Errata

Page 17, 15th line: "demonstrates no activity at the AMPA" for "demonstrates little activity at the AMPA"

Page 95, legend of graph: "10µM" for "100µM"

Page 109, 19th line: "cultured in NMDA with B27" for "cultured in NBM with B27"

Page 124, 17th line: " two way ANOVA" for "two way ANOVA and a Newman-Keuls post test"

Page 140, 3rd line: " did not increase PI" for "did not significantly increase PI"

Page 171, 2nd line: "KA produced" for "KA (300µM) produced"

Page 182, 2nd line: "as BAX is central" for "as BAX seems to be central"

Page 195, 6th line: "by the kinase inhibitors" for " by the kinase inhibitors (24h)"

NEUROPHARMACOLOGY

OF KAINATE RECEPTOR-MEDIATED

EXCITOTOXICITY

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

BY

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SUMMARY

This thesis examines the neuropharmacology of the kainate (KA) subtype of receptors for L-glutamate (Glu), the major excitatory amino acid in the mammalian central nervous system. Overstimulation of ionotropic Glu receptors causes neuronal loss that has been implicated in various neurodegenerative and neurological conditions. Glu receptor-mediated neurotoxicity occurs by two types of cell death, apoptosis (programmed cell death) and/or necrosis. Apoptosis is a complex mechanism that requires protein and DNA synthesis, implying an intracellular "death" pathways are activated controlling the demise of the cell. This thesis describes the establishment of an *in vitro* system used to examine Glu receptor-mediated neuronal death. A primary culture of cerebellar granule cells (CGCs) was developed and maintained in a serum-free environment, where non-neuronal cell populations accounted for less than 5% of the total number of cells. The primary goal of this study was to investigate KA receptor-mediated neurotoxicity, and especially its pharmacological characteristics, the involvement of apoptosis and related aspects of cellular signaling.

Initially, neurotoxicity elicited by the ionotropic Glu agonists, KA, *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), were examined in primary cultures of CGCs. NMDA and KA produced a concentration-dependent cell death, whereas agonists for the AMPA receptor failed to cause any neurotoxicity. NMDA and KA receptor-mediated neuronal loss was attenuated by MK-801 and CNQX, antagonists for NMDA and KA receptors, respectively. The neuronal loss that was induced by KA was largely apoptotic in nature, however at higher concentrations some minor necrosis is evident. Toxicity induced by NMDA and Glu was largely necrotic in nature, but at lower concentrations some apoptosis was evident.

Studies conducted in Chapter Three, examined putative low-affinity KA receptor agonists at native receptors, without the interference from functional AMPA receptors, in CGCs. These studies demonstrated mixed activity of the compounds at the KA and NMDA receptors, although their neurotoxicities were shown to be dependent upon concentration-dependent. (S)-5-iodowillardiine (IW) and (RS)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid (ATPA) seemed most likely to exert their toxicity through action on the low-affinity KA receptors, whereas (2S,4R,6E)-2-amino-4-carboxy-7-(2-naphthyl)hept-6-enoic acid (LY339434) is likely to be a NMDA receptor agonist. 4-methylglutamate (4-MG) had complex actions, most likely due to its activity on Glu transporters and uptake systems, and while it was an extremely potent neurotoxin, very little cell loss was mediated via the NMDA or KA receptor subtypes.

The involvement of the cell cycle in neuronal apoptosis has recently emerged with novel studies showing not only cell cycle gene expression, but neuronal proliferation. To investigate whether KA receptor-mediated apoptosis was associated with the cell cycle the expression of cyclins A, B1 and D1, were examined after KA receptor-mediated insult. BAX, a well known pro-apoptotic member of the Bcl-2 family, protein expression was also examined to further characterize the mechanisms of cell death mediated by KA. A remarkable, time- and concentration-dependent expression of cyclin D1 and BAX protein expression was noted, with maximal expression at 2h and 12h after KA exposure for cyclin D1 and BAX, respectively. Analysis of the cyclins, by reverse transcriptase polymerase chain reaction (RT-PCR), also demonstrated a early increase in cyclin D1 expression, but no change in cyclins B1 or A.

