et al.*, 1998) which is clearly not the case for CGA induced microglial death. The fact that mitochondrial swelling, a hallmark of the PT, can occur without necessarily causing apoptosis (de Gannes *et al.*, 1998; Minamikawa *et al.*, 1999) implies that not all pro-apoptotic factors including cytochrome c may be released during pore opening. Rather the release of another protein such as the apoptosis inducing factor (AIF) (Susin *et al.*, 1996) could be involved in CGA mediated apoptosis though recently this has been shown to itself cause the release of cytochrome c and may actually act directly on nuclei, by-passing the need for caspases (Susin *et al.*, 1999). Evidence also suggests that while the permeability transition pore may open to let cytochrome c out of the mitochondrion this is not the actual mechanism by which it occurs during stimulus induced apoptosis involving cytochrome c release (Green and Reed, 1998). Recently it has been suggested that the voltage dependent ion channel

(VDAC), a component of the pore, exists alone in the mitochondrial membrane and can undergo a conformational change upon binding of Bax, a member of the Bcl-2 family, to allow cytochrome c release (Shimizu *et al.*, 1999). However, BH3, the Bcl-2 homology 3 domain, which is involved in the death inducing properties of molecules such as Bax and Bak, induces apoptosis without detectable translocation of cytochrome c from mitochondria to cytosol (Holinger *et al.*, 1999). It may also be that CGA does not activate the cytosolic factor CIF (cytochrome c efflux inducing factor) which is a requirement for cytochrome c dependent apoptosis (Han *et al.*, 1998, 1999).

Cytochrome c independent or dependent apoptosis may occur as the result of differences in the nature of the caspase pathways activated downstream of mitochondrial effects. The involvement of cytochrome c in apoptosis appears to correlate with caspase-3 activation (Liu *et al.*, 1996; Uehara, *et al.*, 1999; Bossy-Wetzl *et al.*, 1998). However, during CGA induced microglial apoptosis the downstream effects of mitochondrial depolarisation are mediated by caspase-1 since YVAD-CHO is still protective if added following significant mitochondrial depolarisation. This contrasts with studies indicating a biphasic profile of caspase-1 and caspase-3 activation during apoptosis (Krohn *et al.*, 1998; Susin *et al.*, 1997). The caspase-1 inhibitor was unable to prevent staurosporine induced microglia apoptosis whereas a caspase-3 inhibitor was protective leading further credence to the fact that cytochrome c release mediates caspase-3 dependent pathways.

4.6.5 Conclusions

In conclusion, previous studies have shown that microglia may be induced to undergo apoptosis *in vitro* in response to a variety of stimuli that may exist in the toxic milieu

found during neurodegenerative and immune mediated brain disease. The release of factors that are self toxic may act to limit tissue damage. For instance, the clinical benefits of TNF-α in limiting demyelination may be mediated in part through the elimination of activated microglia functioning as the main antigen presenting cell (Spanaus *et al.*, 1998). To date the study of caspases during microglial apoptosis has been limited (Takai *et al.*, 1998) and this is the first evidence for the involvement of mitochondria in this process. Recent experiments suggest that in situations of oxidative stress a key mitochondria enzyme, α-ketoglutarate dehydrogenase complex (KGDHC) may be inactivated in microglia and alter the inflammatory response of these cells (Park *et al.*, 1999). This may have important implications in Alzheimer or Parkinson's disease where it is known that this enzyme activity is reduced (Gibson *et al.*, 1988; Mizuno *et al.*, 1994). Thus these results provide insight into the pathways controlling microglial activation and may point to routes for the modulation of microglial evoked neurotoxicity.

5. SOLUBLE FACTORS RELEASED FROM CGA ACTIVATED MICROGLIA INDUCE NEURONAL APOPTOSIS

5.1 Introduction

Both beneficial and detrimental effects may arise from communication between microglia and neurones. Morphological changes during "microglial activation" may be accompanied by the secretion of a number of either neurotrophic or neurotoxic substances that can affect the survival of adjacent neurones. For instance, microglia may play a role in neuronal survival and differentiation by releasing neurotrophins and stimulating their production from other glial cell types (Giulian *et al.*, 1993). In contrast microglia may also contribute to the pathogenesis of neurodegeneration in diseases such as Alzheimer's (McGeer and McGeer, 1996; Barger and Harmon, 1997), the dementia associated with AIDS (Giulian *et al.*, 1996) and the neuronal cell death following an ischaemic insult (Lees, 1993). Under *in vitro* conditions, lipopolysaccharides and various combinations of proinflammatory cytokines (Chao *et al.*, 1995) have been used to activate microglia. Different neurotoxic pathways may be induced depending upon the stimulatory molecules used to treat the microglia and the agent released by activated microglia. Thus activated microglia may release nitric oxide (Kim and Tauber, 1996), arachidonic acid (Mori *et al.*, 1996), quinolinic acid (Espey *et al.*, 1997), cytokines (Giulian *et al.*, 1994), glutamate (Piani and Fontana, 1994) and other small molecular weight toxins acting through the NMDA receptor (Giulian *et al.*, 1993) and other unidentified proteases (Flavin *et al.*, 1997).

CGA can activate microglia (Taupenot *et al.*, 1996) and there is an increased expression of CGA in a number of neurodegenerative diseases with particular prominence in the senile plaques associated with Alzheimer's disease (Yasuhara *et al.*, 1994). This chapter aims to identify some of the signalling pathways involved. The main neuronal system used was the cerebellar granule cell which are widely used to study the pathways

leading to neuronal death, particularly those involving excitotoxins such as glutamate acting through the NMDA receptor. Glutamate can cause either a fast acute necrotic death or a delayed route to apoptosis (Ankarcrona *et al.*, 1995). Similarly, apoptosis can be induced in cerebellar granule cells by removal of the high concentration of K^+ necessary for survival in culture (Gallo *et al.*, 1997). This involves the activation of caspases (Eldadah *et al.*, 1997; Armstrong *et al.*, 1997). Disruption of $[Ca^{2+}]_i$ homeostasis may play a role in cytotoxicity (Choi, 1988) since a number of calcium mediated processes such as activation of proteases and free radical production are important in cell death and there may be a central involvement of mitochondria as the executioners of apoptosis (Kroemer *et al.*, 1998). In addition the mouse clonal hippocampal HT22 cell line (Davis and Maher, 1994) was used to examine the effects of factors secreted from CGA activated microglia. HT22 cells are particularly sensitive to glutamate induced apoptosis which is mediated by oxidative stress mechanisms (Lezoualc'h *et al.*, 1996; Tan *et al.*, 1998a and b). In addition, loss of hippocampal cell occurs during Alzheimer's disease (Van Hoesen *et al.*, 1991), so these cells may be relevant to microglial mediated effects.

During the course of this work it was reported that microglia activated with CGA could cause neuronal damage using a cortical cell culture system though the molecules involved and signalling pathways activated remain to be identified (Ciesielski-Treska *et al.*, 1998).

Summary of results

Conditioned medium from microglia activated with CGA is toxic to cultures of cerebellar granule neurones. The neuronal death is apoptotic in nature and results in DNA fragmentation and involves caspase-3 activation. Measurement of mitochondrial membrane potential ($\Delta\psi_m$) with the dye JC-1 indicated that there was mitochondrial depolarisation. Neuronal cell death was not mediated by nitric oxide but was partially mediated through ionotropic glutamate receptors since MK801 and CNQX were neuroprotective. The neurotrophin, BDNF was equally neuroprotective. Conditioned medium which had been boiled was less toxic and when incubated in combination with ionotropic glutamate receptor antagonists there was a restoration of cell viability to control levels. Potential cytokines such as TNF-α and IL-1β, which may be sensitive to the effects of denaturation, were unable to induce apoptosis. Conditioned medium also resulted in perturbations of calcium which may have consequences for neuronal survival. Conditioned medium induced apoptosis in the HT22 hippocampal cell and also involved caspase-3 activation. Neurotoxicity was also attenuated with boiling treatment suggesting the involvement of a protein molecule. Cathepsin B a cysteine protease released from CGA activated microglia (see Chapter 3) was able to induce apoptosis in HT22 cells and the neurotoxic effects of conditioned medium could be blocked with a specific cathepsin B inhibitor.

5.2 CGA activated microglia induce neuronal apoptosis

Microglia were stimulated with a range of CGA concentrations known to activate the cells and the conditioned medium from these added to cultures of cerebellar granule neurones. The viability of the neurones was then assessed using FDA and PI staining 24 h later. Cultured cerebellar granule neurones at 10-12 DIV have an extensive neurite network (Fig. 5.2.1A) and contained approximately 10% dead cells (Fig. 5.2.1B and C). When neurones were treated with conditioned medium from non activated microglia (MNCM) there was no change in the morphology of the neurones (Fig. 5.2.1D) and no significant increase in the number of dead cells (15 ± 1.5%, Fig. 5.2.1E and F). However, after 24 h of exposure to medium from microglia exposed to 10 nM CGA for 24 h (MACM), neurites became spindly (Fig. 5.2.1G) and there was a high number of dead cells (53.76 ± 1.9%, Fig. 5.2.1H and I). Furthermore, the effect of using medium from non activated microglia and then applying 10 nM CGA directly to the neurones did not in itself result in a significant change in neuronal viability (91.7 ± 5.1% compared with 89.4 ± 1.6% live cells in control cultures), indicating that neuronal death was mediated by a factor released by activated microglia rather than CGA itself. The response to conditioned medium from activated microglia was dependent upon the age of the neuronal cultures. Cerebellar granule cells at 4 DIV were unaffected by treatment with conditioned medium whereas the neurones at 10 DIV which had developed an extensive neurite network and formed large clumps of cell bodies were responsive (Fig. 5.2.2). A concentration of 10 nM CGA was used because this was previously shown to have the maximum effect on microglial activation in terms of products released e.g. nitric oxide and glutamate. Thus conditioned medium from activated microglia prepared from neonate rats was neurotoxic but CGA alone had no

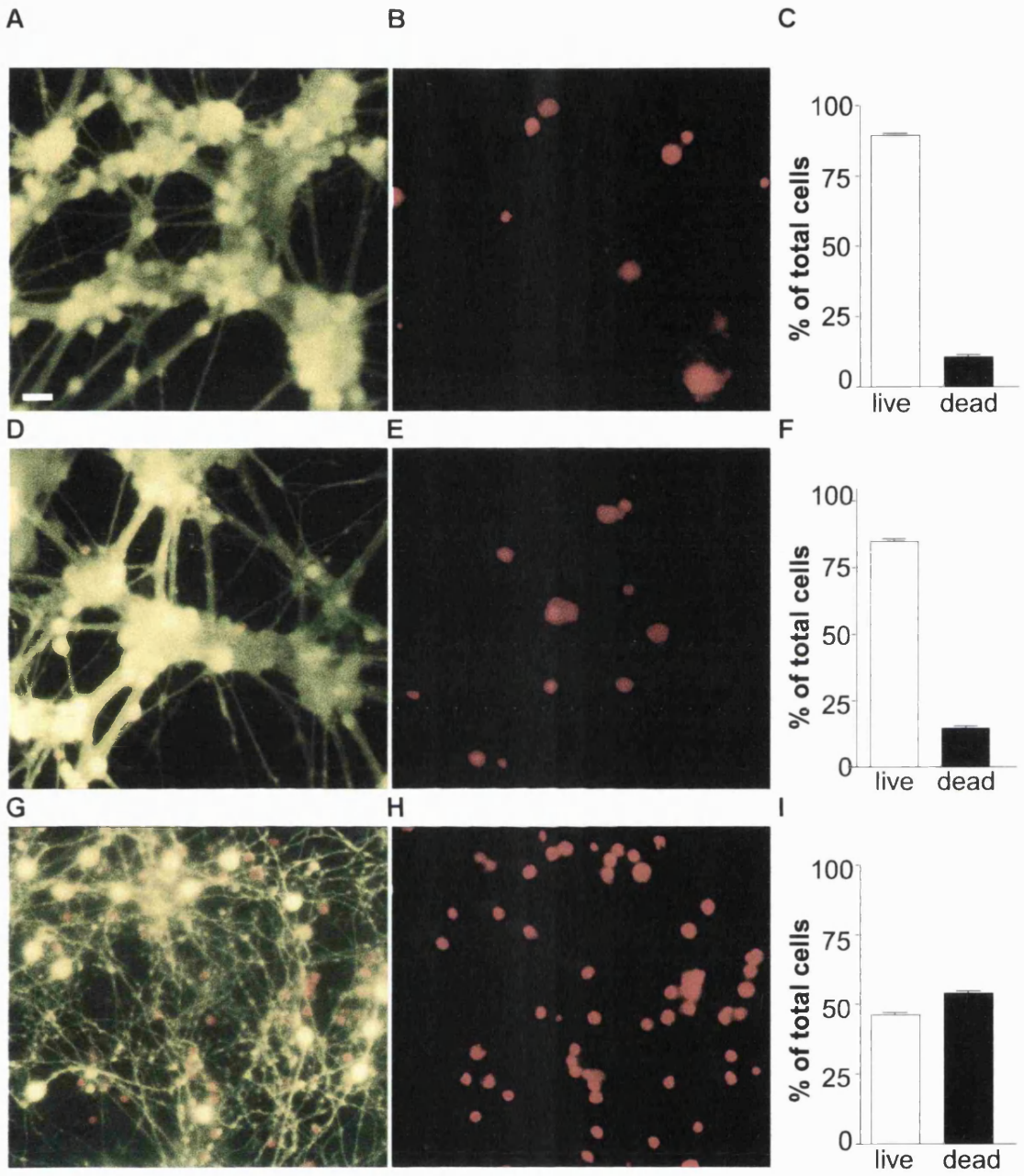


Fig. 5.2.1 Conditioned medium from activated microglia induces cell death in primary cultures of rat cerebellar granule neurones. (A) Control cultures at 10 DIV stained with FDA (live) display an extensive network of thick cable neurites and few propidium positive (dead) cells **(B)**. Neurones exposed for 24 h to 50% medium from untreated microglia show both a similar morphology and number of dead cells **(D and E)**. Following 24 h treatment of granule cells with 50% conditioned medium from microglia activated with 10 nM CGA for 24 h there was an extensive loss of neurites **(G)** and an increase in the number of dead cells **(H)**. The absolute number of live and dead cells stained with FDA or propidum respectively were counted and expressed as a percentage of the total cell number in control cultures, and those exposed to medium from untreated or CGA activated microglia **(C, F, and I)**. Cells were counted in 10 discrete regions from 2 coverslips per condition and 3 independent cell preparations (n = 60) and data are the mean ± SEM values. Scale bar = 20μM.

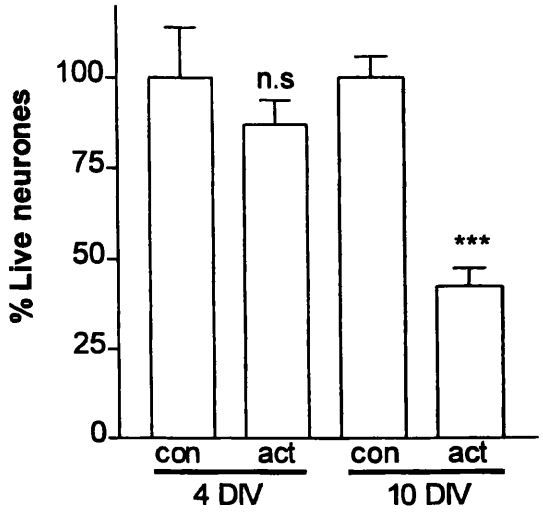


Fig. 5.2.2 The toxic effect of conditioned medium is dependent upon the age of neuronal cultures. Cerebellar granule cells at either 4 DIV or 10 DIV were exposed to 50% conditioned medium from microglia treated with 10 nM CGA for 24 h (act) and the percentage live cells counted using the live/dead assay and compared with control cultures (normalised to live = 100%) at the same stage of development (con). Data represents the mean percent live neurones ± SEM (n = 32). ***p<0.001 statistically different from control cultures at the same age, n.s not significantly different. Thus all subsequent experiments were performed on mature neuronal cultures (10-12 DIV) unless otherwise stated.

effect (Fig. 5.2.3). Microglia were also treated with LPS (1 μg/ml) for 24 h and the conditioned medium transferred to neurones. This was toxic resulting in a reduction in viability to 80.23 ± 3.65% live cells compared with control cultures (Fig. 5.2.3). LPS alone had no significant effect on neuronal viability. Microglial cells were also prepared from adult rat brains and human tissue (see Materials and Methods) and treated with 10 nM CGA. There was no difference between the levels of neuronal death in cultures exposed to medium from untreated microglia and microglia activated with CGA (Fig. 5.2.3). This was probably due to the increased activation status of these cell preparations (see Chapter 3) and thus all further studies used conditioned medium from neonate rat brain cultures to elucidate the signalling molecules involved. Thus it could be shown that the degree of neuronal death was proportional to the CGA concentration used to activate the microglia (from 0.1-10 nM) with a maximal death of 49.9 ± 1.61% when microglia were exposed to 10 nM CGA for 24 h (Fig. 5.2.4A).

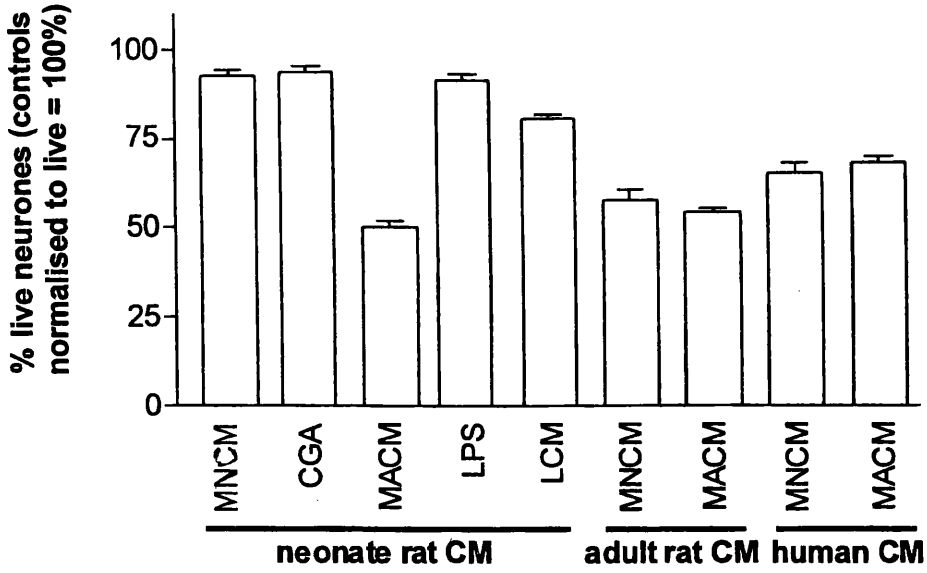


Fig. 5.2.3 The effect of microglial activation status on the toxicity of conditioned medium. Microglia were prepared either from neonate rats, adult rats (>30 days old) or human epileptic tissue and cultured for 24 h. At 1 DIV the cells were either left untreated (MNCM) or exposed to 10 nM CGA (MACM) or 1 μg/ml LPS (LCM) for 24 h and the medium transferred to cultures of cerebellar granule cells (10-12 DIV) for 24 h. The effect of 10 nM CGA (CGA) and 1 μg/ml LPS (LPS) added directly to neurones was also assessed 24 h later using the live/dead assay and is expressed relative to the respective controls which are normalised to live = 100%.

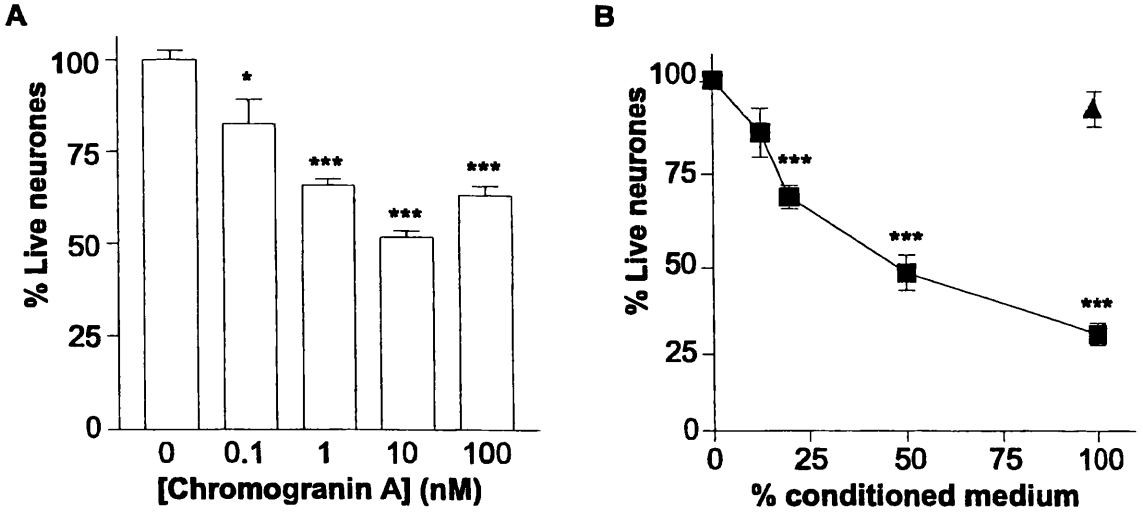


Fig. 5.2.4 Neurotoxicity is dependent on the amount of conditioned medium added and the concentration of CGA used to activate microglia. (A) Cerebellar granule neurones (10-12 DIV) were exposed to 50% conditioned medium from microglia activated with 0.1-100 nM CGA for 24 h and the number of live and dead cells counted 24 h later. Data are expressed as the mean percent live cells ± SEM (n = 60 separate fields of cells) compared with cultures exposed to medium from untreated microglia (normalised to live = 100%). *p<0.05, **p<0.01, ***p<0.001 statistically different from these cultures. **(B)** Neurones were exposed to increasing amounts of conditioned medium from microglia activated with 10 nM CGA for 24 h (squares) or 100% medium from untreated microglia (triangle) and the number of live and dead cells counted. Data are the mean percent live ± SEM (n = 60). ***p<0.001 statistically different from cultures not exposed to conditioned medium.