To further examine the role the cell cycle plays in KA receptor-mediated apoptosis, various inhibitors of the cell cycle, cyclin-dependent kinases (CDK) and mitogen activated protein (MAP) kinases were co-exposed to cultures treated with KA. Olomoucine, a mixed

MAP and CDK inhibitor, was the most effective inhibitor, and completely attenuated neurotoxicity induced by KA. Other MAP kinases were partially effective, and only at lower concentrations of KA ($< 100\mu M$). The cell cycle inhibitors were completely ineffective against KA receptor-mediated toxicity, whereas inhibiting CDK activity partially attenuated toxicity.

The combined results presented in this thesis adds to the knowledge of KA receptor pharmacology, and the intracellular pathways that mediate neuronal apoptosis, demonstrating an involvement for both the cell cycle and MAP kinases in KA receptor-mediated apoptosis.

DECLARATION

The studies contained in this thesis have not been previously submitted by myself or any other person for a degree at this or any other university.

The experiments and analyses involved in this thesis represent my own original work, except where due acknowledgement has been made.

This thesis is less than 100,000 words, excluding tables, figure legends, appendices and bibliography.

Sarah Filippa Giardina

PUBLICATIONS

COMMUNICATIONS ARISING FROM THIS WORK

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ABBREVIATIONS

ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	analysis of variance
ATPA	(RS)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid
BrdU	5-bromo-2'-deoxy-uridine
BSA	bovine serum albumin
cDNA	complimentary DNA
CGCs	cerebellar granule cells
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CTZ	cyclothiazide
DAB	2,2' diaminobenzidine
div	days *in vitro*
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNQX	6,7-dinitroquinoxaline-2,3-dione
Dom	domoate
EDTA	ethylenediaminetetraacetic acid
FCS	foetal calf serum
FITC	fluorescein-isothiocynate
FW	(S)-5-fluorowillardiine
GABA	γ-aminobutyric acid

GFAP	glial fibrillary acidic protein
Glu	L-glutamate
Gln	L-glutamine
GYKI 52466	1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine HCL
HBS	HEPES balanced salt solution
HBSS	Hank's balanced salt solution
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[ethanesulfonic acid]
HRP	Horse radish peroxidase
IgG	immunoglobulin G
iGluR	ionotropic glutamate receptors
iso-olomoucine	6-Benzylamino-2-(2-hydroxyethylamino)-7-methylpurine
IW	(*S*)-5-iodowillardiine
KA	kainate
LY33494	(2*S*,4*R*,6*E*)-2-amino-4-carboxy-7-(2-naphthyl)hept-6-enoic acid
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MAP2	microtubule-associated protein 2
4-MG	(2*S*,4*R*)-4-methylglutamate
mGluR	metabotropic glutamate receptor
M-801	(5*R*,10*S*)-(+)-5-methyl-10-11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate
mimosine	β-[N-(3-Hydroxy-4-pyridone)]-α-aminopropionic acid
mRNA	messenger ribonucleic acid
MTT	3(-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

MW   molecular weight

NaCl   sodium chloride

NBM   Neurobasal™ medium

NMDA   *N*-methyl-D-aspartate

olomoucine   2-(2-Hydroxyethylamino)-6-benzylamino-9-methylpurine

PBS   phosphate buffered saline

PD98059   2-Amino-3-methoxyflavone

PI   propidium iodide

roscovitine   2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-

isopropylpurine

rpm   revolutions per minute

RT-PCR   reverse transcriptase polymerase chain reaction

SB203580   4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-

                  imidazole

SDS   sodium dodecyl sulfate

TBS   Tris-buffered saline

TEMED   N,N,N',N'-tetramethyl-ethylenediamine

TRITC   tetramethyl rhodamine isothiocyanate

TUNEL   terminal deoxynucleotidyl tranferase (TdT)-mediated dUTP-biotin

                  end labeling

TX-100   Triton X-100

U0126   1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene

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In loving memory
of Dad

and

for Mum,
for her unconditional
love and support

CHAPTER ONE

GENERAL INTRODUCTION

1.1 NEUROPHARMACOLOGY OF L-GLUTAMATE

1.1.1 *Glutamate and the Central Nervous System*

L-glutamate (Glu) is the principal excitatory amino acid in the mammalian central nervous system (CNS) where it plays roles in numerous normal physiological functions including synaptic plasticity, learning and memory, nociception and cardiovascular regulation (Collingridge & Singer, 1990). Glu was found to act as a neuroexcitant in the early 1960's in studies where it was applied to neurones by microiontophoresis (Curtis & Watkins, 1960). However, studies conducted before this time concluded that Glu, as an ubiquitous amino acid, only played a role in nitrogen metabolism as a non-essential amino acid (Erecinska & Silver, 1990; Curtis & Johnston, 1974). Glu mediates various biochemical functions, including fatty acid and protein synthesis, energy metabolism, and is a precursor for various endogenous small molecules including folic acid, and for the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Erecinska & Silver, 1990). All these factors mitigated against early attempts to establish Glu as a neurotransmitter. General acceptance of Glu being a major neurotransmitter did not come until the 1980's, when Glu uptake systems (Balcar & Johnston, 1972), various Glu receptors (Fonnum, 1984), and vesicular storage were identified (Storm-Mathisen *et al.*, 1995).

1.1.2 *Glutamate Synthesis and Metabolism*

Glu is a non-essential amino acid that is readily synthesized by the body. Under normal physiological conditions Glu is not able to cross the blood brain barrier, and therefore plasma levels do not reflect levels in the CNS (Fonnum, 1984). Glu required for

neurological functions is synthesized within the CNS (Fonnum, 1984) and remains at a relatively constant concentration (~10mM) (Erecinska & Silver, 1990).

While Glu synthesis can occur via various pathways, its seems that Glu is mainly synthesized from glucose by the process of glycolysis, where glucose is broken down to pyruvate, which in turn enters the tricarboxylic acid cycle (TCA) where Glu is produced from α-ketoglutarate. L-Glutamine (Gln) too can be converted to Glu by phospho-activated glutaminase, an action that largely depends upon glial cells. Glu is taken up by glial cells and converted to Gln, in an ATP-dependent manner, where it is released into the extracellular space and rapidly taken up by neurones. Neurones then convert the Gln to Glu in the synaptic terminals (Erecinska & Silver, 1990; Nicholls & Attwell, 1990) and Glu is then transported into vesicles for storage (Naito & Ueda, 1985) (Figure 1.1). Glu release is believed to be via a classical exocytotic, calcium-dependent pathway utilizing synaptic vesicles and requiring membrane depolarization (Nicholls & Attwell, 1990).