The degree of neuronal death was dependent on the percentage of activated microglial medium to which the neurones were exposed; increasing volumes of medium from CGA activated microglia resulted in the maximum death of 67.4 ± 2.9% following 24 h exposure (Fig. 5.2.4B). Replacing the media on the granule cells with 100% non-activated supernatant resulted in an approximate 10% increase in cell death (Fig. 5.2.4B). To determine at which time point microglial activation leads to the production of neurotoxins, microglia were activated with 10 nM CGA and the conditioned medium removed over a period of 0-24 h. This media was then incubated with cerebellar granule neurones for 24 h. The onset of release of the neurotoxin(s) was essentially immediate and followed a linear profile in contrast to the lag period prior to NO production by microglia (Fig. 5.2.5A). There was significant neuronal death using medium from microglia activated for 10 h with CGA which is before any increase in glial cell death suggesting that neuronal cell death is not merely due to the lysis of microglia. However, the rate of neuronal death did increase significantly at approximately 18 h suggesting that glutamate release may contribute to neuronal death (Fig. 5.2.5A). Again there was no significant loss of viability up to 24 h in the neurones exposed to medium from non activated microglia (Fig. 5.2.5A). Increasing times of exposure of cerebellar granule neurones to conditioned medium from CGA exposed microglia lead to increasing neuronal death from approximately 50% at 24 h to 80% at 72 h (Fig. 5.2.5B). There was also a small increase in the number of dead neurones in the presence of medium from microglia not exposed to CGA suggesting these cells may release low levels of neurotoxin(s) (Fig. 5.2.5B). Thus in all subsequent experiments neurones were exposed to conditioned medium for 24 h to enable determination of any protective effects on neuronal survival from pharmacological intervention.

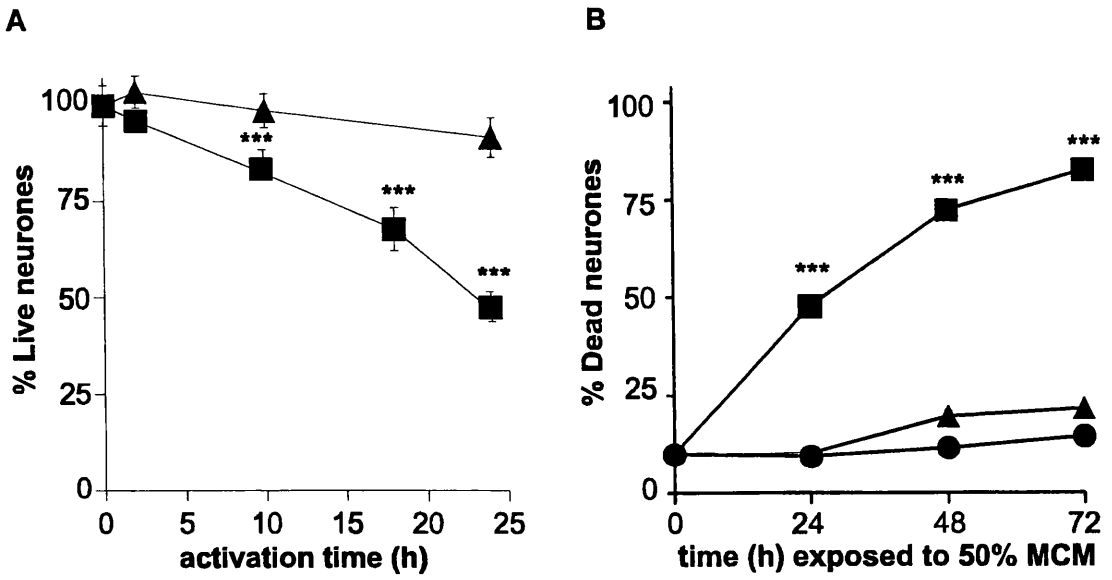


Fig. 5.2.5 Neurotoxicity is dependent on the microglial activation time and length of exposure to conditioned medium. **(A)** Medium from untreated microglia (triangles) or microglia exposed to 10 nM CGA (squares) was collected from 0 to 24 h following exposure and added to granule cell cultures at a 50:50 ratio. Neuronal viability was assessed 24 h later using the live/dead assay. Data are the mean percent live neurones ± SEM (n = 60). ***p<0.001 statistically different from control cultures (normalised to live = 100%). **(B)** Neuronal viability was determined between 24 h and 72 h following exposure to non activated microglial medium (triangles) or medium from microglia activated with CGA for 24 h (squares). Control cultures are also shown (circles). Data are mean absolute number of dead cells stained with ethidium ± SEM (n = 40). ***p<0.001 statistically different from control cultures.

The apoptotic cell marker, Hoechst 33342 was used to determine the nature of this cell death. In control cultures not exposed to microglial conditioned medium, 3.5 ± 1.5% of the cells were apoptotic (Fig. 5.2.6A and C); this was not significantly increased in cultures exposed to medium from untreated microglia (4.81 ± 1.7%) or in cells in which fresh medium was added (4.5 ± 2.4%). Granule cells exposed for 24 h to medium from microglia activated with 10 nM CGA for 24 h showed a high number of apoptotic cells (57.4 ± 6.7%, Fig. 5.2.6B and D). Furthermore, the number of apoptotic neurones correlated well with those stained with propidium iodide and the degree of neuronal death was proportional to the amount of medium added from activated microglia (Fig. 5.2.7A).

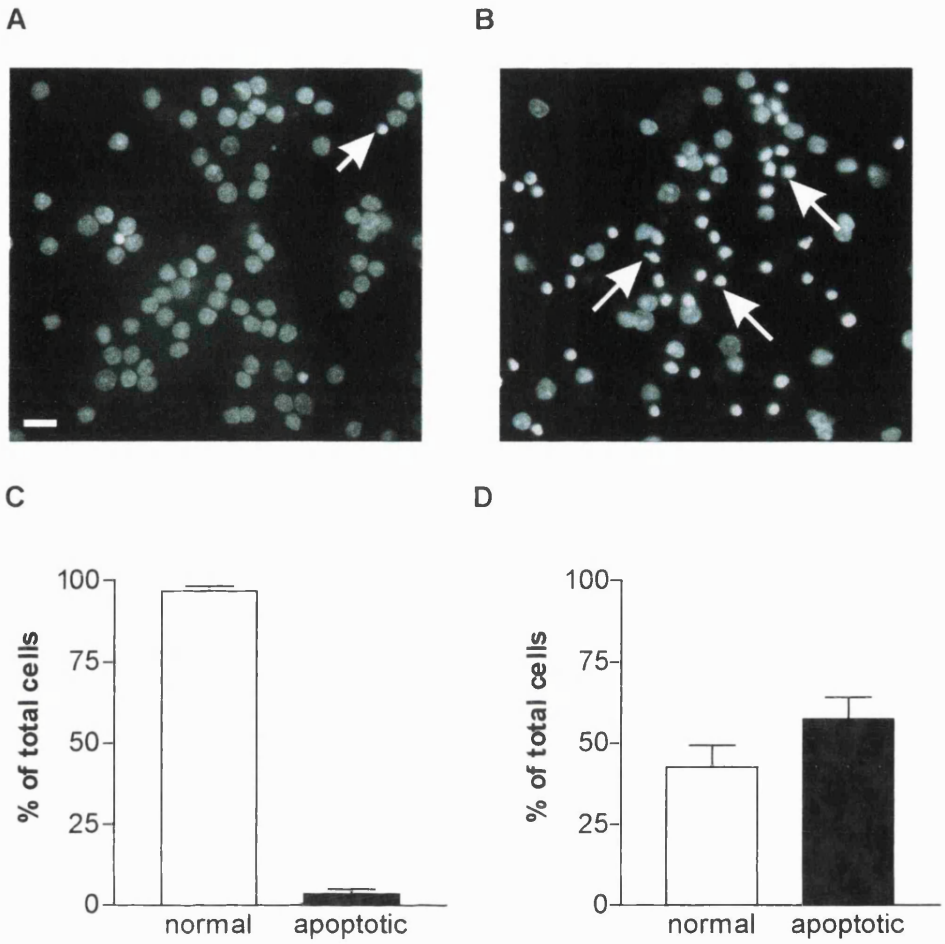


Fig. 5.2.6 Neurones exposed to medium from microglia activated with CGA display apoptotic nuclei. Cultures of cerebellar granule cells at 10 DIV were stained with Hoechst 33342 to indicate pyknotic nuclei. **(A)** Control cells display few apoptotic nuclei (arrows) whereas neurones exposed for 24 h to 50% conditioned medium from microglia activated with CGA for 24 h show a large number of brightly staining condensed nuclei **(B)**. The number of cells displaying pyknotic nuclei (apoptotic) in control **(C)** and cultures exposed to 50% conditioned medium **(D)** were counted in 10 discrete regions from 2 coverslips per condition and 3 independent cell preparations (n = 60) Data are the mean ± SEM values. Scale bar = 20μM.

The involvement of two different caspase sub families was investigated using specific peptide inhibitors of caspase-3 (CPP32-like family) and caspase-1 (ICE-like family), z-DEVD-fmk and YVAD-CHO respectively (Hilibi *et al.*, 1997; Eldadah *et al.*, 1997). Pre-incubation of cerebellar granule cells with z-DEVD-fmk (50 μM) for 1 h before exposure to activated microglial medium significantly reduced neuronal cell death whereas YVAD-CHO (100 μM) had no effect suggesting that microglia induce neuronal death by the activation of specific subfamilies

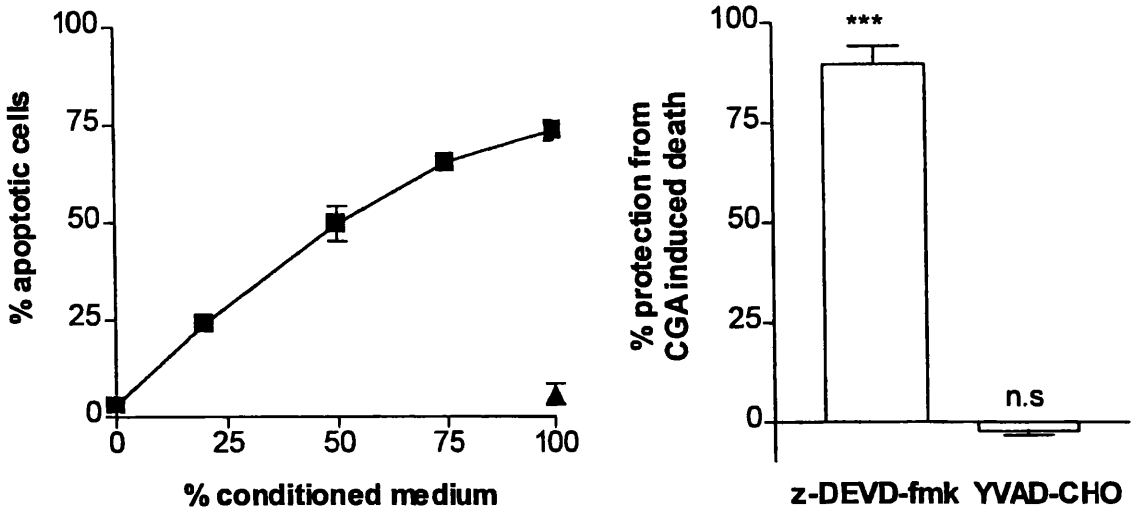


Fig. 5.2.7 Neurotoxicity evoked by conditioned medium can be blocked by a caspase-3 inhibitor. (A) Granule cells were exposed for 24 h to increasing amounts of conditioned medium from microglia which had been activated with 10 nM CGA for 24 h (squares) and then stained with Hoechst 33342 to display apoptotic cells. The number of pyknotic, highly condensed nuclei were counted and are displayed as a percentage of the total cell number ± SEM (n = 60). Triangle indicates the number of apoptotic cells in neuronal cultures exposed to 100% medium from non treated microglia. **(B)** Conditioned medium from microglia activated with 10 nM CGA for 24 h was applied to neuronal cultures for 24 h in the presence of the caspase inhibitors, z-DEVD-fmk (50 μM) or YVAD-CHO (100 μM). The number of live cells in the absence and presence of these compounds was counted using the live/dead assay and data expressed as the percentage survival ± SEM above that in the cultures exposed to conditioned medium alone (n = 60). ***$p<0.001$ significantly different from cultures exposed to conditioned medium alone; n.s, not significantly different.

of caspases (Fig. 5.2.7B). DNA was prepared from granule cells treated with conditioned medium and this showed a significant laddering pattern which suggests the induction of DNA fragmentation another characteristic of apoptosis. This was prevented in the presence of z-DEVD-fmk (Fig. 5.2.8A). Previous studies have shown that a fall in mitochondrial membrane potential ($\Delta\psi_m$) can occur during cerebellar granule cell apoptosis (Tanabe *et al.*, 1998). JC-1, a mitochondrial specific dye, was used to record this. Cerebellar granule cells were loaded with JC-1 at various time points following treatment with conditioned medium from activated microglia and the number of cells exhibiting red punctate staining mitochondria (polarised) or a green diffuse fluorescence (depolarised) was recorded. In control untreated cultures, cells mainly fluoresced red

and the number with depolarised mitochondria (green) accounted for 12.5 ± 1.3% of the total cell population (Fig. 5.2.8B). This number increased slightly over time following treatment with medium from untreated microglia but was significantly elevated to 78.2 ± 7% in the presence of medium from microglia activated with CGA (Fig. 5.2.8B). This effect preceded the appearance of apoptotic nuclei suggesting that mitochondrial depolarisation might be involved in the cell death pathway (Fig. 5.2.8B).

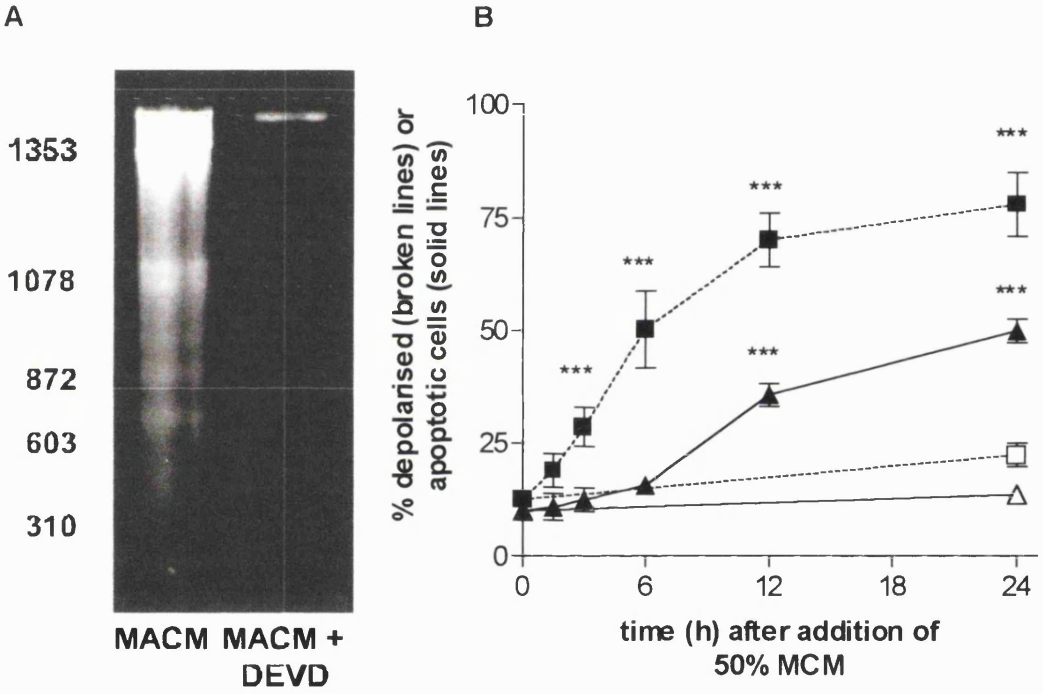


Fig. 5.2.8 Conditioned medium induces DNA fragmentation and mitochondrial depolarisation in cerebellar granule neurones. **(A)** DNA was extracted from cerebellar granule cells exposed for 24 h to conditioned medium from microglia activated with 10 nM CGA for 24 h in the absence (MACM) and presence of 50 μM z-DEVD-fmk (MACM + DEVD) and separated on a 1% agarose gel for 2 h at 125V. The gel was then viewed under UV illumination with ethidium bromide staining. DNA molecular weight markers are shown to the left. **(B)** Cerebellar granule cells were either exposed to conditioned medium (CM) from microglia activated for 24 h with 10 nM CGA (closed symbols) or medium from non treated microglia (open symbols) for the times indicated and then the cells stained with Hoechst 33342 or loaded with JC-1. Either the number of cells that showed brightly stained condensed nuclei indicative of apoptosis or the number of cell soma which had discrete mitochondria fluorescing red and those with depolarised mitochondria which appeared green were counted. The absolute numbers of cells labelled red and green were counted in 8 fields per coverslip and the degree of mitochondrial depolarisation expressed as the percentage green of total cells counted (n = 32). ***P<0.001 significantly different to control at time 0 h.

5.3 Mechanism of microglial induced neuronal apoptosis

Neuronal death may be mediated by NO (Uehara *et al.*, 1999; Bonfoco *et al.*, 1996). Thus cerebellar granule cells were exposed to conditioned medium for varying lengths of time and then assayed for nitrite accumulation using the Griess reagent. There was no significant difference in the nitrite levels between control cultures and those exposed to conditioned medium for up to 24 h (Fig. 5.3.1) suggesting that the cell death is not mediated by NO.

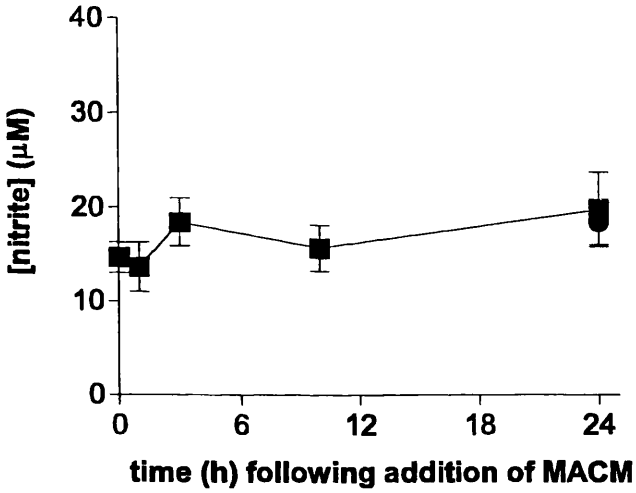


Fig. 5.3.1 Cerebellar granule neurones treated with microglial conditioned medium do not generate significant levels of nitric oxide. NO release was determined with Griess reagent by estimation of nitrite levels. NO release was measured in neurones exposed to 50% conditioned medium for 0-24 h (squares) and also control untreated neurones at the end of the 24 h period (circle). Data are mean ± SEM values of triplicate determinations for two different cell preparations (n = 12).

This was further confirmed by pre-incubating neurones with the NOS inhibitors, L-NNA and AMT-HCl prior to exposure to conditioned medium. There was no significant protective effect (Fig. 5.3.2). However, the level of neuronal death was to a small but significant degree lower in the presence of conditioned medium taken from microglia which have been treated with NOS inhibitors suggesting that prevention of NO dependent glutamate release from microglia is important. Thus since glutamate

released from microglia may play a role it was determined whether the NMDA receptor channel blocker, MK801, and/or CNQX, the AMPA/Kainate receptor antagonist could afford protection from conditioned medium. The combined effect of these two compounds reduced the neuronal toxicity by 37.3 ± 2.6 % indicating that both types of receptor are activated (Fig. 5.3.2). Alone these antagonists attenuated neuronal cell death by 25 ± 2% (MK-801) and 10 ± 3% (CNQX). Since these compounds only

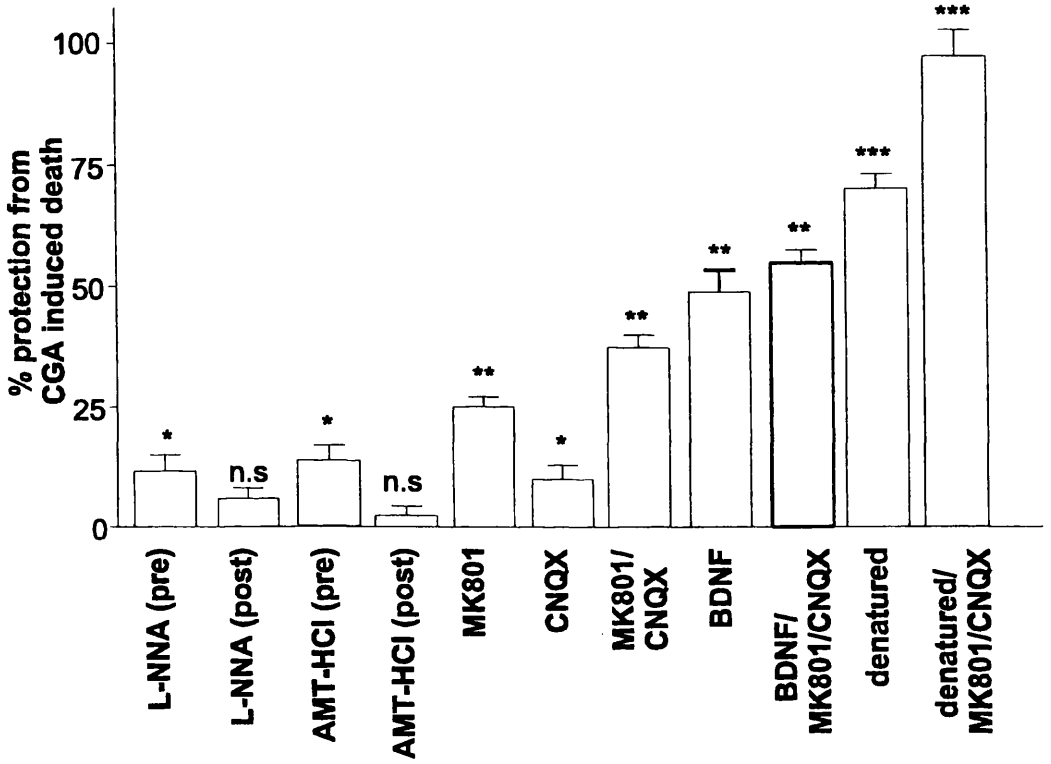


Fig. 5.3.2 Attenuation of neuronal death induced by conditioned media from CGA exposed microglia. Conditioned medium from microglia activated with 10 nM CGA for 24 h was added to cerebellar granule cells at a ratio of 50:50 conditioned medium:neuronal medium. Neuronal viability was assessed 24 h later using live/dead staining and expressed as a percentage of the survival above that in cultures exposed to 50% conditioned medium without drugs. Neurones were incubated with the following compounds during exposure to microglial conditioned medium: the NOS inhibitors, L-NNA (1 mM) or AMT-HCl (150 nM) either added to microglia before CGA induced activation (pre) or added to neurones only (post), 10 μM MK-801, 20 μM CNQX, a combination of MK-801/CNQX, 10 ng/ml BDNF, BDNF plus MK-801 and CNQX. Microglial conditioned medium was also boiled for 1 h and then added to cerebellar granule cells in the absence (denatured) or presence of MK-801 and CNQX (denatured/MK-801/CNQX). Data are the mean ± SEM values (n = ≥ 40 separate fields of cells) from at least two independent experiments. *p<0.05, **p<0.01, ***p<0.001 significantly different from cultures treated with conditioned medium alone. n.s not statistically different.

protected the cells by approximately a third this suggests other factors released by activated microglia determine the survival of neurones. Thus to assess the nature of these molecules the conditioned medium was boiled for 1 h which should denature any proteins such as cytokines released by activated microglia. This reduced the level of cell death by a significant level and when combined with the glutamate receptor antagonists the cells exposed to conditioned medium showed a similar level of viability to control cultures (Fig. 5.3.2). This suggests that more than one factor may be released by CGA activated microglia which affects neuronal cell survival. The ability of the neurotrophic factor, BDNF, to protect neurones was also assessed. This afforded a similar degree of protection to the glutamate receptor antagonists (Fig. 5.3.2). Glutamate (100 μM) was neurotoxic to CGCs resulting in approximately 40% cell death and this was not significantly different when the glutamate had been boiled for 1 h (Fig. 5.3.3A). Cytokines can influence the damage caused by glutamate in the CNS so it was determined whether conditioned medium would have a similar potentiating effect on NMDA mediated cerebellar granule cell death. Granule cells exposed to 100 μM NMDA in magnesium free buffer for varying lengths of time exhibited a time dependent increase in cell death when measured using the live/dead assay 24 h after replacing the cell culture medium (Fig. 5.3.3B). Using an intermediate time point of 15 min of NMDA stimulation, medium from microglia activated with 10 nM CGA for 24 h was added for a further 24 h and resulted in a significant decrease in the number of live neurones compared with control (Fig. 5.3.3B). Non activated microglial medium had no effect. Thus this may be due to either the increased levels of glutamate in the medium of treated microglia or other as yet unidentified proteins.