1.1.3 Glutamate Uptake Systems

Pioneering studies conducted by Curtis and colleagues demonstrated that Glu responses were enhanced by the addition of the membrane transport inhibitor p-chloromercuriphenylsulphonate in feline Renshaw cells (McCulloch *et al.*, 1974). Earlier investigations had failed to provide evidence for the augmentation of responses to excitatory amino acids with the use of various enzyme inhibitors, implying Glu was not degraded after release (Curtis & Watkins, 1960). These studies were the first to demonstrate uptake mechanisms for excitatory amino acids *in vivo* in brain tissue. Recently a family of Glu transporters (GluTs) have been cloned and characterized pharmacologically (Vandenberg, 1998).
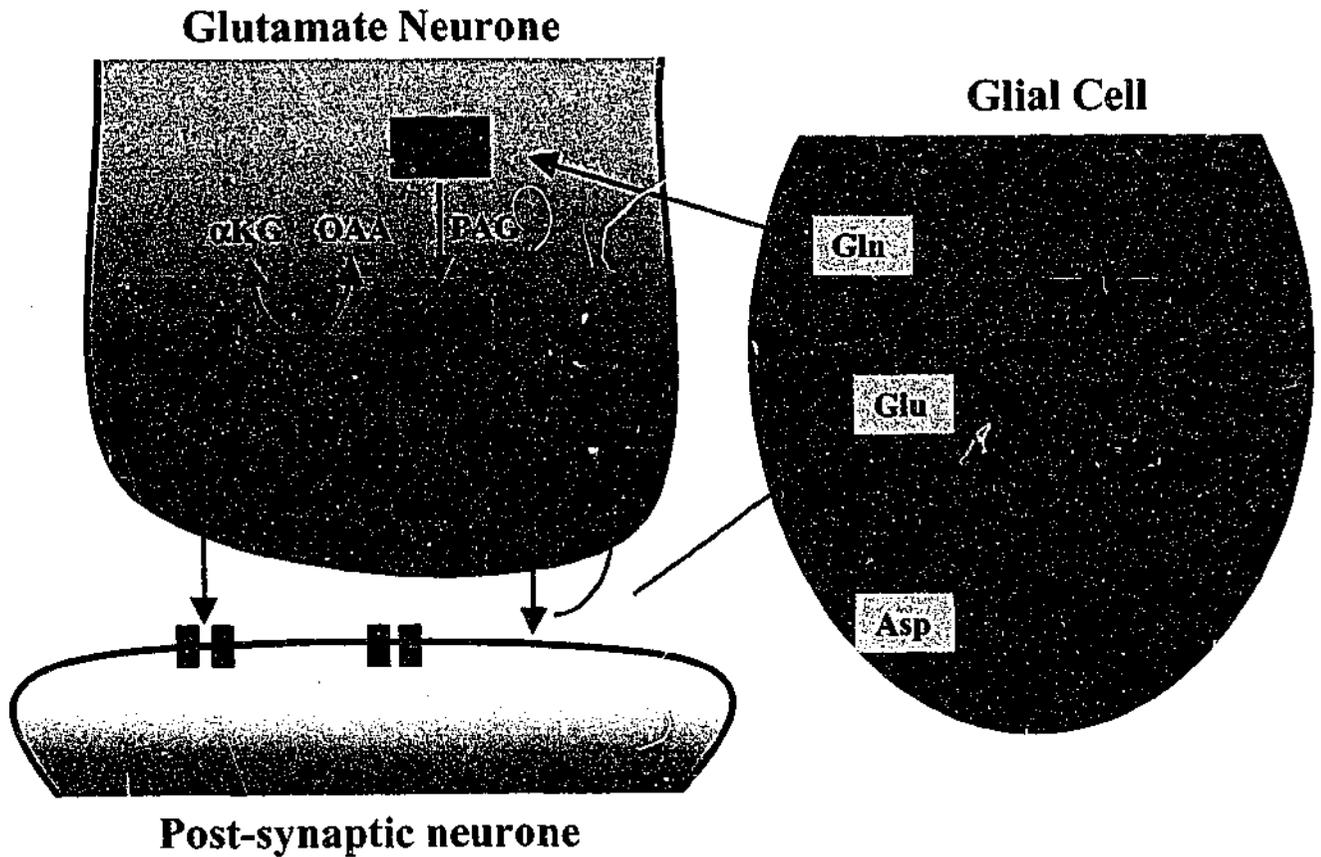
Figure 1.1 Pathways of Glutamate synthesis

α-Ketoglutarate, a TCA cycle intermediate, is transaminated to Glu, which is subsequently converted to Gln in glia. Gln is taken up by neurones and converted to Glu via phosphate activated glutaminase (PAG). Glu is stored in synaptic vesicles, for subsequent transmitter release, or acts as an intermediate in other metabolic cycles. Following release Glu is taken up by neurones and recycled or converted to Gln in glia. Abbreviations: OAA, oxaloacetate; Asp-AT, aspartate aminotransferase.

Glu is transported across the plasma membrane against its concentration gradient and the resultant concentration within the extracellular space is maintained at approximately 1µM, some several thousand fold less than the concentration within the cell (approximately 10mM) (Gegelashvili & Schousboe, 1997). Once Glu is released from nerve terminals it is quickly removed from the extracellular space by GluTs. To date four transporters have been characterized in rodent brain: the Glu/L-aspartate transporter (GLAST), Glu transporter 1 (GLT1), excitatory amino acid carrier 1 (EAAC1) and excitatory amino acid carrier 4 (EAAC4) (Vandenberg, 1998). In the human brain 5 homologues have been cloned and are termed EAAT1-5 (Sims & Robinson, 1999). Other members of this family are likely to be discovered as the transporters cloned thus far are predominantly glial in localization and early evidence suggests there is transport into presynaptic nerve terminals (Beart, 1976). The localization of the various GluTs varies depending on the region of the brain and cell type. Table 1.1 demonstrates the cellular location of the transporters and their relative affinities for Glu.

As Glu is a potent neurotoxin (Meldrum & Garthwaite, 1990), rapid removal from the extracellular space is required to avoid neuronal damage. Various disease states may be linked to abnormal functioning of various GluTs (Vandenberg, 1998). Significant changes in GLAST or GLT1 expression are not evident in post-mortem Alzheimer's diseased brain when compared to aged matched controls, although binding studies have demonstrated a decrease in GLT binding in Alzheimer's patients by 34% (Masliah *et al.*, 1996). There is also evidence to suggest that the transporters are differentially regulated due to lower ratio of N-terminal immunoreactivity when compared to that of central immunoreactivity of GLT in post-mortem brains of Alzheimer's patients (Beckstrom *et al.*, 1999). Additionally, there is direct evidence to suggest that the glial transporter EAAT2 is down-regulated by up to 95% in the motor cortex and spinal cord of patients suffering amyotropic lateral

Glu Transporter	GLAST (EAAT1)	GLT1 (EAAT2)	EAAC1 (EAAT3)	EAAT4	EAAT5
Cellular Localization	Glia	Glia	Neurones	Neurones	Unknown
Tissue Distribution	Brain, Heart, Lung, Placenta, Skeletal Muscle	Brain, Liver	Brain, Intestine, Kidney, Liver, Heart	Cerebellum, Placenta	Retina
Affinity for Glu (Km)	18-20μM	18μM	30μM	2.5μM	64μM

Table 1.1 Characteristics of Glu transporters

(Adapted from Kanai *et al.,* 1997; Vandenberg, 1998).

sclerosis (ALS), possibly due to aberrant RNA splice variants (Lin *et al.*, 1998). Studies employing antisense oligonucleotides and the production of knockout mice have demonstrated the importance of GLAST, GLT1 and EAAC1 in synaptic transmission as these animals develop epilepsy (Meldrum *et al.*, 1999). Therefore, defective functioning of Glu uptake mechanisms may be a common attribute in neurodegenerative diseases, where excess Glu remains in the extracellular space and by receptor overstimulation causes neuronal injury (Masliah *et al.*, 1996).

1.1.4 Glutamatergic Pathways

Historically, identification of pathways that utilize Glu as a transmitter has been difficult, since immunohistochemistry could not be correlated with neurotransmitter levels, as Glu is also a metabolic intermediate. Thus, identification of glutamatergic pathways came from studies involving high affinity uptake, selective lesioning of neurones and monitoring endogenous release and retrograde transport (Fagg & Foster, 1983; Fonnum *et al.*, 1981). Numerous pathways utilize Glu as their transmitter, including those originating from neocortical pyramidal cells, several intrahippocampal pathways and parallel fibres of the cerebellum (Feldman *et al.*, 1997; Storm-Mathisen *et al.*, 1995; Fonnum, 1984). Glutamatergic efferents from various areas of the neocortex project to many regions of the brain including to the nucleus accumbens (Walaas, 1981), the amygdala and thalamus (Walker & Fonnum, 1983). Also, efferent pathways exist that originate in the cerebellum and project to the inferior olive and within the cerebellum there are parallel fibres present within the cerebellum that arise from the granule cells (Storm-Mathisen *et al.*, 1987).