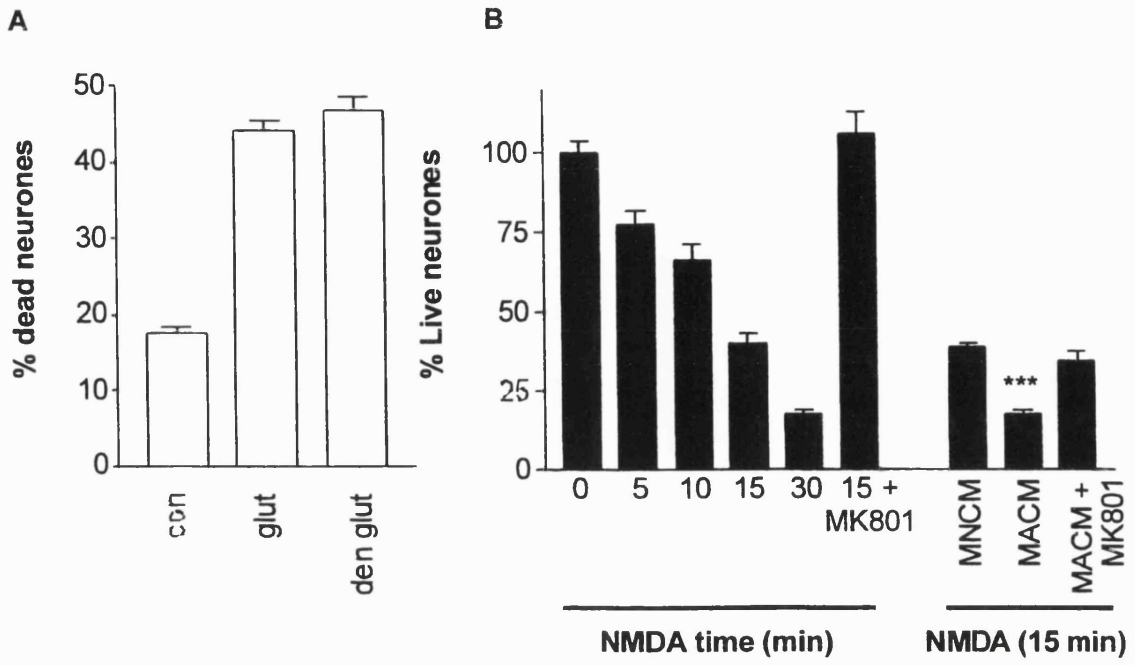


Fig. 5.3.3 Glutamate and NMDA mediated neurotoxicity. (A) The number of dead neurones stained with propidium were counted in control cultures (con) and those exposed to 100 μM glutamate (glut) or 100 μM glutamate which had been boiled for 1 h (den glut) after 24 h. **(B)** CGCs (10-12 DIV) were exposed to 100 μM NMDA in Mg^{2+} free basic medium for 0-30 min and then incubated for a further 24 h in normal culture medium at which time the number of live and dead cells were counted using live/dead staining. Similarly, neurones were exposed to 100 μM NMDA for 15 min but then incubated in 50% medium from untreated microglia (non-act media) or 50% conditioned medium from microglia activated with 10 nM CGA for 24 h in the absence (act media) or presence of 10 μM MK-801 (act + MK-801). Data are expressed as the percentage live neurones ± SEM normalised to control equals 100% live (n = 32). ***p<0.001 significantly different from cultures treated with only NMDA for 15 min.

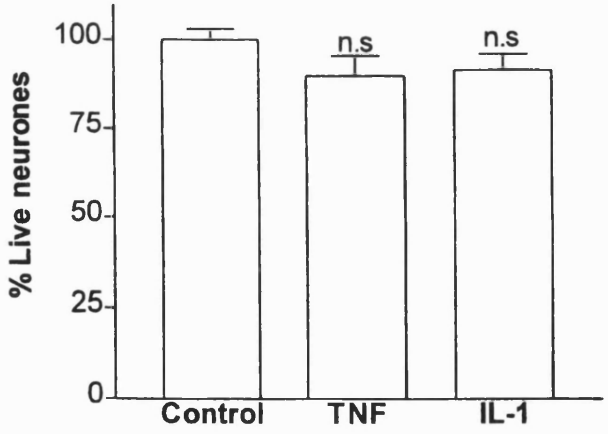


Fig. 5.3.4 The effect of cytokines on neuronal cell viability. Cerebellar granule cells were exposed to either TNF-α (100 ng/ml) or IL-1β (50 ng/ml) for 24 h and then the number of live and dead cells counted. Data are expressed as the percentage live neurones ± SEM where control cultures are normalised to live = 100% (n = 32). n.s, not significantly different from control cultures.

Since the proteins released from microglia by CGA activation might conceivably be cytokines the role of two such proteins, TNF-α and IL-1β on neuronal viability was assessed. Cells exposed to these cytokines showed no significant reduction in viability (Fig. 5.3.4) suggesting either these are not responsible or they act in concert with other factors released by activated microglia.

5.4 Intracellular Ca^{2+} responses to neurotoxic factors in granule cells

A critical event in promotion of apoptosis may be the disruption of cellular calcium homeostasis (Nicotera *et al.*, 1992). For example, the peptide β-amyloid has been shown to cause increases in $[Ca^{2+}]_i$ in hippocampal neurones prior to the appearance of apoptotic cells (Mark *et al.*, 1995). Hence, it was examined whether such alterations were involved in the apoptotic pathway elicited by treatment of neuronal cultures with conditioned media from CGA-activated microglia. The intracellular free calcium concentration, $[Ca^{2+}]_i$, in individual neurones was measured using fura-2. Direct application of CGA to neurones and also the application of medium from untreated microglia had no significant effect upon $[Ca^{2+}]_i$ (Fig. 5.4.1A and B). However, with addition of microglial conditioned medium to the cells there was an immediate small rise in $[Ca^{2+}]_i$ which was then maintained as a plateau phase (Fig. 5.4.1C). This effect was similar to that observed when 100 μM NMDA was acutely added (Fig. 5.4.1D). The calcium response to microglial conditioned medium was further dissected. It was shown that if the conditioned medium was applied to cells in magnesium free basic medium the magnitude of the response was greatly elevated (Fig. 5.4.2B). Furthermore, the overall increase in $[Ca^{2+}]_i$ could be prevented

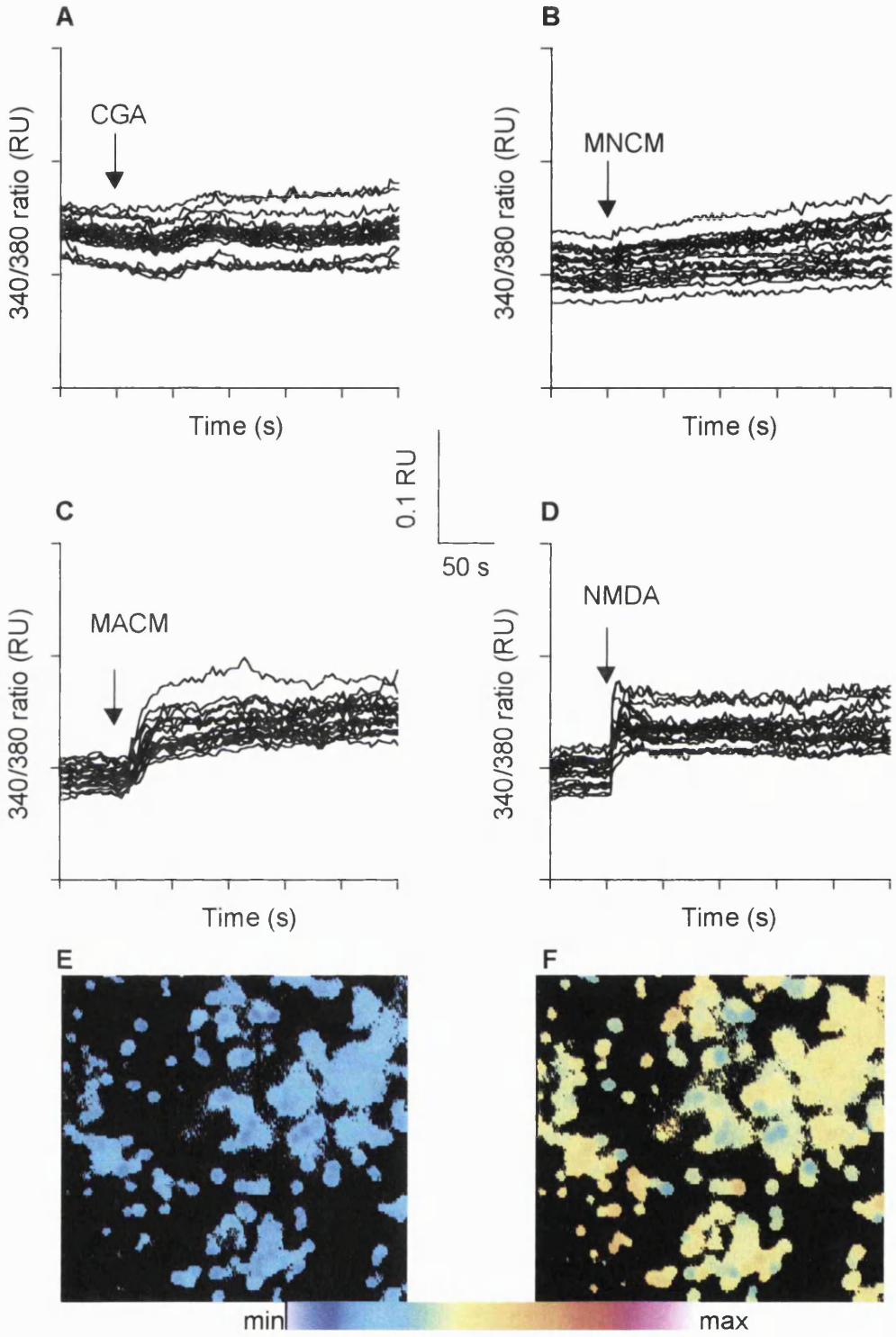


Fig. 5.4.1 Effect of conditioned medium on $[Ca^{2+}]_i$ in cerebellar granule neurones. Cells were loaded with fura-2 in basic medium for 30 min. Individual cells were randomly selected and their fura-2 responses monitored following additions as indicated by arrows. Traces show the effects of **(A)** CGA alone **(B)** non conditioned medium (MNCM) **(C)** conditioned medium (MACM) and **(D)** 100 μM NMDA and are recorded as the 340/380 fluorescence ratios from 20 cells. False colour images are displayed of control cells **(E)** and those immediately following addition of conditioned medium **(F)** with the scale bar of minimum to maximum intensity from left to right.

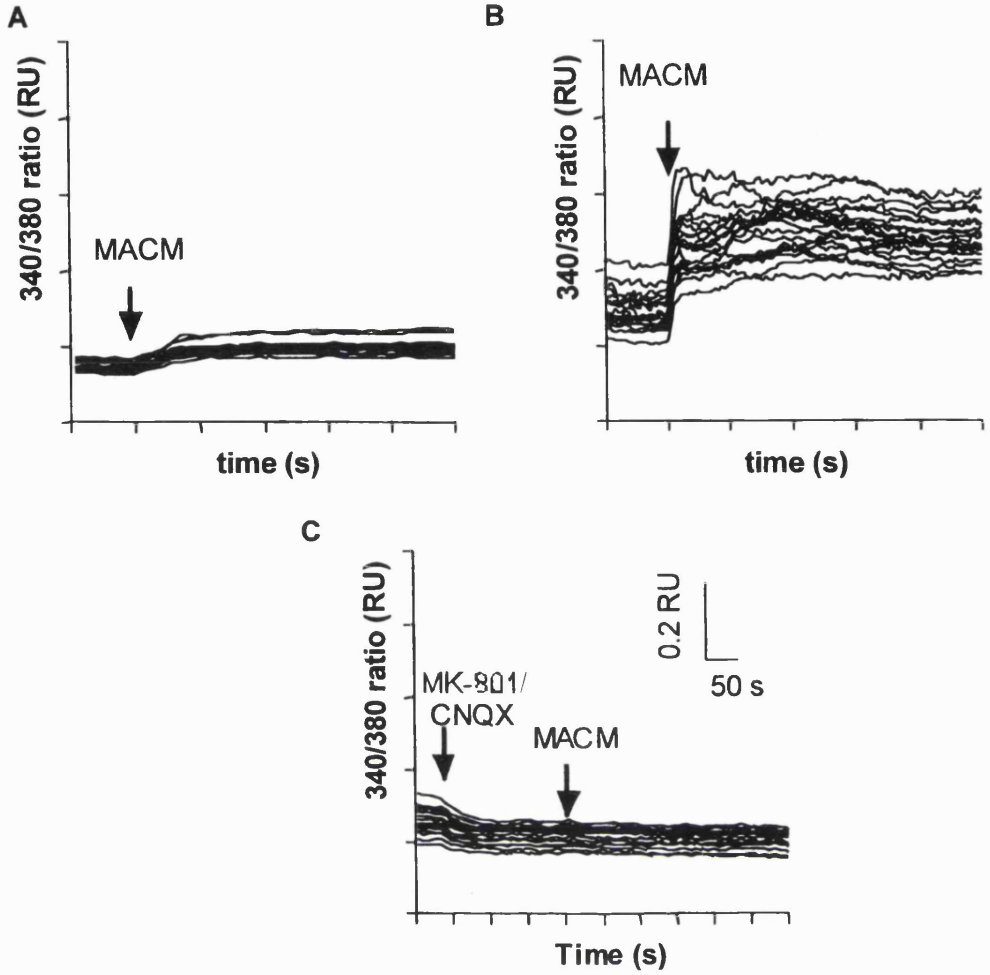


Fig. 5.4.2 The role of glutamate receptors in the $[Ca^{2+}]_i$ elevation. Cerebellar granule cells were loaded with fura-2 and calcium imaging performed as described in Chapter 2. Traces show the effects of conditioned medium (MACM) in **(A)** magnesium containing basic medium **(B)** magnesium free medium **(C)** the presence of the glutamate receptor antagonists MK-801 (10 μM) and CNQX (20 μM) and recorded as the 340/380 fluorescence ratios from 20 cells.

by the application of MK801 and CNQX suggesting this effect to be mediated via the ionotropic glutamate receptors (Fig. 5.4.2C). It has been reported that cytokines can influence calcium responses to various forms of stimulation. Thus depolarisation induced changes were investigated in cells exposed to conditioned medium for a period of 5 h. There was no noticeable difference in both the profile or magnitude of the response to the addition of 50 mM KCl between control and MACM treated cells

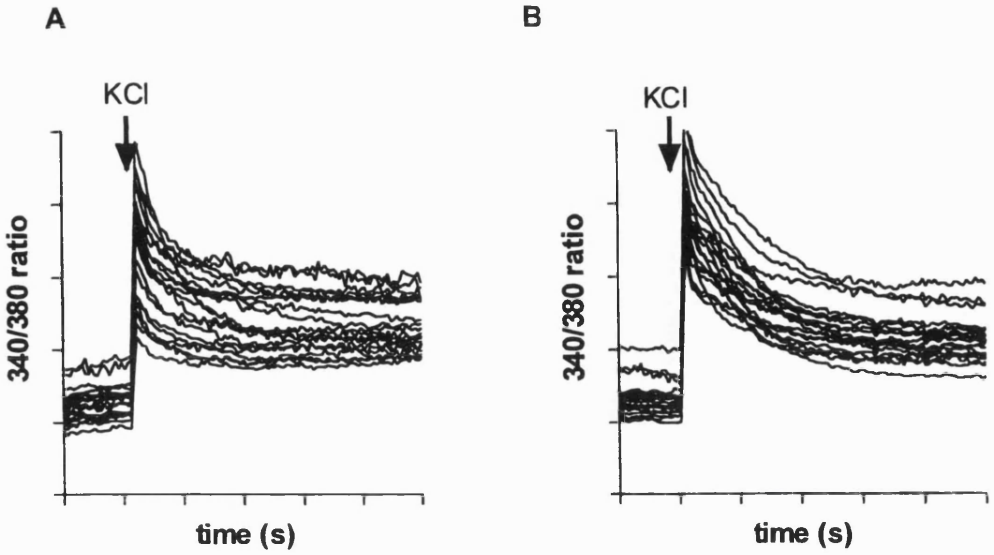


Fig. 5.4.3 Effect of conditioned medium on KCl evoked calcium signals in neurones. Cerebellar granule cells were loaded with Fura-2 and calcium imaging performed as described in chapter 2. Traces show the effects of the addition of 50 mM KCl in **(A)** control cells and **(B)** cells exposed to conditioned medium for 5 h and recorded as the 340/380 fluorescence ratios from 20 cells.

(Fig. 5.4.3A and B) suggesting that calcium deregulation may not occur until later time points in the apoptotic cascade.

5.5 Cathepsin B induces neuronal apoptosis

Previously in Chapter 3 it was shown that microglia activated with CGA could increase the secretion of cathepsin B, an event which has also been observed in BV-2 microglial cells treated with LPS (Ryan *et al.*, 1995). Thus, it was of interest to determine whether cathepsin B could induce neuronal apoptosis. A different neuronal model system was used for this, the immortalised hippocampal cell line, HT22 (Davis and Maher, 1994). The addition of conditioned medium from activated microglia to these cells, resulted in increasing neuronal death with the amount of medium applied and only a small volume was needed to elicit significant cell death (Fig. 5.5.1A). Medium from untreated microglia had no effect on cell viability (Fig. 5.5.1A). A time course using 50% conditioned medium revealed that there were significant increases in cell death

following 8 h of treatment (Fig. 5.5.1B). This cell death could also characterised as apoptotic since 46 ± 2.34% of the cells displayed condensed nuclei with Hoechst 33342 staining, versus 49 ± 2.78% of the cells that were stained with propidium iodide. Furthermore neuronal death was significantly reduced in the presence of the caspase-3 inhibitor, z-DEVD-fmk (Fig. 5.5.1C). Thus these cells appeared to mirror the response of primary cultures of granule cells to conditioned medium. Similarly, medium which had been boiled for 1 h was significantly less toxic (Fig. 5.5.1C). Thus the possibility that cathepsin B might be responsible was tested by using an inhibitor, z-FA-fmk. Pre-incubation with 100 μM z-FA-fmk significantly reduced the toxic effects of microglial conditioned medium (Fig. 5.5.1C). Addition of cathepsin B (0.1-10 μM) to HT22 for a period of 24 h resulted in a dose dependent increase in the number of dead cells stained with propidium iodide (Fig. 5.5.2A). There was also a significant increase in the number of apoptotic cells detected with Hoechst 33342 which could be prevented by pre-incubation with the caspase-3 inhibitor, z-DEVD-fmk, or cathepsin B inhibitor, z-FA-fmk (Fig. 5.5.2B). Furthermore, boiling cathepsin B for 1 h resulted in a significant reduction in neurotoxicity (Fig. 5.5.2B). Similar results were obtained when CGCs were exposed to 10 μM cathepsin B for 24 h (Fig. 5.5.2C). Application of 10 μM cathepsin B to HT22 cells caused a faster onset of cell death when compared with the effects of MACM which suggests other factors present in MACM may interact to control the rate of cell death (Fig. 5.5.3A). This rapid cell death was reflected in the ability of the caspase 3 inhibitor to reduce cell death. There was a small time window of 2-4 h following addition of cathepsin B at which the compound was neuroprotective indicating early activation of the caspase (Fig. 5.5.3B). The caspase-1 inhibitor, YVAD-CHO was ineffective (Fig. 5.5.3B).

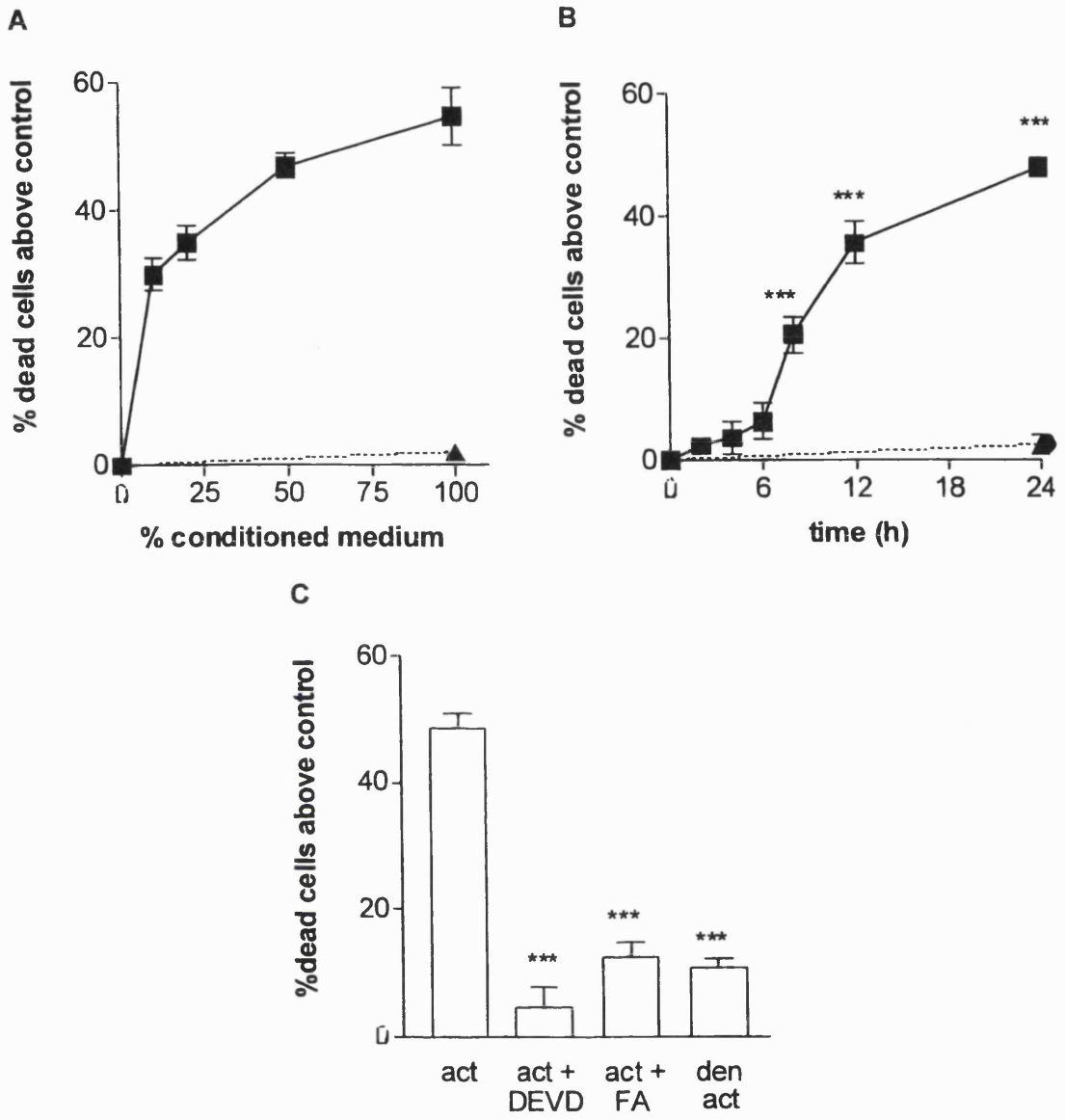


Fig. 5.5.1 Microglial conditioned medium is neurotoxic to the HT22 cell line. (A) Neurones were exposed to increasing amounts of conditioned medium for 24 h from microglia activated with 10 nM CGA for 24 h (squares) or 100% medium from untreated microglia (triangle) and the number of live and dead cells counted. Data are the mean percent live ± SEM (n = 32). **(B)** Neurones were exposed to 50% MACM (Squares) and the number of dead cells counted at the times indicated and in control cultures (circles) and cultures treated with medium from non activated cells (triangle) at 24 h. Data are expressed as the mean percentage dead cells ± SEM above control (n = 32), ***p<0.001 significantly different to conditioned medium alone. **(C)** HT22 cells were treated with 50% conditioned medium in the absence (act) and presence of 50 μM z-DEVD-fmk (act + DEVD), or 100 μM z-FA-fmk (act + FA) or with medium which had been boiled for 1 h (den act) and the number of dead cells counted after 24 h. ***p<0.001 significantly different to act alone.

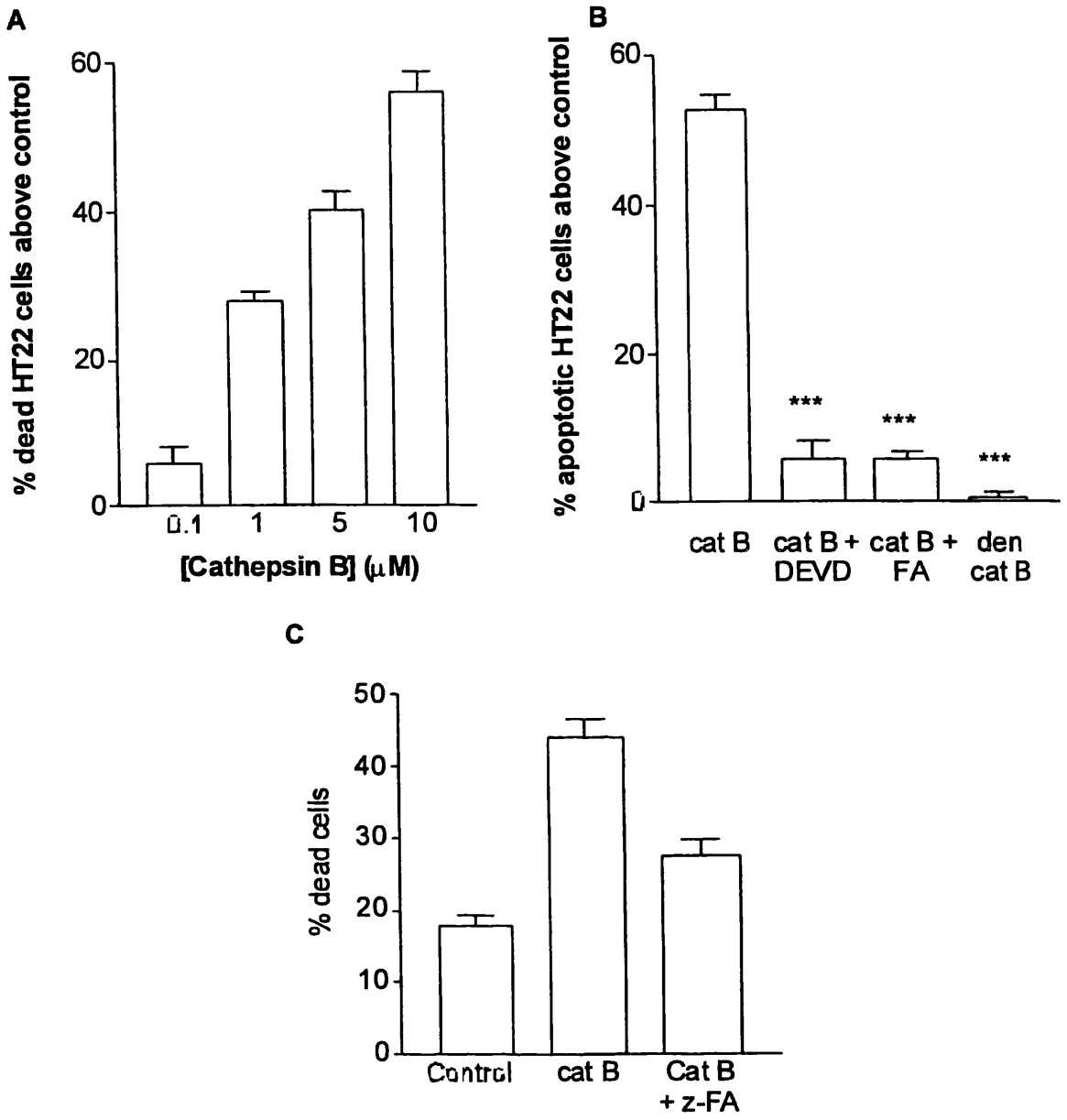


Fig. 5.5.2 Cathepsin B induces apoptosis in HT22 cells and CGCs. (A) HT22 cells were exposed to increasing concentrations of cathepsin B (0.1-10 μM) for 24 h and then number of dead neurones counted using the live/dead assay (n = 32). **(B)** The number of apoptotic HT22 cells were counted using Hoechst 33342 staining in control cultures and those exposed to 10 μM cathepsin B in the absence (cat B) and presence of 50 μM caspase inhibitor z-DEVD-fmk (cat B + DEVD), 100 μM cathepsin B inhibitor, z-FA-fmk (cat B + FA) or cathepsin B which had been boiled for 1 h (den cat B) (n= 32). ***$p<0.001$ significantly different to cultures in the presence of cathepsin B alone. **(C)** Cerebellar granule cells (CGCs) were either left untreated (control) or exposed to 10 μM cathepsin B in the absence (cat B) or presence of 100 μM cathepsin B inhibitor (cat B + z-FA).

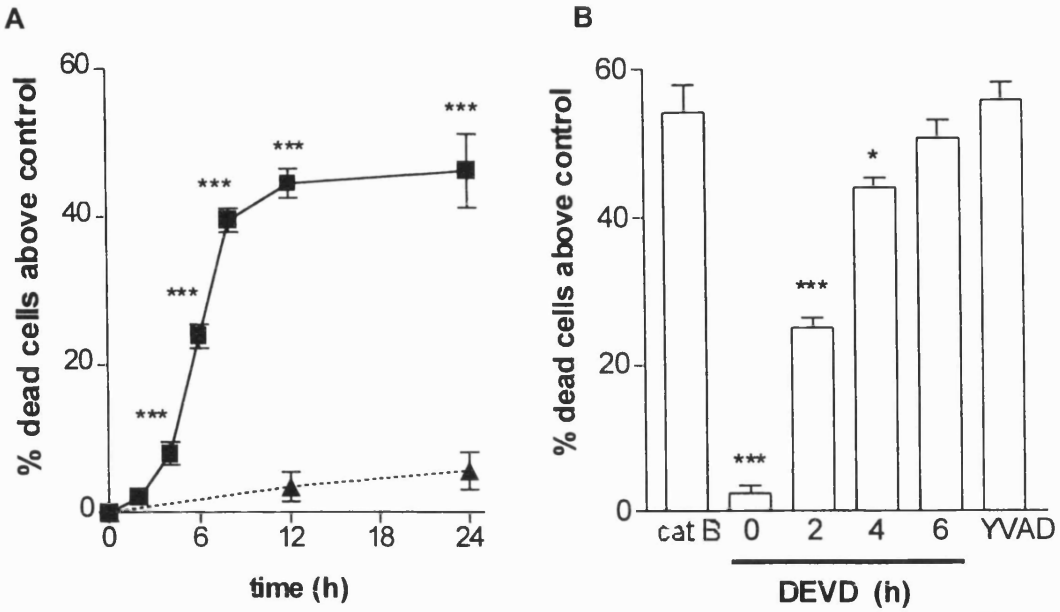


Fig. 5.5.3 Time course of neuronal death and caspase activation. (A) The number of live and dead cells were counted using FDA and PI at various time points following the addition of 10 μM cathepsin B to HT22 cells (squares) or in untreated cultures (triangles). Data are expressed as percentage dead above control at time 0 h. **(B)** Cells were treated with 10 μM cathepsin B for 24 h in the absence (cat B) or presence of 50 μM z-DEVD-fmk which was added either with cathepsin B (0) or 2, 4, or 6 h later. The effect of 10 μM YVAD-CHO co-applied with cathepsin B is also shown (YVAD). Data are expressed as a percentage dead above control (n = 32). ***p<0.001, *p<0.05 significantly different to cultures treated with cathepsin alone.

To determine whether mitochondrial depolarisation was involved, HT22 cells were loaded with JC-1 and the number of cells displaying a green fluorescence recorded as a measure of a fall in $\Delta\psi_m$. There was only a significant increase in the number of cells with depolarised mitochondria at 24 h following treatment with either 50% conditioned medium from activated microglia or 10 μM cathepsin B, suggesting that this phenomenon was not responsible for induction of apoptosis as cell death occurs earlier (Fig. 5.5.4A). Oxidative stress has been proposed as a model for glutamate induced neuronal apoptosis in the HT22 cell line (Tan *et al.*, 1998). Thus the levels of nitrite were determined in the supernatants of HT22 cells exposed to either 50% conditioned medium from activated microglia or 10 μM cathepsin B. There was an increase in those treated with MACM but this effect was as a result of the increased NO production by

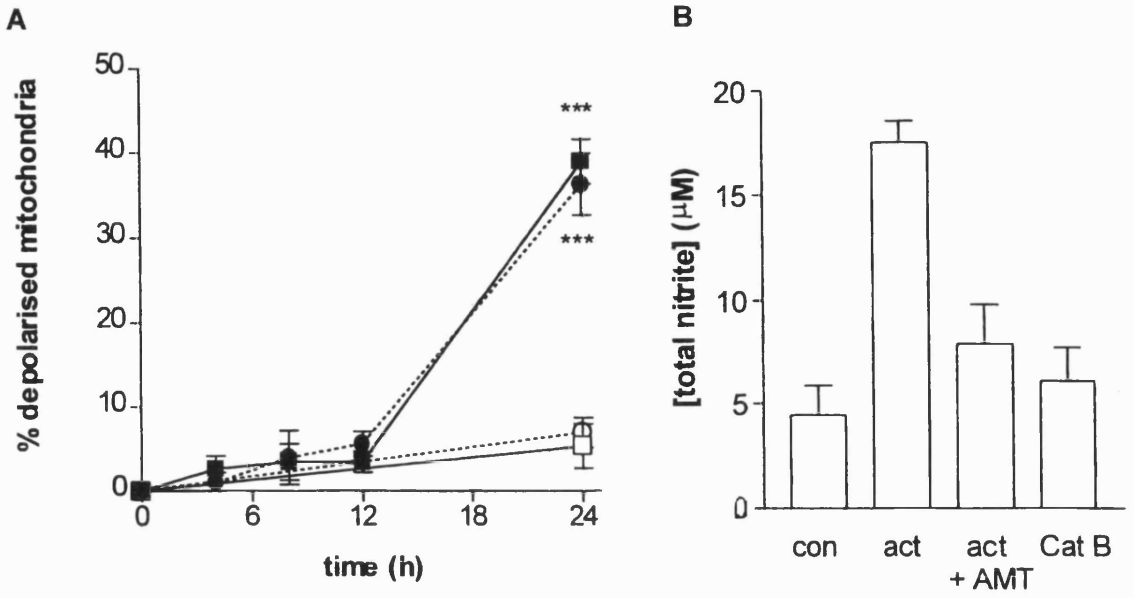


Fig. 5.5.4 Neither mitochondrial depolarisation or nitric oxide production are involved in conditioned medium or cathepsin B induced HT22 cell death. (A) HT22 cells were loaded with JC-1 and the number of neurones with either red fluorescing punctate mitochondria or a diffuse green fluorescence were counted at various time points following treatments. Cells were either exposed to 50% conditioned medium from microglia activated with 10 nM CGA (closed squares) or non activated microglia (open squares) or 10 μM cathepsin B (closed circles). Untreated HT22 cells are also shown (open circles). Data are expressed as the percentage of cells displaying depolarised mitochondria (green), n = 32. ***p<0.001 significantly different to controls at time 0 h. **(B)** The total levels of nitrite were determined with the Griess assay in supernatants from control HT22 cells (con) or those exposed to 50% microglial conditioned medium from CGA activated microglia (act), microglial conditioned medium in which microglial NO production had been prevented with AMT-HCl (act + AMT) or 10 μM cathepsin B (cat B). n = 12 determinations from 3 independent preparations.

t activated microglia since MACM from cells which had been incubated with the iNOS inhibitor, AMT-HCl, showed no significant increase above control levels (Fig. 5.5.4B). Cathepsin B had no effect on the levels of NO (Fig. 5.5.4B). Finally with a view to dissecting the signalling cascades prior to caspase activation and cellular apoptosis the effect of phosphorylation was investigated. Genistein, a non specific inhibitor of tyrosine kinases significantly reduced the toxicity elicited by microglial conditioned medium – other kinase inhibitors, H-7 (serine/threonine kinases) and LY294002 (PI3-kinase) were ineffective (Fig. 5.5.5A). These results were mirrored in HT22 cells treated with cathepsin B (Fig. 5.5.5B).

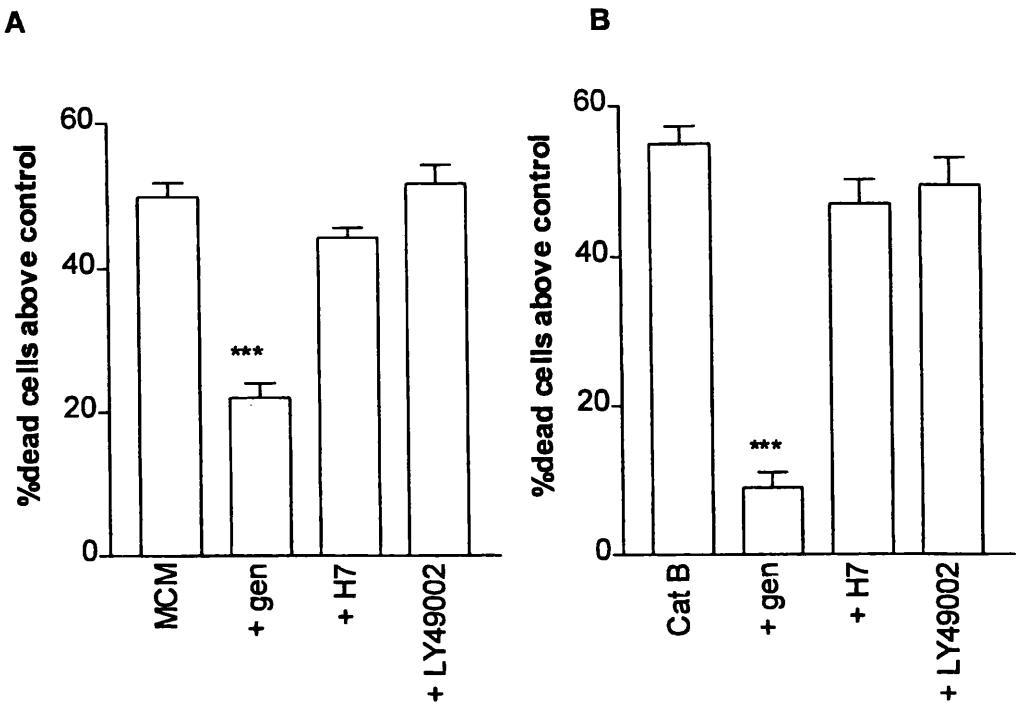


Fig. 5.5.5 The effect of kinase inhibitors on HT22 cell death evoked by conditioned medium and cathepsin B. **(A)** Neurones were exposed to 50% conditioned medium from microglia activated with 10 nM CGA/24 h, in the absence (MCM) and presence of genistein (200 μM), H7 (200 μM) or LY294002 (1 μM) and the number of dead cells counted at 24 h. **(B)** Cells were exposed to 10 μM cathepsin B in the absence (cat B) and presence of genistein (200 μM), H7 (200 μM) or LY294002 (1 μM) and the number of dead cells counted at 24 h. Data are the mean number of dead cells ± SEM (n = 32), ***p<0.001 significantly different to cultures in the absence of genistein.

5.6 Discussion

Observations both *in vivo* and *in vitro* suggest that activated microglia can cause extensive neuronal damage. Activated microglia are associated with the lesions seen in brain disorders such as ischaemia (Streit, 1993), degenerative diseases and demyelination (Guilian, 1987, 1992) and cell culture studies suggest that neurotoxicity can be mediated by the secretion of mediators such as nitric oxide (Dawson *et al.*, 1994), glutamate (Piani *et al.*, 1994) and other unidentified molecules (Giulian et *al*, 1994). However, the signalling cascades from microglial activation through to neuronal loss are poorly understood since most studies have used LPS, a microglial modulator

that is unlikely to be present within the normal brain. Chromogranin A has been localised to both neurones and glia and is widely distributed throughout the brain (Mahata *et al*, 1991; Munoz *et al.*, 1990) and thus may play an important role in CNS signalling. Furthermore, there is evidence that CGA accumulates during the pathologies of diseases such as Alzheimers (Munoz, 1991) localising to the characteristic β-amyloid plaques. The results presented here suggest that CGA may act as a natural inflammatory agent and cause neuronal death. Treatment of both primary cultures of rat cerebellar granule neurones and the HT22 hippocampal cell line with conditioned medium from microglia activated with CGA (MACM) resulted in neuronal apoptosis which involved caspase-3 dependent pathways. Neurotoxicity was mediated by both ionotropic glutamate receptor agonists and protein molecules released by activated microglia.

5.6.1 Microglial conditioned medium is neurotoxic

Results show that microglia activated with CGA can induce neuronal cell death which bears the characteristics of apoptosis including chromatin condensation, DNA fragmentation and caspase activation. However, the direct application of CGA to the neuronal cultures was not deleterious and neither was medium from untreated microglial cultures. Previous reports have shown that control macrophages/microglia show similar neurotoxic activity to those that are stimulated (Zietlow *et al.*, 1999; Flavin *et al.*, 1997; Thery *et al.*, 1993; Piani *et al.*, 1992). This may be a result of the increased spontaneous activation state of the cell used. Such results are similar to those obtained using adult rat brain and human tissue untreated or activated with CGA. These preparations were shown to exhibit markers indicative of an increased activation state (chapter 3). Furthermore, if microglia are co-cultured with neurones (Piani *et al.*, 1992) they are subject to neuronal activating factors such as TGF-β (Dobbertin *et al.*, 1997).

The ability of MACM to induce neuronal death was found to be dependent upon the age of the granule cell cultures. During time in culture the neurones acquire the morphological and biochemical characteristics of differentiated neurones (Harrold *et al.*, 1997). At 4 DIV the cells display a limited neurite network and at this stage are unaffected by MACM. By 10-12 DIV neurites are more developed and resemble thick cables linking large 3D-clumps of cell bodies and the neurones are sensitive to MACM. This morphological transition is accompanied by changes in the profile of channels and receptors including the voltage gated Ca^{2+} channels (Harrold *et al.*, 1997), adenosine receptors (Sanz *et al.*, 1996), glutamate receptors (Gallo *et al.*, 1987, Santi *et al.*, 1994; Hack *et al.*, 1995) and cell surface molecules such as NCAM (Meier *et al.*, 1984). Glutamate is necessary for the maturation of granule cells early in culture but becomes toxic to the mature neurones and this is mediated by entry of Ca^{2+} through the NMDA receptor (Levi *et al.*, 1982; Balazs *et al.*, 1988b). Thus the differences in sensitivity to MACM between 4 DIV and 12 DIV may be a result of changes in receptor profile or a switch in the nature of the molecule(s) concerned from survival promoting to death inducing.