1.1.5 Glutamate Receptor Subtypes

The majority of neurones and astrocytes possess Glu receptors (Fonnum, 1998; Lipton & Rosenberg, 1994; Hosli & Hosli, 1993) and the most studied and understood actions of Glu are mediated via the ionotropic, ion-gated Glu receptors (iGluRs), which are named after their preferring agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) (Fletcher & Lodge, 1996; Bettler & Mulle, 1995; Collingridge & Singer, 1990; Honore, 1989). More recently several G-protein coupled Glu receptors have been discovered and collectively termed metabotropic Glu receptors (mGluRs) (Nakanishi *et al.*, 1998; Bockaert *et al.*, 1993; Schoepp & Conn, 1993).

The identification of receptors for Glu was largely hindered by the lack of selective antagonists. Early studies identified differing sensitivity to Glu and Asp of spinal neurones, suggesting the existence of more than one receptor subtype (Duggan, 1974). Further studies demonstrated that KA, a structurally restricted Glu analogue, was more potent than Glu itself in stimulating spike discharges in cortical neurones. However if the concentration of KA was increased the amplitude of the discharge decreased before being completely abolished, an effect attributed to receptor desensitization (Shinozaki & Konishi, 1970). Furthermore, NMDA and KA differed in potency when applied to spinal interneurones and Renshaw cells (McCulloch *et al.*, 1974). While the evidence for different Glu receptors was growing the lack of selective antagonists hindered the full characterization of these receptors. Evans *et al.* (1977) employed the first selective antagonist, Mg^{2+}, to block NMDA receptor function demonstrating for the first time a clear distinction between NMDA and non-NMDA receptors. Studies that first identified two forms of non-NMDA receptors, the quisqualate (QA) and KA receptors, did not come from the use of selective antagonists, but rather the identification of differing regional potencies

(Davies & Watkins, 1981). However, nearly 5 years elapsed before radioligand binding studies and the development of AMPA, a QA analogue (Krogsgaard-Larsen *et al.*, 1985; Krogsgaard-Larsen *et al.*, 1982), aided the further characterization of the non-NMDA receptors. Like the functional electrophysiological and pharmacological studies, the binding studies with [^{3}H] AMPA supported the concept of three different iGluRs, the KA, AMPA and NMDA receptors (Greenamyre *et al.*, 1985; Monaghan *et al.*, 1984; Monaghan *et al.*, 1983).

The development of selective iGluR antagonists led to further differentiation between the NMDA and non-NMDA receptor subtypes. The first series of selective antagonists were longer chain D-analogues of Glu, which led to the synthesis of the selective NMDA receptor antagonists ω-phosphonoates (Davies & Watkins, 1981). The development of the quinoxalinediones (eg. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) further advanced the understanding of the non-NMDA receptors and defined their importance in many neurological functions (Honore *et al.*, 1988). Studies conducted in cultured neurones have led to invaluable insights into the Glu receptors (Choi *et al.*, 1987). Early studies demonstrated altered levels of second messengers when neurones and glia were stimulated with Glu. While this phenomenon was initially believed to only involve a secondary response subsequent to the stimulation of iGluRs (Smart, 1989), direct application of AMPA, KA or NMDA could not replicate results found with Glu itself increasing phospholipase C (PLC) turnover (Nicoletti *et al.*, 1986; Sladeczek *et al.*, 1985). Hence, studies conducted in the 1980's demonstrated the direct coupling of Glu receptors to phosphoinositide hydrolysis (Sugiyama *et al.*, 1987; Nicoletti *et al.*, 1986). Since then, various other Glu receptors coupled to second messenger systems have been discovered, and collectively termed the

mGluRs (Miller *et al.*, 1995; Pin & Duvoisin, 1995; Bockaert *et al.*, 1993; Schoepp & Conn, 1993).