Further characterisation of the neurotoxicity showed that it was both dependent upon the concentration of CGA used to activate the microglia and the amount of MACM to which the neurones were exposed. Thus increasing concentrations of CGA progressively activated microglia and thus released more neurotoxin(s) to which the neurones were sensitive. Neurotoxicity was not due microglial lysis since there was a linear correlation with increasing activation time indicating neurotoxins are released at an early stage when microglia are still viable. However, at late time points there was an increased rate of neuronal death which correlated with loss of microglial viability and increased microglial glutamate release. Thus there may be a number of neurotoxins

responsible for cerebellar granule cell death and this may be important when devising neuroprotective strategies.

5.6.2 Microglial conditioned medium induces apoptosis and mitochondrial depolarisation in cerebellar granule neurones

Conditioned medium from activated microglia causes neuronal apoptosis. Apoptosis is a physiologically occurring form of cell death which is important during formation of brain structure. Early in development there is a major loss of granule cells in the cerebellum which can be attributed to apoptosis (Wood *et al.*, 1993). Granule cells cultured from neonate rats have become extensively used to investigate the mechanisms of apoptosis (Yan *et al.*, 1994; Eldadah *et al.*, 1997). Furthermore, there is growing evidence supporting a role for apoptosis in neurodegenerative diseases such as Alzheimer's. *In vivo* studies have indicated higher levels of pro-apoptotic genes in the brains of susceptible patients and DNA fragmentation studies on post mortem samples implicate apoptosis in these diseases which are often associated with increased microglial activity (Su *et al.*, 1997; Lassmann *et al.*, 1995). Thus microglia activation may mediate neuronal apoptosis rather than promoting the clearance of dying neurones. Cerebellar granule cell death was assessed using Hoechst 33342. The number of apoptotic neurones was found to correlate almost exactly with the proportion of dead cells observed with PI staining, suggesting that the predominant form of death was apoptotic. Caspases may be involved in cerebellar granule cell apoptosis (Armstrong *et al.*, 1997; Eldadah *et al.*, 1997). The peptide inhibitor of caspase-3, z-DEVD-fmk, was found to exert a protective effect from MACM but z-YVAD-CHO a blocker of caspase-1 did not increase neuronal viability significantly. Caspase-3 has been strongly implicated in granule cell apoptosis using the K^+/serum deprivation model (Armstrong

et al., 1997; Eldadah *et al.*, 1997). A common marker for active caspase-3 is the ability to cause the cleavage of poly-(ADP-ribose)-polymerase (PARP) an enzyme involved in DNA repair (Gu *et al.*, 1995). However, PARP cleavage in granule cells upon K^+ deprivation is undetectable (Armstrong *et al.*, 1997; Taylor *et al*, 1997) but it does occur in staurosporine induced apoptosis which is not sensitive to z-DEVD (Taylor *et al*, 1997). Thus, it can be concluded that distinct signalling pathways exist depending upon the nature of apoptotic stimulus and this is likely to be the case for MACM.

Evidence suggests that loss of mitochondrial membrane potential ($\Delta\psi_m$) might be responsible for apoptosis in cerebellar granule cells (Tanabe *et al.*, 1998) and especially in glutamate stimulated neurones mitochondrial depolarisation may act as one of the earliest signals specific to excitotoxin exposure (White and Reynolds, 1996). Compared with controls, neurones treated with conditioned medium from CGA activated microglia contained an elevated number of depolarised mitochondria when assessed by JC-1 fluorescence staining indicating this may play a role in the apoptotic pathway. Mitochondrial depolarisation occurs quickly suggesting this event is upstream of caspase activation as observed following the depletion of serum (Tanabe *et al.*, 1998).

5.6.3 Neurotoxicity is mediated by glutamate receptor agonists and proteins

The component molecules responsible for the neurotoxicity observed with conditioned medium were investigated. Glutamate released in the proximity of vulnerable neurones has been proposed as a cause of the neurotoxicity observed in numerous human diseases many of which are associated with activated microglia (Lipton and Rosenberg, 1994). Since it had already been shown that CGA activated microglia released glutamate the neuroprotective effect of the ionotropic glutamate receptor antagonists, MK801 and

CNQX was determined. These drugs were found to afford a small degree of protection from the conditioned medium suggesting that the glutamate may be toxic to the granule cells. Previous studies have indicated that glutamate is the major determinant of microglial evoked neurotoxicity (Piani *et al.*, 1991; 1992). This was not the case for CGA activated microglia since there was still a large proportion of cells that were killed in the presence of the glutamate receptor antagonists. However the fact that conditioned medium activates caspase-3 is in agreement with recent reports of excitotoxin induced apoptosis in granule cells (Du *et al.*, 1997; Nath *et al.*, 1998). Glutamate induced neuronal death was originally believed to be entirely due to necrosis and there are still recent reports that glutamate induced death does not show the classical signs of apoptosis such as DNA fragmentation (MacManus *et al.*, 1997). However, it has become clear over the last few years that an important determining factor is the level of excitotoxin to which the neurones are exposed (Ankarcrona *et al.*, 1995; Bonfoco *et al.*, 1995). Thus, using a low concentration of glutamate it has been shown that neuronal apoptosis is mediated by caspase-3 (Du *et al.*, 1997). One anomaly is that when conditioned medium from CGA activated microglia was added to cerebellar granule neurones there was no significant increase in nitric oxide production as determined by nitrite levels. It is generally accepted that glutamate acting through NMDA receptors induces neurotoxicity by the production of NO and other oxygen free radicals via a Ca^{2+} dependent process. Activation of NO synthase, phospholipase A(2) and other enzymes like xanthine oxidase contribute significantly to this response (Gunasekar *et al.*, 1995; Atlante *et al.*, 1997). It has thus been proposed that the generation of both NO and ROS results in the formation of peroxynitrite, which causes the cellular damage, and this can be blocked with antioxidants (Gunasekar *et al.*, 1995; Ciani *et al.*, 1996). Therefore the cell viability of granule neurones exposed to MACM incubated with nitric oxide

synthase inhibitors, L-NNA and AMT-HCl was assessed. There was no significant difference between the neurotoxicity evoked in the absence and presence of these compounds which is at odds with the role of glutamate. Only when microglial cells were incubated with the NOS inhibitors was there a slight reduction in the neurotoxicity observed with MACM suggesting that production of microglia derived neurotoxin(s) may be dependent upon microglial NO production. This might suggest it is glutamate as it was shown previously the secretion of glutamate by CGA activated microglia was dependent upon NO (see chapter 3).

Thus part of the neurotoxicity caused by MACM is mediated via ionotropic glutamate receptors. Rather than glutamate itself other molecules could act at the NMDA receptor. For instance, quinolinic acid is released by activated microglia (Espey *et al.*, 1997), and is a known agonist of the NMDA receptor and generally regarded as an excitotoxin (Reinhard Jr *et al.*, 1994). In the case of HIV infection, a neurotoxic amine, NTox, may cause NMDA receptor mediated damage (Giulian *et al.*, 1996). Furthermore, senile plaques stimulate microglia to release a neurotoxin similar to that found in Alzheimer brain (Giulian *et al.*, 1995). However, it is not known whether these neurotoxins cause the generation of nitric oxide or the effects can be prevented by treatment with NOS inhibitors.

Neurotrophins including BDNF may be involved during cerebellar granule cell differentiation (Gao *et al.*, 1995). BDNF can also protect against the apoptosis induced by switching to low K^+ (Kubo *et al.*, 1995) and neurotoxicity induced by glutamate (Lindholm *et al.*, 1993). BDNF was partially neuroprotective towards conditioned medium from CGA activated microglia. Approximately 50% of the neurones were protected in the presence of BDNF and when neurones were treated with BDNF and the

ionotropic glutamate receptor antagonists there was no further protection suggesting that the neurotrophin protects against glutamate receptor mediated neurotoxicity.

Other likely molecules released by CGA activated microglia which could be neurotoxic are the cytokines (Morganti-Kossmann *et al.*, 1992). Thus MACM was boiled for 1 h to denature any proteins secreted by CGA activated microglia (boiling had no effect on the neurotoxicity evoked by glutamate). Boiling significantly protected the granule neurones from the toxic effects of MACM and furthermore when it was added in the presence of the glutamate receptor antagonists there was a total block of the neurotoxic activity. Previous studies of the effects of cytokines on granule cells have focused on their potential to mediate neuronal survival. The age of cerebellar granule cell cultures can determine whether cytokines are neuroprotective (De Luca *et al.*, 1996) and thus it may be that exposure of granule cells to cytokines at 10-12 DIV will result in cell death, a finding consistent with the age dependent neurotoxicity of MACM. Cytokines levels are known to be elevated in a number of CNS disorders including Alzheimer's (Fillit *et al.*, 1991) and molecules such as TNF-α and IL-1 found at the sites of inflammation have been proposed to cause neuronal damage (Chao *et al.*, 1995). To date most *in vitro* studies have examined the effects of cytokines in systems where there is a mixture of glia and neuronal cell types and implicated NO and NMDA receptor activation in neurotoxicity (Chao *et al.*, 1995; 1996). However, the direct actions of cytokines on neurones is unknown. Thus the effect of treating mature granule cell cultures with TNF-α or IL-1β for a period of 24 h was examined. Neither of these cytokines had a significant effect on neuronal viability suggesting that they either do not act directly to initiate neuronal death or that they require the presence of other factors that may be present in the MACM (Brown, 1999). Since CGA caused glutamate release from

microglia and glutamate is also likely to be found at sites of neuronal damage the effect of MACM on NMDA induced neurotoxicity in granule cell cultures was investigated. Using 100 μM NMDA, a time course for neurotoxicity established that 15 min of treatment followed by 24 h in culture medium gave approximately 50% neuronal death. The effect of subsequently culturing the cells with medium from untreated or CGA exposed microglia was investigated. It was found that untreated microglial medium showed no significant change in viability from NMDA treated controls while conditioned medium from CGA activated microglia significantly potentiated the NMDA induced neurotoxicity. These observations contrast with recent studies of the effect of TNF-α on glutamate induced neurotoxicity. Blockade of endogenous TNF-α in focal ischaemic injury was neuroprotective but TNF-α did not affect the sensitivity of neurones to glutamate or oxygen radicals (Barone *et al.*, 1997). However, in a different system of human foetal brain neurones it was found that TNF-α could potentiate glutamate neurotoxicity which was blocked by MK801 and 2-APV (Chao and Hu., 1994). Other studies contradict these observations and suggest that TNF-α can protect hippocampal neurones against NMDA induced damage (Prehn, 1996).

5.6.4 Microglial conditioned medium raises $[Ca^{2+}]_i$ in CGCs

Since changes in Ca^{2+} homeostasis have been reported to be a factor in neurodegeneration the effect of MACM on Ca^{2+} within cerebellar granule cells was investigated. There was a rise in $[Ca^{2+}]_i$ in the cells upon acute addition of MACM which was more pronounced when the experiments were performed in minus Mg^{2+} medium which allows the activation of NMDA receptors. Since there is an elevated concentration of glutamate in this media it is likely that this response is due to activation of the glutamate receptors. The fact that a signal is observed in the presence of Mg^{2+}

suggests that multiple receptor subtypes may be stimulated including the metabotropic receptors. Trans-ACPD an agonist of these receptors has been shown to elicit the formation of inositol phosphates in cerebellar granule cells (Gorman *et al.*, 1995) which can thus liberate Ca^{2+} from internal stores. However, when the cells are relieved of the Mg^{2+} block the ionotropic receptors contribute the majority of the rise in $[Ca^{2+}]_i$ since MK801 and CNQX could totally abolish this response. Therefore when the cells are under normal culture conditions glutamate may interact with both receptor types to control the status of the cell. For instance, mGluR5 has been proposed to control the developmental onset of apoptosis in these cells (Copani *et al.*, 1998) and mGluR agonists can influence NMDA cell currents (Yu *et al.*, 1997). Furthermore, following NMDA receptor activation a Ca^{2+} induced Ca^{2+} release mechanism may exist and play a role in neurotoxicity (Simpson *et al.*, 1993). The effect of MACM on the rise in $[Ca^{2+}]_i$ induced by KCl was investigated since proteins such as cytokines may influence calcium signalling processes. While the influence of cytokines on glial signalling have been investigated in detail (Koller *et al.*, 1996; Whittemore *et al.*, 1993; Holliday and Gruol, 1993) only a few studies have shown a modulation of Ca^{2+} currents in neurones (Szucs *et al.*, 1995; Soliven and Albert, 1992). However, cytokines can influence the other agonist induced Ca^{2+} signalling mechanisms in neurones such as elevating NMDA responses (Qiu *et al.*, 1995). There was no difference in either the peak response or inactivating properties of the traces after 5 h exposure to MACM by which time the effect of any disturbance on Ca^{2+} homeostasis should have begun to take effect with respect to the time course of apoptosis further suggesting that the toxicity elicited by MACM is not dependent on IL-1β or nitric oxide since it has been shown that following 2 h exposure to NO donors cerebellar granule cells lose their ability to respond to KCl depolarisations (Bonfoco *et al.*, 1996).

5.6.5 Microglial conditioned medium and cathepsin B are neurotoxic to HT22 neurones

The effects of MACM on a different cell model, the HT22 cell line, were also examined. Previous studies have shown that treatment of these cells with glutamate results in apoptosis and that this occurs as a result of oxidative stress (Tan *et al.*, 1998a and b). Since microglial conditioned medium contains elevated levels of glutamate it might be expected to produce similar effects. Exposure of HT22 cells to conditioned media from CGA activated microglia resulted in cell death that could be blocked with an inhibitor of caspase-3 but not caspase-1. This was in contrast with the observation that glutamate dependent apoptosis in the HT22 cell line involves caspase-1 activation (Tan *et al.*, 1998a). It may be that other factors in the medium predominate in activating different caspase pathways. In the HT22 cell line, MACM did not induce mitochondrial depolarisation until after apoptosis had been initiated which is in contrast to the effect on cerebellar granule neurones. In this instance it appears that mitochondrial damage is a consequence rather than the cause of apoptosis (Barbieri *et al.*, 1998). It has also been shown that mitochondrial depolarisation is not necessary for staurosporine evoked neuronal apoptosis (Krohn *et al.*, 1999) or for PC12 cells exposed to oxidative stress (Satoh *et al.*, 1997). During oxidative stress pathways, it is possible that upstream caspases become activated immediately and are responsible for the production of any reactive oxygen species (ROS) which can subsequently activate caspase-3 (Krohn *et al.*, 1998). In this instance, there may be a requirement for the mitochondrial electron transport chain to be functional since it, itself, is the source of the ROS. For example, if FCCP is used to dissipate the $\Delta\psi_m$ in HT22 neurones then the cells are protected from glutamate toxicity (Tan *et al.*, 1998). Thus distinct apoptotic pathways initiated by the same molecules may exist in different cell types and modes of

death. Finally, HT22 cells exposed to MACM do not release NO suggesting that ROS such as H_2O_2 may be more important (Maher and Davis, 1996; Lezoualc'h *et al.*, 1996).

Results from chapter 3 indicated the release of a protein from CGA activated microglia with a molecular weight of approximately 25 kDa that corresponded to cathepsin B. It has previously been reported that gamma interferon causes the induction of cathepsin B within macrophages (Lah *et al.*, 1995; Li and Bever Jr, 1997). However, it has also been reported that both the BV-2 microglial cell line and human monocyte derived macrophages can release cathepsin B into the extracellular medium (Ryan *et al.*, 1995; Reddy *et al.*, 1995). More recently evidence exists that cathepsin B can be released by exocytosis in both epithelial cells and a glioblastoma cell line (Linebaugh *et al.*, 1999). Thus the fact that cathepsin B can be processed and subsequently secreted suggests a potential role for this protease in neurotoxicity. This was investigated by applying cathepsin B directly to the HT22 hippocampal cell line. This resulted in a dose dependent increase in neuronal apoptosis which could be blocked with an inhibitor of caspase-3 consistent with the findings of the effects of conditioned medium on the cells. In a previous study of microglial induced neuronal apoptosis, it was proposed that hippocampal cell death could be mediated by a protease (Flavin *et al.*, 1997), however the mechanism was never elucidated. There is evidence for a link between cathepsin B and processing of caspases within a cell. Cathepsins have been shown to directly cleave pro-caspases 1, 7, and 11 thus initiating apoptotic pathways (Vancompernolle *et al.*, 1998; Zhou and Salvsen, 1997). However, there are no reports of this effect by extracellular cathepsins. It is has been shown in culture that cathepsin B can work efficiently at neutral pH and is responsible for the degradation of extracellular matrix (ECM) proteins (Buck *et al.*, 1992; Reddy *et al.*, 1995). The link between the ECM and caspase activation is poorly understood. However, there is evidence that the ECM

provides survival signals to a cell and when components such as the adherins e.g focal adhesion kinase (FAK), are cleaved by caspases in response to toxins, apoptosis can result (Bannerman *et al.*, 1998; Levkau *et al.*, 1998). This may have consequences for diseases involving proteolytic tissue destruction. For instance multiple sclerosis involves the degradation of myelin (Berlet and Ilzenhofer, 1985; Whitaker *et al.*, 1982). Furthermore, cathepsin B is a constituent of senile plaques in Alzheimer's disease and may be responsible for the irregular cleavage of amyloid precursor protein (Cataldo and Nixon, 1990). The effects of cathepsin B mirrored that of MACM since it was unable to induce mitochondrial depolarisation or result in the production of neuronal NO. However, at the concentration of cathepsin B used in most experiments (10 μM) the onset of neuronal apoptosis was quicker than MACM. This may be due to other factors found in the microglial conditioned medium that inhibit the action of this protease. For instance, cystatin C, a potent inhibitor of cathepsin B is present within microglia (Zucker-Franklin *et al.*, 1987), though it is significantly down-regulated following activation of macrophages (Chapman *et al.*, 1990).

5.6.6 Conclusions

In conclusion it appears that microglia activated with CGA can secrete a number of neurotoxic mediators including glutamate/glutamate receptor agonists and heat sensitive molecules such as cathepsins that can activate apoptotic pathways in two different neuronal cell types and also affect the Ca^{2+} homeostasis of these cells. Thus chromogranin-A might be a natural factor that can trigger the microglial response in regions of neurodegeneration and contribute to the pathology observed in diseases such as Alzheimer's.

6. GENERAL DISCUSSION

6.1 Tissue culture – a suitable system to investigate microglial-neuronal interactions

The work in this thesis describes a model with which to study microglial-neuronal interactions using a tissue culture system. With the individual isolation of microglia and neurones it is possible to dissect the signalling pathways following microglial activation through to the effects of released microglial factors on neuronal survival and function. Though cells may behave differently in isolation, since it is impossible to provide a culture medium that absolutely mimics the physiological milieu, much data can be extrapolated to the *in vivo* situation. For instance, any technique used to isolate microglia will result in a degree of cellular activation but once in culture the cells can adopt a resting phenotype. Upon exposure to activating factors they are observed to undergo a morphological change to an ameboid active form which mirrors the processes occurring *in vivo*. However, even though the cells may appear non activated there are many studies that show untreated microglia can induce neuronal death suggesting they are still secreting toxic factors. Most preparations are from mixed glial cultures which have been grown for up to 2 weeks (McCarthy and de Vellis, 1980). Thus the microglia will have been exposed to factors from other glial cells and their biochemical properties may have changed from those found immediately on isolation as they were in the *in vivo* situation. The experiments described in this thesis have utilised microglia isolated directly from rat neonate brain by homogenisation rather than enzyme dissociation which can also result in activation and changes in the membrane proteins responsible for microglial function (Ford *et al.*, 1996; Havenith *et al.*, 1998). The microglia isolated were found to adopt a resting morphology and were quiescent for the first few days in culture thus allowing the process of activation to be followed. It was not possible to obtain either adult rat brain microglia or human microglia in a non activated state. It may be that since the cells are derived from the mature brain and are likely to be fully

ramified any insult will cause activation. Also in the instance of human tissue it is likely that post-mortem time will determine how activated the cells are. However, any effects observed in neonate microglia were mirrored in the adult, albeit from a higher basal level of activation. Thus these cultures provided a suitable model from which to draw conclusions which might be relevant for adult disease states. Furthermore, in order to confirm that any effects observed by activating primary cultures were due to microglia and not contaminating glia (95% of the cells were OX-42 positive) the N9 microglial cell line was also used.

Most *in vitro* studies have involved activating microglia with lipopolysaccharide (LPS) but this is unlikely to be a natural activator in situations where there is injury without infection. LPS is also non specific since it can affect other cells such as astrocytes. Thus chromogranin A was used, a peptide that is widely expressed throughout the normal CNS (O'Connor *et al.*, 1984; Munoz *et al.*, 1990). The importance of this as a microglial activator has been shown in tissue culture studies (Taupenot *et al.*, 1996; Ciesielski-Treska *et al.*, 1998) and *in vivo* where it accumulates in the senile plaques of Alzheimer's disease in regions where activated microglia have been found (Munoz, 1991; Itagaki *et al.*, 1988; McGeer *et al.*, 1988). Furthermore, at the concentrations of CGA used to activate microglia in these studies it was found that CGA did not affect neuronal viability directly, thus any changes in cell survival/function could be attributed to factors secreted by the microglia. The cerebellar granule cell and HT22 hippocampal cell line were used to investigate this. CGCs are a well established model for examining pathways of neuronal apoptosis and excitotoxicity. Granule cells constitute the majority of neurones in the brain and cell cultures can provide a homogenous population free of other neuronal types and with minimal contaminating glia, which is

necessary to be able to study the effects of microglia alone. Typically cultures prepared in this laboratory displayed 3.5% GFAP positive cells and 0.95% OX-42 positive cells when used at 10-12 DIV. There is also evidence that the cerebellum is affected in Alzheimer's disease though it is believed that other regions of the brain such as the hippocampus are damaged more readily (Larner, 1997; Van Hoesen *et al.*, 1991). Thus the effects of microglial factors were also investigated in the hippocampal HT22 cell line. Both systems provided evidence that activated microglia may be deleterious to neuronal survival.