Cloning experiments have played a central role in the understanding of the various Glu receptor subunits, which include GluR1-4 (AMPA); NR1, NR2A-D (NMDA); KA1, KA2 (high affinity KA receptors) and GluR5-7, (low affinity KA receptors); and mGluR1-8 (mGluRs) (Figure 1.2) (Hollmann & Heinemann, 1994). There are three main subfamilies of mGluRs determined by their selective agonists. Group 1 receptors (mGluR1 and mGluR5) are coupled to phopholipase C (PLC) and via stimulation of protein kinase C (PKC) elevate intracellular Ca^{2+}. Group 2 receptors (mGluR2 and mGluR3) are negatively coupled to adenyl cyclase resulting in a decrease in cyclic AMP generation. While the group 3 receptors (mGluR4 and mGluR6-8) are also negatively coupled to adenyl cyclase, they possess different selectivity for agonists when compared to those of group 2 (Pin & Duvoisin, 1995; Hollmann & Heinemann, 1994; Lipton & Rosenberg, 1994).

The various iGluR subunits do not share sequence homology with any other ligand-gated ion channel receptors, however they are believed to assemble into receptors in a similar way to the nicotinic acetylcholine receptor (nAChR). nAChR, and possibly the iGluRs, are comprised of five multiple subunits around a central cation-permeable pore (Wenthold *et al.*, 1992). Some iGluR subunits can form functional homomeric receptors, however most native receptors are heteromeric (Jorgensen *et al.*, 1995; Wenthold *et al.*, 1992). Two proposed models of iGluR topology exist based on hydropathy profiles of the receptors. iGluRs have a hydrophobic domain at the N-terminus and four transmembrane domains. While the C-terminus was generally believed to be extracellular (Hollmann & Heinemann, 1994; Jorgensen *et al.*, 1995), there now is some evidence that it is indeed intracellular (Fletcher & Lodge, 1996).