6.2 Microglial cell death in culture: a model for *in vivo* control of activated cells?

There is evidence that the morphological changes that occur during the activation of microglia might be influenced by the levels of free radicals since by either adding antioxidants directly (Heppner *et al.*, 1997) or culturing microglia on a layer of radical scavenging astrocytes (Sievers *et al.*, 1994) produces ramification. Microglia separated from their normal intracellular mix of cells might be particularly susceptible to the effects of endogenously produced free radicals and this could result in a self activating process that leads to the loss of viability that is regularly observed over time in culture. This was found to be the case for microglia treated with CGA. Cell death corresponded to elevated levels of NO and could be prevented with inhibitors of iNOS. There is evidence that NO participates in the pathology of Alzheimer's disease in which microglial activation is involved (Colton and Gilbert, 1987; McDonald *et al.*, 1997). However, microglial activation in these diseases is also accompanied by the release of various other factors which can either propagate the NO release or inhibit it (Pfeilschifter, 1991; Hopkins and Rothwell, 1995). Thus the extracellular milieu may determine whether the microglia becomes apoptotic. It was found that exogenously

applied NO could not induce microglial cell death suggesting that NO alone was not deleterious. Rather it was glutamate that was released subsequent to endogenous NO production that killed the cells. Studies have shown that β-amyloid enhances macrophage production of glutamate and suggest that this may contribute to the pathogenesis of Alzheimer's disease (Klegeris and McGeer, 1997). CGA treated microglia released significantly higher levels of glutamate in comparison with the β-amyloid study suggesting CGA to be a particularly relevant molecule during activation of microglia in this disease. The main release occurs through the Na^+-independent x_c^- transport system (Piani and Fontana, 1994) but a proportion may be released vesicularly since it can be blocked by bafilomycin. This may provide a function for the recently identified SNAP-25 homologue found in microglia (Hepp *et al.*, 1999). Interestingly, the activity of the transporter has been shown to be enhanced by the levels of oxygen free radicals (Bannai and Ishii, 1988) further suggesting a co-operative effect of NO and glutamate. Thus glutamate released by activated microglia might feed back to the microglia and results indicate that it does so, through a metabotropic glutamate receptor. Both in situ studies and work with primary cultures show that glia respond to glutamate through both ionotropic (Berger, 1995; Halzwarth *et al.*, 1994) and metabotropic receptors (Conn and Pin, 1997). However, there appears to be little in the literature suggesting a role for mGluRs in eliciting cell death. Since it was only shown recently that microglia contain mGluR (Biber *et al.*, 1999), the receptor subtypes and endogenous activators involved needs further investigation. Thus in culture, microglial cells die as a result of the release of NO and glutamate following activation with CGA but can these results be correlated with *in vivo* observations?

Since the cell death is an ordered process this suggests that it does not involve lysis and is not necrotic in nature. Thus in order to limit further cellular damage it is likely that an apoptotic pathway is involved. The importance of this is observed when microglia are responsible for restructuring the CNS during development (Moore and Thanos, 1996). It could be via the release of factors from microglia themselves that act as a control mechanism to dampen the activation status of the cell (Jones *et al.*, 1997). For instance, *in vivo* studies using the rat model of MS, EAE, suggest that microglia are particularly sensitive to apoptosis (White *et al.*, 1998) and this may involve microglial derived cytokines which are upregulated during the acute phase of the disease (Renno *et al.*, 1995; Merrill *et al.*, 1992). Also studies of the Alzheimer brain show there is an association of fragmented DNA with activated microglia (Lassmann *et al.*, 1995). The cell death in culture displays characteristics of apoptosis – CGA induces chromatin condensation and DNA fragmentation. It can also be blocked with an inhibitor of caspase-1. Generally, there is little support for a role of caspase-1 in apoptosis. Rather it is proposed to be merely involved in the production of interleukin 1β during the inflammatory response (Black *et al.*, 1989). However, it does exhibit the property of all other caspases in that it can degrade key cellular proteins suggesting it may have a dual biological role (Yuan *et al.*, 1993). Furthermore, since IL-1β is a product of activated microglia a feed back mechanism might operate whereby there is a switch in function. Incidentally, the few reports that do exist of caspase-1 mediated apoptosis all involve cells of the macrophage lineage (Hilbi *et al.*, 1997; Bermudez *et al.*, 1999 Singhal *et al.*, 1998). Also in the only other study to describe caspase activation in cultured microglia, apoptosis could be inhibited by YVAD-CHO (Glinka *et al.*, 1997). Thus, the release of microglial factors may sensitise the cells to the toxic effects of other molecules. For instance, glutamate might only be toxic in the presence of NO rather like the

observation that TNF-α can make microglia susceptible to Fas ligand induced apoptosis (Spanaus *et al.*, 1998).

There is evidence that changes in mitochondrial function may play a critical role in determining the fate of the cell. In the majority of the experiments described, JC-1 a mitochondrial specific potentiometric dye was used to investigate this aspect. With this probe it is possible to visualise easily, both cells that contain normal and depolarised mitochondria, and this makes it the preferred choice over other dyes such as rhodamine that bind with high affinity to both forms (Johnson *et al.*, 1981). Results indicated that upon exposure to CGA there was a fall in mitochondrial membrane potential and that this was dependent on NO and it preceded the appearance of apoptotic microglia. This correlates with previous reports that suggest mitochondrial depolarisation constitutes an irreversible step of programmed cell death (Zamazami *et al.*, 1995). This particularly applies to effector cells such as lymphocytes which are commonly removed by apoptosis and therefore is a likely mechanism by which activated microglial cell numbers may be controlled. A fall in $\Delta\psi_m$ is likely to have consequences for mitochondrial complex activity since the flow of electrons through the respiratory chain will be diminished. It would be interesting to investigate which complexes are sensitive to the effects of CGA and this could be carried out by a simple spectrophotometric assay. Recent experiments suggest that in situations of oxidative stress, α-ketoglutarate dehydrogenase complex (KGDHC) may be inactivated in microglia (Park *et al.*, 1999). This may have important implications in Alzheimer's disease where it is known that the activity of this enzyme is reduced (Gibson *et al.*, 1988). The process of mitochondrial depolarisation was shown to be involved in the phenomenon known as the permeability transition. CGA caused mitochondrial swelling which was sensitive to cyclosporin A

but did not evoke the release of cytochrome c. Thus other, unidentified factors may be necessary for caspase activation. This is not surprising since the apoptotic pathway described is novel and it appears that cytochrome c release is more generally associated with caspase-3 dependent pathways (Liu *et al.*, 1996; Uehara, *et al.*, 1999; Bossy-Wetzl *et al.*, 1998) and as illustrated by staurosporine evoked microglial apoptosis.

Thus following CGA treatment there is an induction of NOS, followed by glutamate release and mitochondrial depolarisation, and subsequent caspase activation resulting in apoptosis. The main section left to investigate would be the lag phase prior to NO release during which the signalling processes are unknown. This might involve phosphorylation events since initial experiments have shown that tyrosine kinase inhibitors prevent NO release and that a number of low molecular weight proteins are phosphorylated after CGA treatment. Previous studies have shown the importance of similar changes which can affect gene transcription and the level of neurotoxicity evoked by an activated microglial cell (Bhat *et al.*, 1998; Combs *et al.*, 1999).

Microglial apoptosis in culture can be prevented by inhibition at various points in the death cascade. Both inhibitors of NOS and glutamate release act at the level of the effector molecules, cyclosporin A acts at the mitochondrial level and caspase inhibitors during the execution phase of apoptosis. Whether or not prevention of apoptosis would be beneficial in treating diseases is dependent on the balance of factors being released by activated microglia. If the cells were secreting neurotoxic factors such as NO or glutamate then by blocking apoptosis it might potentiate the neurotoxic effect since there would be no mechanism to control the activated cells. Alternatively, preventing microglial apoptosis could aid regeneration if the cells were secreting high levels of growth factor.

6.3 Activated microglia in culture are toxic to neurones: a link between CGA and neurodegenerative disease?

Microglia may have both beneficial and detrimental effects on neuronal survival and function. They can either release neurotrophins and growth factors (Elkabes *et al.*, 1996) or contribute to the pathogenesis of neurodegeneration in diseases including Alzheimer's (McGeer and McGeer, 1996; Barger and Harmon, 1997) by secreting neurotoxic factors such as nitric oxide or glutamate (Chao *et al.*, 1992; Piani *et al.*, 1991). Much of the previous work has utilised LPS as microglial activator therefore it was interesting to see whether a molecule like CGA could act as a natural inflammatory molecule. Microglia were activated with CGA and the conditioned medium transferred to either cultures of primary cultures CGCs or a hippocampal cell line. This allowed the investigation of stable soluble factors that might be released following activation and meant that the role of NO could not be determined. It would be interesting to examine the effects of co-culture where NO would have an effect since it is known that NO donors induce apoptosis in CGCs (Bonfoco *et al.*, 1996). Nevertheless, the results showed that conditioned medium was toxic to neuronal cultures. The cell death was apoptotic in nature resulting in DNA fragmentation and involving caspase-3 activation. Apoptosis was originally believed to occur only in physiological conditions such as during development of the CNS (Raff *et al.*, 1993). For instance, during formation of the mature cerebellum a large number of granule cells are lost by apoptosis (Wood *et al.*, 1993). However, evidence has accumulated to suggest that apoptosis might be involved in the pathological conditions involved in neurodegenerative disorders such as Alzheimer's disease. Much of this work has come from studies of post-mortem tissue using TUNEL labelling to detect single stranded DNA breaks (Tatton *et al.*, 1998). Studies have shown that there is an increase in TUNEL staining in both neurones and glia in the AD brain (Smale *et al.*, 1995; Lassman *et al.*, 1995; Dragunow *et al.*, 1995).

Since this can only show the consequences of the disease, cell culture models can be used to elucidate the signalling pathways and molecules involved and may provide clues to therapeutic areas.

Thus it was shown that neurotoxicity was due to multiple factors secreted from activated microglia including both glutamate or glutamate receptor agonist and heat sensitive protein molecules. Glutamate released in the proximity of vulnerable neurones, which in this case might be neurones over expressing CGA, has been proposed as a cause of the neurotoxicity observed in numerous human diseases many of which are associated with activated microglia (Lipton and Rosenberg, 1994). Rather than glutamate itself it is possible that other molecules secreted by CGA activated microglia may act at the glutamate receptors. Quinolinic acid is released by activated microglia (Espey *et al.*, 1997) and is a known excitotoxic agonist of the NMDA receptor (Reinhard Jr *et al.*, 1994). Senile plaques can also stimulate microglia to release a neurotoxin with NMDA-R properties, similar to that found in Alzheimer brain (Giulian *et al.*, 1995). Additionally, microglia associated with Alzheimer plaques may release NAAG, which besides it role as mGluR agonist may also affect neurotransmission through NMDA-R (Sekiguchi *et al.*, 1992; Westbrook *et al.*, 1986). The presence of elevated levels of glutamate may be particularly important in CGCs which are a good model for studying excitotoxicity and provides further evidence that excitotoxins can in fact cause apoptosis rather than necrosis (Portera-Cailliau *et al.*, 1995; Pollard *et al.*, 1994). However, glutamate can also kill HT22 cells probably via oxidative mechanisms not involving the NMDA receptor suggesting that factors secreted by microglia may influence many types of neurone and effect different forms of death. Also, experiments using JC-1 showed that the same molecules responsible for neuronal apoptosis could either evoke mitochondria dependent or independent pathways depending on cell type.

To date there is little direct evidence for decreases in $\Delta\psi_m$ being responsible for human neurodegenerative disease. However, one recent study shows that mitochondria in fibroblasts taken from Parkinson's disease patients show significantly lower levels of $\Delta\psi_m$ suggesting mitochondrial defects might be responsible for the disease (communicated in Tatton and Olanow, 1999). Rather, most evidence comes from post-mortem analysis of diseased brain that suggests there may be changes in terms of mitochondrial complex activity (Reichmann *et al.*, 1993). Thus a more thorough investigation of this aspect of microglial evoked neuronal apoptosis is required. For instance it will be important to determine whether the permeability transition is operating and the nature of the molecules involved in caspase-3 activation may provide clues to how mitochondria are by-passed in some cell types.

The major component of the microglia evoked neurotoxicity is the protein molecule. The level of cytokines may be elevated in a number of conditions including Alzheimer's disease (Fillit *et al.*, 1991) and molecules such as TNF-α and IL-1 found at the sites of inflammation have been proposed to cause neuronal damage (Chao *et al.*, 1995). However, there is little evidence that they can directly cause neuronal damage and when CGCs were treated with either of these cytokines there was no affect on neuronal survival. Instead it was found that CGA activated microglia released cathepsin B a protease that has been hypothesised to play a role in disease (Cataldo and Nixon, 1990; Berlet and Ilzenhofer, 1985; Whitaker *et al.*, 1982). This was found to cause apoptosis in HT22 cells and displayed characteristics similar to that of conditioned medium.

Calcium imaging experiments in CGCs demonstrated that the conditioned medium evoked a small rise in $[Ca^{2+}]_i$ that was similar to the response observed with glutamate.

Increases in $[Ca^{2+}]_i$ might only be important in an excitotoxic model so further experiments are needed to determine the importance of this in other systems. Apoptosis in CGCs can be blocked with inhibitors of glutamate receptors, addition of BDNF which probably prevents glutamate toxicity and also a caspase-3 inhibitor, z-DEVD-fmk. Thus these might provide suitable therapies when treating neurodegenerative disease. Work from studies of glutamate toxicity *in vitro* has now been extended to finding clinically tolerated antagonists of glutamate receptors (reviewed in Lipton, 1993). Also at one time it was believed that neurotrophins held the answer to curing CNS disease. However, with studies showing that some growth factors such as NGF can in fact themselves cause apoptosis it will be necessary to find the correct balance between factors that promote and suppress apoptosis. This leaves caspase inhibitors as the most likely source to treat disease. To date there are very few effective inhibitors of proteases in the clinic. This is because it is very difficult to produce a non-peptide molecule small enough to penetrate membranes, which is also specific enough to target a particular enzyme. Studies of caspase inhibition *in vitro* will aid the design process. Nevertheless, whether anti-apoptotic therapy would be suitable for treating chronic disease remains to be seen. Since neuronal death by apoptosis may occur as a protective mechanism to minimise further inflammatory reactions the long term effects of caspase inhibitors are unknown. For instance, a recent study of microglial induced neuronal apoptosis showed that blockade of a caspase-3 dependent pathway shifted cell death to a non apoptotic route (Tanabe *et al*, 1999). Interestingly, this model used Bcl-2 over expression which to date is probably the most advanced of all techniques aiming to treat neurodegeneration.

6.4 General conclusions

A scheme for the role that CGA might play in neurodegeneration is summarised in Fig. 6.4.1. During diseases such as Alzheimer's, certain neurones may accumulate elevated levels of CGA (Lassmann *et al.*, 1992) such that they cause neighbouring microglia to become activated. This results in the induction of NOS and the release of glutamate receptor agonists and cathepsin B. Glutamate may have diverse effects both affecting microglial apoptosis which in the presence of NO proceeds via mitochondrial depolarisation or causing neuronal apoptosis. Cathepsin B can also directly influence neuronal survival. These processes result in the destruction of the neurones containing elevated levels of CGA and act to control the level of microglial activation thus preventing excessive neuronal loss.

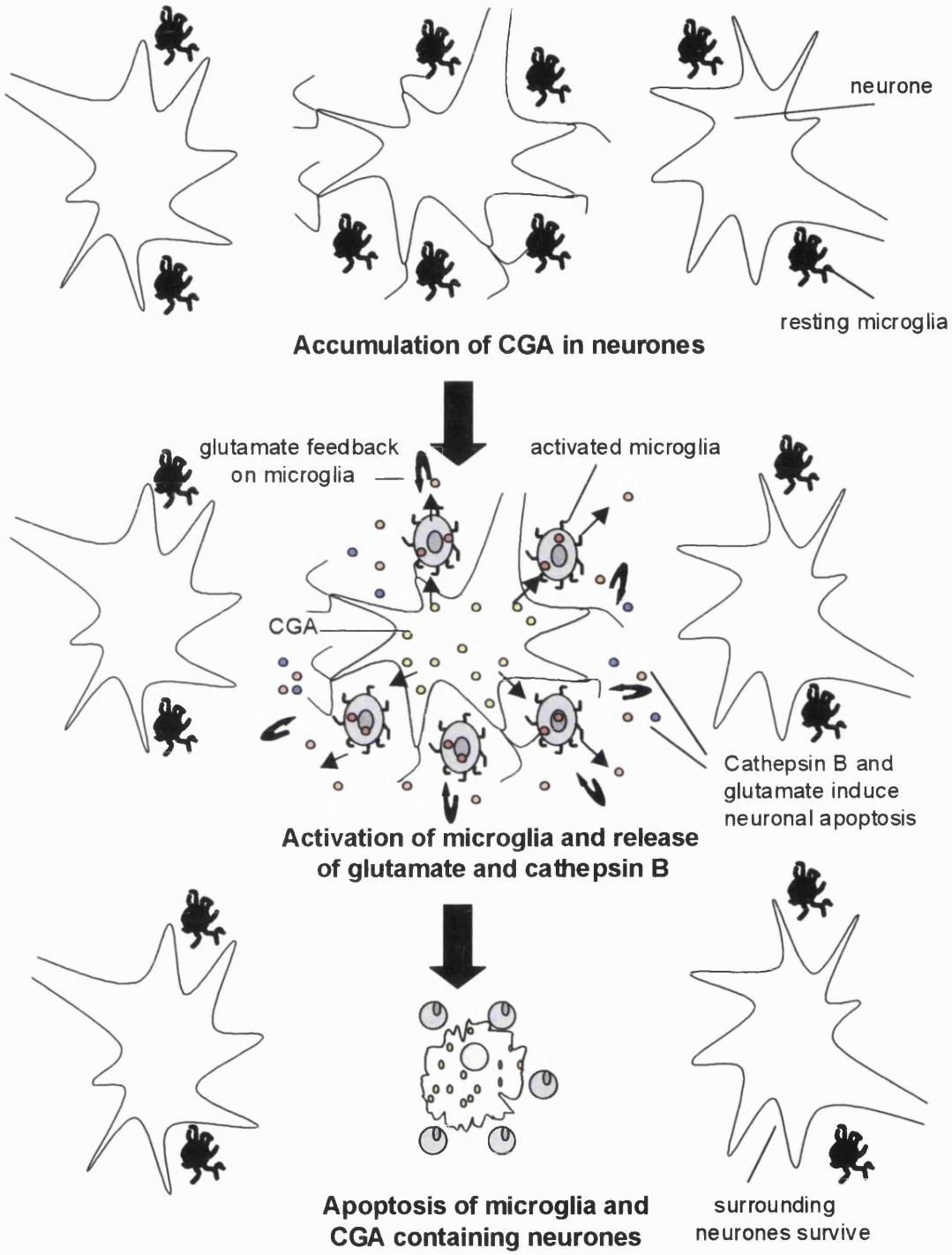


Fig. 6.4.1 Schematic of the role CGA may play in neurodegenerative disease. See text for details.

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Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Apoptotic Pathways Mobilized in Microglia and Neurones as a Consequence of Chromogranin A-Induced Microglial Activation

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Abstract: Senile plaques of Alzheimer's brain are characterized by activated microglia and immunoreactivity for the peptide chromogranin A. We have investigated the mechanisms by which chromogranin A activates microglia, producing modulators of neuronal survival. Primary cultures of rat brain-derived microglia display a reactive phenotype within 24 h of exposure to 10 n*M* chromogranin A, culminating in microglial death via apoptotic mechanisms mediated by interleukin-1β converting enzyme. The signalling cascade initiated by chromogranin A triggers nitric oxide production followed by enhanced microglial glutamate release, inhibition of which prevents microglial death. The plasma membrane carrier inhibitor aminoadipate and the type II/III metabotropic glutamate receptor antagonist (*RS*)-α-methyl-4-sulphonophenylglycine are equally protective. A significant amount of the released glutamate occurs from bafilomycin-sensitive stores, suggesting a vesicular mode of release. Inhibition of this component of release affords significant microglial protection. Conditioned medium from activated microglia kills cerebellar granule cells by inducing caspase-3-dependent neuronal apoptosis. Brain-derived neurotrophic factor is partially neuroprotective, as are ionotropic glutamate receptor antagonists, and, when combined with boiling of conditioned medium, full protection is achieved; nitric oxide synthase inhibitors are ineffective.
Key Words: Macrophages—Caspases—Alzheimer's disease—Neurotoxicity—Cerebellar granule cell.
J. Neurochem. **73,** 538–547 (1999).

There is increasing evidence of a closely coupled bidirectional interaction between neuronal and glial signalling systems (Giulian et al., 1993; Tsacopoulos et al., 1997; Bezzi et al., 1998; D'Ambrosio et al., 1998). Microglia are the resident macrophages of the CNS and play an important role in determining neuronal survival and differentiation by releasing neurotrophins and stimulating their production from other glial cell types (Giulian et al., 1993). Microglia act as the immunoeffector cells of the CNS, killing invading microorganisms and removing dead and dying cells (Kreutzberg, 1996). In contrast, microglia may also contribute to the pathogenesis of neurodegeneration, for example, in multiple sclerosis (Diemel et al., 1998), Alzheimer's disease (McGeer and McGeer, 1996; Barger and Harmon, 1997), the dementia associated with AIDS (Giulian et al., 1996), and the delayed neuronal death following an ischaemic insult (Lees, 1993). Under in vitro conditions, microglia can be activated by exposure to lipopolysaccharides (LPSs) and proinflammatory cytokines (Chao et al., 1995) and may release nitric oxide (NO), arachidonic acid, quinolinic acid, cytokines, and other unidentified small-molecular-weight toxins acting through the *N*-methyl-D-aspartate (NMDA) receptor.

Recently the secretory protein chromogranin A (CGA) has been shown to be a novel activator of microglia (Taupenot et al., 1996). Expression of CGA is up-regulated in numerous neurodegenerative diseases and is particularly prominent in the senile plaques associated with Alzheimer's disease (Munoz, 1991; Yasuhara et al., 1994). Activated microglia are the predominant glial cell associated with senile plaques (Haga et al., 1989; Itagaki et al., 1989; Larner, 1997). Exposure to β-amyloid, the predominant peptide associated with senile plaques, leads to microglial activation (Meda et al., 1995) and the production of superoxide radicals, which can generate a local inflammatory response (McDonald et al., 1997). Similarly, the activation of microglia with CGA has been shown to cause neuronal degeneration, although no direct link between the release of NO and neuronal degeneration could be established (Ciesielski-Treska et al.,

Received December 23, 1998; revised manuscript received April 12, 1999; accepted April 12, 1999.

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Abbreviations used: Adp, L-α-aminoadipic acid; AMT-HCl, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride; BDNF, brain-derived neurotrophic factor; CGA, chromogranin A; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DIV, days in vitro; GFAP, glial fibrillary acidic protein; iGluR, ionotropic glutamate receptor; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; INT, iodonitrotetrazolium; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; mGluR, metabotropic glutamate receptor; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate; MSPG, (*RS*)-α-methyl-4-sulphonophenylglycine; L-NNA, N^G-nitro-L-arginine; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NO_2^-, nitrite; NOS, nitric oxide synthase; YVAD-CHO, Tyr-Val-Ala-Asp-aldehyde; z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone.

1998). In this study we have investigated the signalling pathways mobilized in both microglia and neurones as a consequence of CGA-induced microglial activation.

MATERIALS AND METHODS

Materials

Foetal calf serum and minimum essential medium with Earle's salts were obtained from Life Technologies Ltd. (Paisley, U.K.). Percoll was obtained from Pharmacia, and tissue culture plasticware was obtained from Greiner (Gloucestershire, U.K.). N^G-Nitro-L-arginine (L-NNA) and the caspase inhibitors benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD-FMK) and Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO) were purchased from Calbiochem-Novabiochem (Nottingham, U.K.). 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT-HCl) and (*RS*)-α-methyl-4-sulphonophenylglycine (MSPG) were purchased from Tocris (Bristol, U.K.). CGA was from Scientific Marketing Associates (Herts, U.K.). Calcein acetoxymethyl ester, ethidium homodimer, and fura-2 acetoxymethyl ester were purchased from Molecular Probes. The glutamate assay kit was purchased from Boehringer Mannheim (Lewes, U.K.). OX-42 (IgG1) was from Dako (Ely, U.K.), ED1 (IgG1) was from Serotec (Oxford, U.K.), and glial fibrillary acidic protein (GFAP; monoclonal clone no. G-A-5) was from Sigma (Dorset, U.K.). All other chemicals were purchased from Sigma. Animals were killed by cervical dislocation and decapitation in accordance with the Scientific Procedures Act 1986.

Cerebellar granule cell culture

Cerebellar granule neurones were isolated from 7-day-old rat pups and prepared as previously described (Pocock et al., 1993). Cells were plated on 13-mm poly-D-lysine-coated glass coverslips at a density of 0.25×10^6 per coverslip and maintained in minimum essential medium with Earle's salts supplemented with 25 m*M* KCl, 30 m*M* glucose, 25 m*M* $NaHCO_3$, 1 m*M* glutamine, 10% foetal calf serum, and penicillin/streptomycin. After 24 h in vitro 10 μ*M* cytosine furanoarabinoside was added to prevent proliferation of nonneuronal cells. The cultures were maintained at 37°C in 5% CO_2, and the medium was changed on day 6. Cells were used at 10–12 days in vitro (DIV).

Microglial cell culture

Microglial cells were isolated from 7-day-old rat pups by a modification of methods previously described (Ford et al., 1995; Slepko and Levi, 1996). Brains were removed, chopped, and enzymatically dissociated with 1 mg of type XI collagenase and 100 U of DNase I. After a 45-min digestion the cells were washed and transferred to a discontinuous Percoll gradient consisting of 2-ml steps of the following densities: 1.088, 1.072, 1.065, and 1.03 g/ml. The gradient was centrifuged at 1,250 *g* for 20 min at 20°C, and cells were collected from the 1.065–1.072 g/ml interface. After washing, the cells were plated onto poly-D-lysine-coated coverslips at a density of 2.0×10^5 cells. Cultures were maintained at 37°C in 5% CO_2, and the medium was changed at 24 h after plating.

Immunocytochemistry

The purity of the isolated microglia was monitored immunocytochemically with OX-42 (an anti-CR3 complement receptor antibody), ED1 (a marker for activated microglia), or anti-GFAP antibody (an astrocytic marker). The cells were fixed and stained with OX-42, ED1, or anti-GFAP (all at 1:200 dilutions) and visualized by biotinylated anti-mouse IgG antibody (1:200 dilution), preformed avidin–biotin–horseradish peroxidase complex, and diaminobenzidine tetrahydrochloride according to previous methods (Hayes et al., 1988). Cerebellar granule cell cultures of 10 DIV were also stained with OX-42 to determine if any microglia were present in these cultures.

Treatment of cultures and assessment of cell viability

Microglial cell cultures were activated at 1–2 DIV by addition of CGA to the culture medium. After 24 h, microglial viability was assessed using a double stain of calcein acetoxymethyl ester (5 μ*M*) and ethidium homodimer (2.5 μ*M*) (Bonfoco et al., 1996). The numbers of live cells with intact plasma membranes (calcein-positive) and of dead cells with permeable membranes (ethidium-positive) were scored in 10 fields per coverslip at a magnification of 40×, on at least two separate cultures, and expressed as a percentage of control values. Conditioned medium was removed from microglial cultures activated with CGA, filtered using a cellulose acetate filter (pore size, 0.2 μm) to remove cellular material, and subsequently transferred to cerebellar granule cells. A further 24 h later neuronal viability was assessed using calcein and ethidium staining.

The number of apoptotic cells was assessed by Hoechst 33342 (bis-benzimide trihydrochloride) staining. Cells were washed in phosphate-buffered saline, fixed for 10 min in 4% paraformaldehyde at 4°C, washed once in distilled water, and stained with Hoechst 33342 (5 μg/ml) for 10–15 min. Nuclear morphology was viewed using a fluorescent microscope with excitation at 365 nm and emission at >490 nm. Apoptotic cells contained small brightly stained nuclei compared with controls (Yan et al., 1994).

DNA laddering

Following treatment of microglia with 10 n*M* CGA for 24 h and of cerebellar granule cells with conditioned medium from microglia for 24 h, cells were lysed in situ, and the DNA was prepared according to the method of Laird et al. (1991). Cells were washed in phosphate-buffered saline and lysed in 1 ml of lysis buffer (100 m*M* Tris-HCl, 5 m*M* EDTA, 0.2% sodium dodecyl sulphate, 200 m*M* NaCl, and 100 μg/ml proteinase K, pH 8.5) for 1 h at 55°C. DNA was precipitated by 1 ml of isopropanol and continuous shaking for 1 h at room temperature. DNA was pelleted by centrifugation at 14,000 rpm for 15 min, and the pellet was resuspended in TE buffer (10 m*M* Tris-HCl and 0.1 m*M* EDTA, pH 7.5). The DNA concentration was determined spectrophotometrically, and DNA (1 μg) was loaded onto a 1% agarose minigel in Tris-borate buffer for 2 h at 125 V. Bands were visualized by ethidium bromide staining.

Measurement of lactate dehydrogenase (LDH) activity in culture supernatants

Levels of LDH in cell supernatants were measured by a colorimetric assay following the conversion of iodonitrotetrazolium (INT) to formazan in the presence of excess lactate according to published methods (Klegeris and McGeer, 1997). Medium from control cells was used as a blank, and a standard curve was constructed by adding known amounts of LDH to culture medium.

Nitrite (NO_2^-) quantification

Nitrate was converted to NO_2^- before Griess assay by incubation with nitrate reductase (0.025 U/ml) and βNADPH (100 μ*M*) for 15 min at 37°C according to the method of Green

et al. (1982). The efficiency of conversion of nitrate to NO_2^- was determined against a standard nitrate curve. To determine NO_2^- concentration, Griess reagent was combined with an equal volume of supernatant from CGA-exposed or nonexposed microglial cell cultures and incubated for 15–30 min at room temperature (Ding et al., 1988). The optical density of this solution was recorded at 540 nm and adjusted for background levels of NO_2^- in the medium.

Measurement of glutamate content of microglial-conditioned medium

Supernatants were assessed for levels of glutamate by a colorimetric method coupled to glutamate dehydrogenase and a formazan end product using a commercially available kit (Boehringer Mannheim). In brief, diaphorase, INT, and conditioned medium were combined in a 96-well plate and incubated for 2 min. Then 2.7 U of glutamate dehydrogenase solution was added, and the absorbance was measured at 450 nm every 3 min until the reaction reached steady state. Medium that had not been exposed to cells was used as a blank. A standard curve was constructed by adding known concentrations of glutamate to culture medium.

RESULTS

Morphological and immunoreactive markers of microglial activation

The average number of microglial cells recovered from 7-day-old rat pups was 1×10^6 cells/g of tissue wet weight. Immediately after plating, the cells were small and rounded, but after 24 h in culture they increased in size and assumed a resting, ramified morphology (Fig. 1A), in accordance with previous reports (Slepko and Levi, 1996). The cells stained positive for OX-42 immunoreactivity (Table 1), suggesting a highly enriched culture of microglia. The majority of the cells did not stain with ED-1 (Table 1), suggesting a resting phenotype. There was no detectable immunoreactivity for GFAP at 2 DIV and <2% by 4 DIV, suggesting minimal astrocyte contamination of these cultures (Table 1). Following incubation with 10 n*M* CGA for 12 h, microglia became activated as indicated by their altered morphology and immunoreactivity for ED-1 (Table 1). Cultures of 12 DIV cerebellar granule cells revealed little immunoreactivity for OX-42 or GFAP (Table 1), indicating that few microglia or astrocytes were present in these cultures.

Microglial activation results in microglial death by apoptosis

Cultures of resting microglia contained a large number of viable cells (Fig. 1A); 91 ± 1.6% of the cells stained positive with calcein (Fig. 1A and C), and 9 ± 1.6% stained positive with ethidium (Fig. 1B and C). Activation of microglia with CGA was time- and concentration-dependent, with maximal death at a concentration of 10 n*M* CGA (Fig. 2A). After 24 h of exposure to 10 n*M* CGA, the cells lost their processes and became round (Fig. 1D). Cell death increased, reducing the number of live cells to 42 ± 0.9% of the total (Fig. 1D and F), providing a window to determine whether modulation of

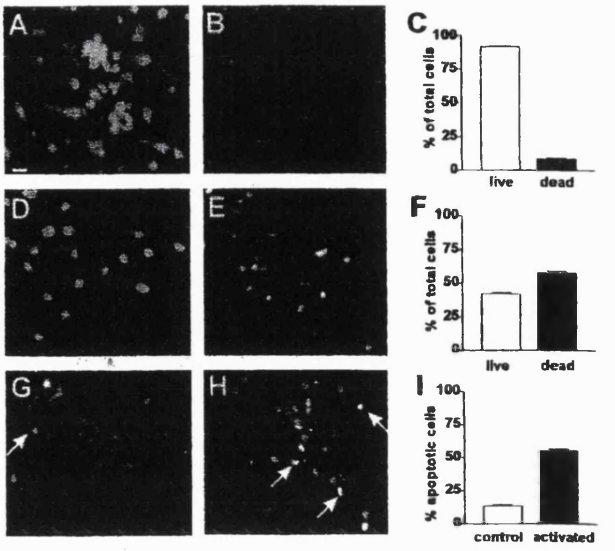

FIG. 1. CGA is toxic to primary cultures of rat microglia. **A** and **B:** Control cells stained with calcein acetoxymethyl ester and ethidium, respectively. Cells display a resting morphology (**A**). **C:** Number of cells as a percentage of the total cell number that stained positive for calcein (live) or positive for ethidium (dead). After a 24-h exposure to 10 n*M* CGA, cells display a rounded morphology (**D**), an increased number stain with ethidium (**E**), and the ratio of live to dead cells falls (**F**). To quantify apoptotic microglia, cells were stained with Hoechst 33342, and the number of brightly stained, pyknotic nuclei was scored: (**G**) control cells and (**H**) cells exposed to 10 n*M* CGA for 24 h. **I:** Number of apoptotic cells as a percentage of the total cell number in control and activated (with 10 n*M* CGA, for 24 h) cultures of microglia. Six coverslips per condition with 10 discrete areas each were scored from three independent experiments. Data are mean ± SEM (bars) values. Bar = 20 μm.

microglial signalling pathways offered protection or further exacerbated microglial death (Fig. 1E and F).

Nonactivated and activated microglia were stained with Hoechst 33342, to allow the identification of cells with pyknotic nuclei, a characteristic of apoptosis (Kerr et al., 1972). Cultures of nonactivated microglia contained relatively few apoptotic nuclei at 2 DIV; 11 ± 2.3% were apoptotic (Fig. 1G). On activation for 24 h with 10 n*M* CGA, the number of apoptotic nuclei increased to 53 ± 2% of the total (Fig. 1H). Analysis of DNA revealed that exposure of microglia to 10 n*M* CGA for 24 h induced extensive DNA laddering indicative of apoptosis (Wyllie, 1980) (Fig. 2B).

The activation of cellular caspases is a prerequisite for the promotion of apoptosis (Lazebnik et al., 1991). The involvement of two caspases, caspase-1 [or interleukin-1β (IL-1β)-converting enzyme] and caspase-3 (or CPP32), was investigated using specific peptide inhibitors of these enzymes, YVAD-CHO and z-DEVD-FMK, respectively (Eldadah et al., 1997; Hilbi et al., 1997). Preincubation of microglia with 100 μ*M* YVAD before stimulation with 10 n*M* CGA significantly prevented microglial death, whereas 50 μ*M* z-DEVD-FMK was ineffective (Fig. 2C).

TABLE 1. *Immunoreactivity of microglial and cerebellar granule neuronal cultures*

Cell type	OX-42 antibody	ED-1 antibody	GFAP antibody
Unstimulated microglia, 2 DIV	95 ± 3.5	18.77 ± 3.29	ND
Activated microglia (12 h, 10 n*M* CGA), 2 DIV	—	92.78 ± 1.89	—
Unstimulated microglia, 4 DIV	92.3 ± 1.1	—	1.65 ± 0.32
Cerebellar neurones, 4 DIV	2.08 ± 0.48	—	2.85 ± 0.85
Cerebellar neurones, 12 DIV	0.95 ± 0.34	—	3.50 ± 2.01

Microglia and cerebellar granule cell cultures were stained for immunoreactivity to OX-42 (a microglial marker), ED1 (a marker for activated microglia), and GFAP (a marker for astrocytes). Data are mean ± SEM values, expressed as the percentage of the cell number in 10 discrete fields in at least two independent cultures that stained with the respective primary antibody following visualization using avidin–biotin peroxidase. ND, not detected.

Microglial activation stimulates NO production

Microglia cells produced NO when exposed to 0.1–100 n*M* CGA (Fig. 2D). Production of NO_2^- was abolished by pretreatment with cycloheximide (Fig. 2E), as was microglial death (Fig. 2F). The increase in microglial death following exposure to CGA for 24 h was proportional to the CGA-induced NO_2^- production (Fig. 2A and D). The NO synthase (NOS) inhibitor L-NNA completely attenuated this cell death, and the specific inducible NOS (iNOS) inhibitor AMT-HCl (150 n*M*) was equally protective (Fig. 2F).

CGA activation stimulates microglial glutamate release

Conditioned medium from microglia activated for 24 h with 0.1–100 n*M* CGA contained increasing amounts of glutamate from a baseline of 37 ± 3.2 μg/mg of protein to a maximum of 139 ± 22.2 μg/mg of protein following exposure to CGA (Fig. 3A). The induction of NOS activity preceded the onset of increased glutamate in the medium, which furthermore preceded the onset of significant microglial death (Fig. 3B). By 21 h glutamate release was 148 ± 14.15% above baseline control levels. Because no significant LDH release and no significant propidium iodide uptake were measurable at this time (Fig. 3B), this suggests that glutamate release is not due to cell lysis. Furthermore, the production of glutamate required new protein synthesis because it could be blocked by 1 μg/ml cycloheximide to a level of 57.9 ± 6 μg/mg of protein, suggesting the induction of a regulated pathway.

Attenuation of microglial cell death and glutamate release

Glutamate release was significantly attenuated by preincubation with the iNOS inhibitor AMT-HCl and with the general NOS inhibitor L-NNA (Fig. 3C). Propentophylline, an inhibitor of microglial activation and suppressor of free radical generation, significantly attenuated glutamate release. Significant attenuation of release was achieved by preincubation with L-α-aminoadipic acid (Adp; 2.5 m*M*), an inhibitor of the cystine/glutamate antiport (Piani and Fontana, 1994), suggesting the involvement of the low-affinity sodium-independent transporter x_c^- pathway (Watanabe and Bannai, 1987). The possible involvement of vesicular glutamate release was investigated with bafilomycin A_1, an inhibitor of the vesicular H^+-ATPase (Bowman et al., 1988). This compound was toxic when added for the full 24 h of CGA exposure and so was added at 16 h, before any significant glutamate release (Fig. 3B). Bafilomycin (1 μ*M*) caused a small but significant inhibition of glutamate release (Fig. 3C). The ionotropic glutamate receptor (iGluR) antagonists (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not significantly reduce glutamate release (Fig. 3C). Likewise, the type II/III metabotropic glutamate receptor (mGluR) antagonist MSPG did not significantly modulate release (Fig. 3C).

Adp or propentophylline significantly attenuated CGA-induced microglial death (Fig. 3D), as did bafilomycin, although to a lesser extent. The iGluR antagonists MK-801 (10 μ*M*) and CNQX (20 μ*M*) did not afford significant protection (Fig. 3D). However, the nonspecific mGluR antagonist MSPG (200 μ*M*) was protective (78.8 ± 13.7%).

Activated microglia induce neuronal apoptosis

Microglia were activated with a range of CGA concentrations, and conditioned medium was added to cultures of cerebellar granule cells. At 24 h later the viability of cerebellar granule neurones was assessed. Cultured cerebellar granule neurones at 10–12 DIV extend neurites (Fig. 4A) and contain approximately 10% dead cells (Fig. 4B and C). When neurones were exposed to supernatant from nonactivated microglia, neuronal morphology (Fig. 4D) and degree of cell death (15 ± 1.5%; Fig. 4E and F) were not significantly different from controls. A 24-h exposure to supernatant from activated microglia resulted in the loss of thick cablelike neurites (Fig. 4G) and a significant increase in neuronal death (46.24 ± 1.9% live cells; Fig. 4H and I). The effect of transferring nonactivated microglial-conditioned medium and applying 10 n*M* CGA directly to the neurones did not in itself result in a significant change in viability (91.7 ± 5.1%), compared with control neurones (89.4 ± 1.6%). In control cultures not exposed to microglial-conditioned medium, 3.5 ± 1.5% of the cells were apoptotic (Fig. 4J and L); this was not significantly increased in cultures exposed to nonactivated microglial medium

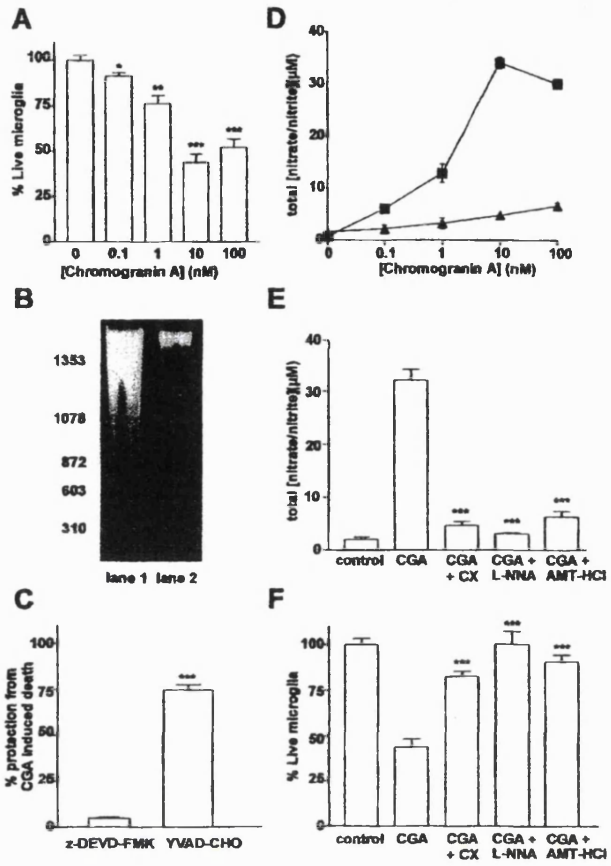

FIG. 2. CGA induces microglial apoptosis that is dependent on NOS induction. **A:** Microglial viability decreased with increasing concentrations of CGA, plateauing at 50% with 10–100 n*M* CGA. In all subsequent experiments 10 n*M* CGA was used. Data are normalized to the control as 100% survival and are mean ± SEM (bars) values (n = 60). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **B:** DNA was extracted from microglia exposed to 10 n*M* CGA for 24 h and separated on a 1% agarose gel for 2 h at 125 V. DNA molecular weight markers (bp) are shown to the left. Lane 1, DNA from CGA-exposed microglia; lane 2, DNA from CGA-exposed microglia preincubated with 100 μ*M* YVAD-CHO. **C:** Cells were preincubated with 50 μ*M* z-DEVD-FMK or 100 μ*M* YVAD-CHO for 1 h before exposure to 10 n*M* CGA for 24 h. The number of live cells (staining positive with calcein acetoxymethyl ester) to dead cells (staining positive with ethidium) was scored in 40 discrete areas in at least four different coverslips from two independent experiments. Data are mean ± SEM (bars) values, expressed as the percent increase in the number of live cells above the number of live cells present in cultures exposed to 10 n*M* CGA (n = 40 separate fields of cells). $^{***}p < 0.001$. The z-DEVD-FMK effect was not significantly different from CGA-treated cultures. **D:** NO release was determined with Griess reagent by estimation of total NO_2^- levels. NO release was measured from microglia in unstimulated cultures (▲) or those exposed to CGA (0.1–100 n*M*) for 24 h (■). Data are mean ± SEM (bars) values of duplicate determinations of two different cell preparations (n ≥ 4). **E:** Preincubation of cells with 1 μg/ml cycloheximide (CX), L-NNA (1 m*M*), or AMT-HCl (150 n*M*) for 1 h before exposure to 10 n*M* CGA maintained NO levels at those observed in control, untreated cells (control). **F:** Preincubation of cells with CX as in E or the NOS inhibitors L-NNA (1 m*M*) and AMT-HCl (150 n*M*) for 1 h prevented subsequent cell death following a 24-h exposure to 10 n*M* CGA. Data are mean ± SEM

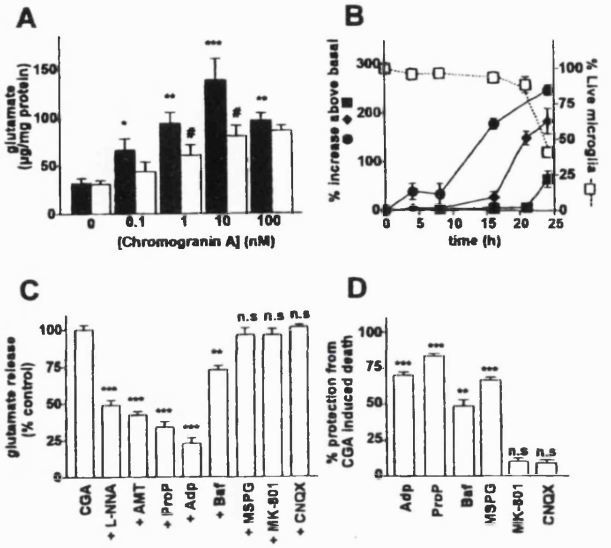

FIG. 3. CGA-activated microglia release glutamate. **A:** Following exposure for 24 h to various concentrations of CGA, supernatants from microglia treated alone (solid columns) or in the presence of 1 m*M* L-NNA (open columns) were assayed for glutamate using the glutamate dehydrogenase diaphorase assay. Data are mean ± SEM (bars) values (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$ significantly different from respective control at the same concentration of CGA but in the presence of 1 m*M* L-NNA. **B:** Cells were exposed to 10 n*M* CGA from 0 to 24 h, and cells were assayed for viability using calcein/ethidium staining (□), or supernatants were assayed for total NO_2^- levels using Griess reagent (●), glutamate levels using a glutamate dehydrogenase diaphorase assay (◆), or release of LDH by following the formation of formazan (■). **C:** Glutamate release from microglia exposed to 10 n*M* CGA was measured in cells in the presence of 1 m*M* L-NNA, 150 n*M* AMT-HCl (AMT), 50 μ*M* propentophylline (ProP), 2.5 m*M* Adp, 1 μ*M* bafilomycin A_1 (Baf), 200 μ*M* MSPG, 10 μ*M* MK-801, or 20 μ*M* CNQX. Data are mean ± SEM (bars) values, expressed as the percentage of the released glutamate from cells in the presence of 10 n*M* CGA (n ≥ 4 coverslips). $^{**}p < 0.01$, $^{***}p < 0.001$; n.s, not significantly different from control (CGA) release. **D:** The degree of microglial death on exposure to 10 n*M* CGA for 24 h was assessed in the presence of 2.5 m*M* Adp, 50 μ*M* ProP, 1 μ*M* Baf, 200 μ*M* MSPG, 10 μ*M* MK-801, or 20 μ*M* CNQX. Data are mean ± SEM (bars) values, expressed as the percentage of cells surviving above baseline death in the presence of 10 n*M* CGA (0% protection) (n = 40). $^{**}p < 0.01$, $^{***}p < 0.001$; n.s, not significantly different from control (CGA) survival.

(4.81 ± 1.7%) or in cells in which fresh medium was added (4.5 ± 2.4%; Fig. 4L). In neuronal cultures exposed to activated microglial medium (with 10 n*M* CGA), 57.4 ± 6.7% of the cells were apoptotic (Fig. 4K and L).

The degree of neuronal death was proportional to the CGA concentration used to activate the microglia (from

(bars) values (n = 40 separate fields of cells from at least four different coverslips from two separate experiments). $^{***}p < 0.001$, significance levels when compared with cell death in cultures exposed to 10 n*M* CGA.

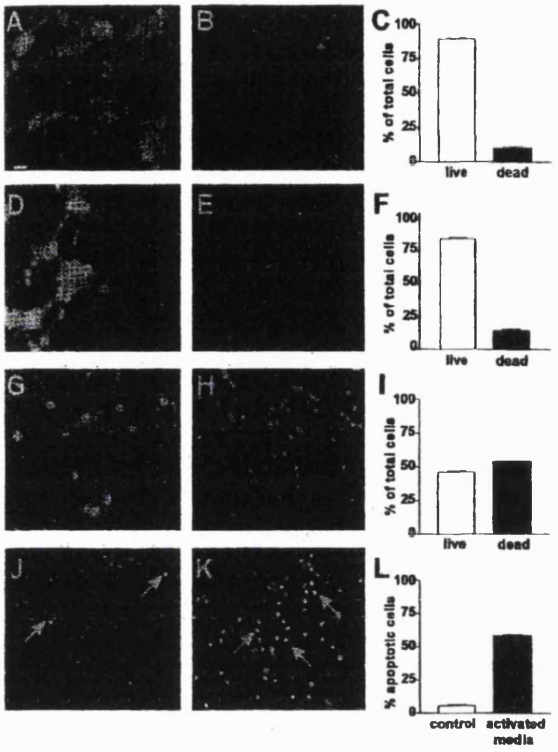

FIG. 4. Conditioned medium from activated microglia induces cerebellar granule cell death. **A:** Cerebellar granule cells at 10 DIV stained with calcein display an extensive network of thick cable neurites. The corresponding field of neurones stained with ethidium to reveal dead neurones (**B**) indicates that ~10% of the cells are dead (**C**). **D** and **E:** Neurones exposed to conditioned medium from nonactivated microglia display similar viability (**F**) to controls. Neurones exposed to conditioned medium from microglia exposed to 10 n*M* CGA for 24 h reveal an extensive loss of neurites (**G**) and decreased neuronal viability (**H** and **I**). The number of cells expressed as a percentage of the total cell number that stained positive for calcein (live) or ethidium (dead) are displayed (C, F, and I). Data are mean ± SEM (bars) values (n = 60 separate fields of cells). Cultures of cerebellar granule neurones were stained with Hoechst 33342 dye to indicate pyknotic nuclei. Neurones unexposed to microglial-conditioned medium (**J**) display few apoptic nuclei (arrows), whereas neurones exposed for 24 h to conditioned medium from microglia activated with 10 n*M* CGA for 24 h (**K**) display numerous brightly staining condensed nuclei (arrows). **L:** The number of cells displaying pyknotic nuclei expressed as a percentage of the total cell number that stained with Hoechst 33342 was assessed in 60 separate fields of cells in control cultures (defined as neurones exposed to nonactivated microglial medium) and those exposed to conditioned medium from microglia activated with 10 n*M* CGA for 24 h (activated medium). Data are mean ± SEM (bars) values. Bar = 20 μm.

0.1 to 10 n*M*), with maximal death of 49.9 ± 1.61% when 10 n*M* CGA was used to activate microglia (Fig. 5A). To gain insight into the point at which microglial activation leads to the production of neurotoxins, we activated microglia with 10 n*M* CGA and then removed conditioned medium over a 0–24-h period. This medium was then incubated with cerebellar granule cells for 24 h (Fig. 5B). The onset of release of the neurotoxin(s) was essentially immediate on microglial exposure to CGA because significant neuronal death was evident following 10 h of microglial activation (Fig. 5B). The rate of neuronal death significantly increased at ~18 h of microglial activation, suggesting that microglial death and glutamate release may contribute to neuronal death. The degree of neuronal death was dependent on the percentage of activated microglial-conditioned medium to which the neurones were exposed; increasing volumes of supernatant from CGA-activated microglia resulted in a maximal loss of 67.4 ± 2.9% cells following a 24-h exposure (Fig. 5C). Furthermore, the number of apoptotic neurones correlates with the number of dead neurones observed with ethidium (Fig. 5C), and analysis of DNA revealed significant DNA laddering in neurones exposed to CGA-activated medium (Fig. 5E, lane 1). Increasing times of exposure of cerebellar granule neurones to conditioned medium from CGA-exposed microglia led to increasing neuronal death from 50% death at 24 h to ~80% death at 72 h (Fig. 5D). We therefore used a 24-h exposure for the majority of experiments because this allowed determination of a protective or detrimental effect on neuronal survival of pharmacological intervention.

Mechanism of microglial-induced neuronal apoptosis

Inhibition of microglial NO production with L-NNA or AMT-HCl resulted in 11.7 ± 3.4 or 13.5 ± 3.1% protection, respectively, above baseline neuronal death (Fig. 5F), indicating that the predominant neurotoxin is not dependent on NO production from microglia. The combined effect of the NMDA receptor antagonist MK-801 and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist CNQX significantly reduced neuronal death by 37.3 ± 2.6%, indicating that both types of receptor are activated. Alone these antagonists attenuated neuronal death by 25 ± 2% (MK-801) and 10 ± 3% (CNQX). Activation of neuronal NMDA receptors can trigger the constitutive isoform of neuronal NOS to synthesize NO, augmenting cell death (Garthwaite et al., 1989). However, preincubation of cerebellar granule cells with L-NNA before incubation with conditioned medium from CGA-stimulated microglia was not neuroprotective (Fig. 5F). Boiling the activated supernatant for 1 h afforded significant protection (70 ± 3.2%) above basal, and when this was combined with MK-801 plus CNQX, neurones were completely protected.

Apoptotic pathways leading to microglial-induced neuronal death

Exposure of cerebellar granule cells to brain-derived neurotrophic factor (BDNF; 10 ng/ml) for 1 h before addition of activated microglial-conditioned medium resulted in significant protection (49.4 ± 3.5% above basal death; Fig. 5F). BDNF in combination with MK-801 and CNQX did not significantly increase neuronal survival (52.85 ± 5%) above that evoked with BDNF alone. The

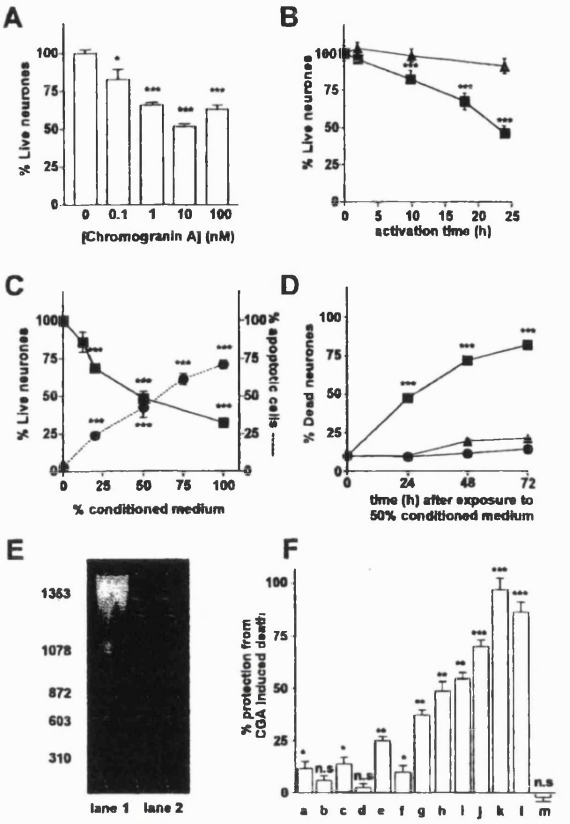

FIG. 5. Characterization of cerebellar granule cell death induced by CGA-activated microglia. **A:** The neurotoxicity of conditioned medium from CGA-activated microglia is dependent on the concentration of CGA to which the microglia are exposed. Data are mean ± SEM (bars) values (n = 60 separate fields of cells). $^{*}p < 0.05$, $^{***}p < 0.001$. **B:** Conditioned medium from nonactivated microglia (▲) or from microglia exposed to 10 n*M* CGA (■) was collected from 0 to 24 h following exposure and added to cultured cerebellar granule cells at a 50:50 ratio. Neuronal viability was assessed 24 h later using calcein/ethidium staining. Data are mean ± SEM (bars) values (n = 60 separate fields of cells). **C:** Neurones were exposed to increasing amounts of conditioned medium from microglia activated with 10 n*M* CGA for 24 h (■). Apoptotic neurones are indicated (●). Data are mean ± SEM (bars) values (n = 60 separate fields of cells). **D:** Neuronal viability was assessed between 24 and 72 h following exposure to nonactivated microglial supernatant (▲) or activated supernatant from microglia exposed to 10 n*M* CGA (■). Control, untreated neurones are also shown (●). Data are mean ± SEM (bars) values (n = 40 separate fields of cells). **E:** DNA from cerebellar granule cells was extracted and separated on a 1% agarose gel for 2 h at 125 V. Lane 1, cells exposed to 50% conditioned medium from 10 n*M* CGA-exposed microglia; lane 2, cells preincubated with 50 μM z-DEVD-FMK before exposure to conditioned medium from 10 n*M* CGA-exposed microglia. DNA molecular weight markers (bp) are shown to the left. **F:** Attenuation of neuronal death induced by conditioned media from CGA-exposed microglia. Conditioned medium from microglia exposed to 10 n*M* CGA for 24 h was added to cerebellar granule cells at a ratio of 50:50 conditioned medium:neuronal medium. Neuronal viability (as assessed with calcein/ethidium staining) was determined 24 h later and expressed as a percentage of the survival above that in cultures exposed to 50% conditioned medium without drugs. Neurones were incubated with the following compounds during exposure to microglial-conditioned medium: (a) 1 m*M* L-NNA (pre), added to microglia before CGA-induced activation; (b) 1 m*M* L-NNA (post), added to neurones only; (c) 150 n*M* AMT-HCl (pre), added to microglia before CGA-induced activation; (d) 150 n*M* AMT-HCl (post), added to neurones only; (e) 10 μM MK-801, (f) 20 μM CNQX, (g) MK-801/CNQX combinations added to neurones in the presence of microglial-conditioned medium; (h) BDNF (10 ng/ml); (i) BDNF plus MK-801 (10 μM) and CNQX (20 μM); microglial-conditioned medium boiled for 1 h and then added to cerebellar granule cells in the (j) absence or (k) presence of 10 μM MK-801 and 20 μM CNQX; and (l) 50 μM z-DEVD-FMK or (m) 100 μM YVAD-CHO for 1 h before exposure for 24 h to microglial-conditioned medium. Data are mean ± SEM (bars) values (n ≥ 40 separate fields of cells from at least two independent experiments). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n.s, not significantly different from survival in the presence of 10 n*M* CGA alone.

most dramatic effects were observed by preincubation with the CPP-32/caspase-3 inhibitor z-DEVD-FMK (50 μM) for 1 h before exposure to activated microglial-conditioned medium; cell death was reduced to control levels (Fig. 5F). The caspase-1 inhibitor YVAD-CHO (100 μM) did not prevent neuronal death, suggesting that microglia induce neuronal death by the activation of specific subfamilies of caspases.

DISCUSSION

Primary cultures of rat brain-derived microglia express a reactive phenotype when exposed to CGA, accompanied by an induction of NOS activity and the stimulation of microglial glutamate release. Apoptotic pathways were triggered that were inhibited by YVAD-CHO and dependent on NOS and glutamate release. Glutamate release triggered microglial apoptosis by activation of an mGluR. When cerebellar neurones were exposed to conditioned medium from activated microglia, a delayed neuronal death occurred, mediated by z-DEVD-FMK-sensitive apoptotic pathways, independent of NOS activity.

Microglial cell death

The induction of iNOS following CGA exposure is in accordance with previous findings (Taupenot et al., 1996). The glioprotection afforded by inhibitors of iNOS indicates that the production of reactive oxygen intermediates from microglia is cytotoxic under conditions of CGA-induced activation. However, there is conflicting evidence about the susceptibility of microglia/macrophages to NO toxicity (Mitrovic et al., 1994; Boggs et al., 1998). In other cell types the ratio between NO and superoxide levels is an important factor in determining cellular viability; an imbalance can shift the cells toward an apoptotic or a necrotic death (Sandau et al., 1997). Caspase-1-mediated apoptosis has been observed in macrophages following exposure to the *Shigella* bacterium (Hilbi et al., 1997) but does not appear to mediate macrophage apoptosis induced by NO (Messmer et al., 1998). Caspase-1 has dual biological roles, where, on the one hand, it participates in the induction of cellular apoptosis, and on the other it processes pro-IL-1β to the

active form (Schumann et al., 1998; William et al., 1998). Thus, it is possible that caspase-1 activation leads to processing of pro-IL-1β to active IL-1β and the release of this cytokine. However, the activation of IL-1β by caspase-1 has been shown to inhibit apoptosis (William et al., 1998); thus, the processing of pro-IL-1β to active IL-1β by caspase-1 may take a secondary role to the induction of apoptosis by caspase-1-mediated pathways. The present results point to an involvement of a caspase of the IL-1β-converting enzyme family in CGA-mediated microglial apoptosis.

Temporally, microglial NO_2^- production precedes the onset of glutamate release. The levels of glutamate release induced by CGA are high (200-fold above low baseline levels) compared with previous reports using amyloid protein as the macrophage activator (Klegeris and McGeer, 1997). Furthermore, we find that stimulation of microglia with LPS results in significantly lower levels of glutamate release than observed with CGA (12.9% above basal). Inhibition of release by Adp suggests that glutamate release occurs via the x_c^- transporter as has been shown to occur when microglia are activated with different bacterial components (Piani and Fontana, 1994). Inhibition of glutamate release by bafilomycin hints at an exocytotic mode of glutamate release, as has been observed in neurones (Pocock et al., 1995); however, bafilomycin may have effects on the microglial respiratory burst (Murphy and Forman, 1993). Although having no effect on glutamate release from activated microglia, the mGluR inhibitor MSPG prevented CGA-induced microglial death. This suggests that the buildup of extracellular glutamate via the x_c^- transporter leads to the activation of type II/III mGluRs on microglia to exacerbate CGA-induced microglial injury, because inhibitors of the transporter or of mGluRs were equally glioprotective.

Cerebellar granule cell death

Microglia activated by CGA produce neurotoxins that kill primary cultures of cerebellar granule neurones within 24 h of exposure and before significant LDH release or propidium iodide uptake is recorded in microglia. Nonactivated microglia do not secrete neurotoxins, and microglia are a prerequisite for CGA-induced neuronal toxicity. Neuronal death induced by CGA-stimulated microglia is apoptotic in nature and inhibited by the putative caspase-3 inhibitor z-DEVD-FMK but not by the putative caspase-1 inhibitor YVAD-CHO. This suggests that the current route to neuronal death is highly distinctive because cerebellar granule cell apoptosis induced by K^+ removal involves caspases other than caspase-3 (D'Mello et al., 1998; Marks et al., 1998) and is mediated by reactive oxygen species (Schulz et al., 1996). Caspase-3 is a member of the CED-3-related caspase family, and thus microglial-evoked apoptosis in cerebellar granule cells is mediated by this subfamily of caspases.

The apoptotic pathway induced in the present conditions would appear to be different from that evoked by NO donors (Bonfoco et al., 1996) because NOS inhibitors did not prevent cerebellar granule cell apoptosis under the present conditions. In agreement with Ciesielski-Treska et al. (1998), who studied cortical neurones, we find that microglial-derived NO breakdown products such as NO_2^- are not themselves neurotoxic. Similar NO-independent neuronal death has been observed for cultured hippocampal neurones exposed to peritoneal macrophage conditioned medium (Flavin et al., 1997) and for cerebellar granule cells in coculture with reactive microglia (Piani et al., 1992). Our data are at odds with other reports where NO was found to mediate neurotoxicity following microglial activation with LPS and cytokines (Boje and Arora, 1992).

Antagonists of iGluRs offered only partial protection from microglial-derived toxins; similar findings for a partial involvement of NMDA receptors in microglial-mediated neuronal death were reported by Boje and Arora (1992) for microglia stimulated with LPS and cytokines. There is considerable variation in the literature as to the degree of microglial-mediated neurotoxicity occurring via the NMDA receptor (Piani et al., 1992; Flavin et al., 1997). Differing degrees of the contributions of oxidative glutamate toxicity versus glutamate exitotoxicity mediated by iGluRs may be one explanation (see Murphy et al., 1989), with the former mediated by apoptotic pathways (Ratan et al., 1994).

High concentrations of glutamate kill neurones within 2–3 h by acute methods and do not induce death with the characteristic features of apoptosis, i.e., DNA laddering and caspase activation, observed with lower glutamate concentrations (Yan et al., 1994; Ankarcrona et al., 1995; Nath et al., 1998). Thus, it would appear that the glutamate concentrations reached in our system are not high enough to cause death by acute methods. Neuronal apoptosis could not be prevented with cycloheximide, suggesting that new protein synthesis was not required. This is similar to apoptosis in cerebellar granule cells induced by staurosporine (Taylor et al., 1997) or low-dose glutamate toxicity (Du et al., 1997), both of which induce caspase-3 activity but do not require new protein synthesis. This suggests that cerebellar granule cells die by activation of preexisting molecules (Taylor et al., 1997).

The protective effect of BDNF on cerebellar granule cells and the fact that no further protection could be afforded by the iGluR antagonists MK-801 and CNQX suggest that BDNF protects cerebellar neurones by preventing apoptotic pathways mediated by activation of glutamate receptors. The effect of BDNF correlates with its reported prevention of caspase-3-mediated apoptosis (Harada and Sugimoto, 1998) and neurotoxicity mediated by glutamate (Lindholm et al., 1993). Partial protection of cerebellar granule neurones from the neurotoxins secreted by activated microglia was obtained by antagonists of the NMDA receptor, suggesting that glutamate certainly contributes to neurotoxicity. Because the combined effect of BDNF and the iGluR antagonists was only partially neuroprotective, other processes leading to apoptosis are involved. The neurotoxicity of acti-

vated microglial supernatant could be significantly attenuated by boiling, suggesting the involvement of a protein. Flavin et al. (1997) reported that macrophage-induced neurotoxicity was mediated via the release of a large species (molecular mass >50 kDa). Other candidates could include the proinflammatory cytokines tumour necrosis factor-α, which is known to be up-regulated following CGA stimulation of microglia (Ciesielski-Treska et al., 1998), and IL-1β (Giulian et al., 1993). Although we found that the application of each of these cytokines alone did not affect neuronal viability, it is possible that factors in combination may be more neurotoxic, as has been recently shown for tumour necrosis factor-β1 and glutamate (Brown, 1999).

The results presented here indicate that CGA, a peptide that is up-regulated in Alzheimer's disease and associated with senile plaques, induces apoptosis of microglia and of neurones as a consequence of microglial reactivity. Apoptosis of the two cell types occurs by different pathways, one dependent and the other independent of NO. The distinct signalling cascades activated suggest possible routes by which microglial and neuronal death may be regulated and point to strategies for intervention.

Acknowledgment: This work was supported by the Medical Research Council, U.K., and the Brain Research Trust.

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