Glu Receptors

Ionotropic ———— **Metabotropic**

Ionotropic:
- Kainate
- AMPA
- NMDA

Metabotropic:
- Group 1
- Group 2
- Group 3

AMPA:
GluR1
GluR2
GluR3
GluR4

Group 1:
mGluR1
mGluR5

Group 2:
mGluR2
mGluR3

Group 3:
mGluR4
mGluR6
mGluR7
mGluR8

NMDA:
NR-1

NR-2A
NR-2B
NR-2C
NR-2D

Kainate:
- High Affinity KA
- Low Affinity KA

High Affinity KA:
KA-1
KA-2

Low Affinity KA:
GluR5
GluR6
GluR7
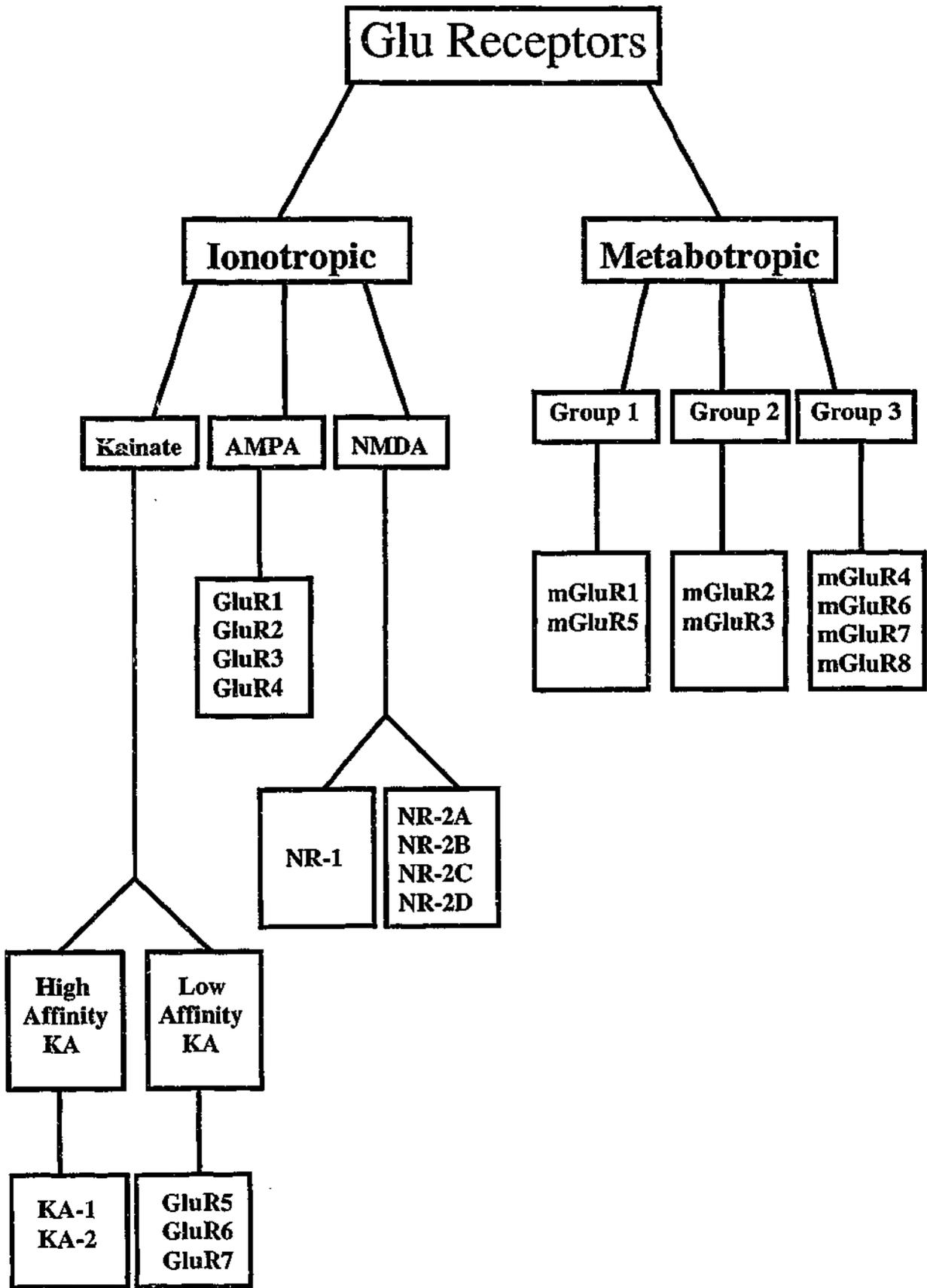
Figure 1.2 Cloned Glutamate Receptors

Phylogenic tree demonstrating the relationship between the glutamate receptors and the subunits which combine to form a specific type of Glu receptor.

1.1.5.1 The Ionotropic Receptor Complexes

1.1.5.1.1 The NMDA Receptor

The NMDA receptor is the best characterized of the amino acid receptor subtypes (Yamakura & Shimoji, 1999; Scatton, 1993). Stimulation of the NMDA receptor permits the influx of both Na^+ and Ca^{2+} ions, however, before the receptor can be activated a voltage-dependent Mg^{2+} block must first be removed. The NMDA receptor complex also contains binding sites for phosphorylation, a modulatory glycine site, a site for divalent cations including Zn^{2+}, polyamines and a site within the channel, known as the phencyclidine (PCP) site, whereby non-competitive antagonists bind, including MK-801. (Hollmann & Heinemann, 1994; McBain & Mayer, 1994) (Figure 1.3A). Sites for phosphorylation are found on the C-terminus of NMDAR1 and thought to play an important role in regulating synaptic plasticity and long term potentiation (Tingley *et al.*, 1993). NMDA receptors are composed of NR1 and NR2 protein subunits, of which there are eight and four splice variants, respectively (Hollmann & Heinemann, 1994; McBain & Mayer, 1994). The exact assembly of the NMDA receptor subunits is yet to be elucidated, however it is believed that they form heteromeric receptors, although the NMDAR1 subunits can form functional homomeric receptors (McBain & Mayer, 1994).

The NMDA receptor-mediated currents are slow in onset and long in duration, due to Glu occupying the NMDA receptor site for a long period of time, and can be terminated immediately by the application of Mg^{2+} (Lester *et al.*, 1990). Radioligand studies have shown that the NMDA receptors are localized postsynaptically and are present in high density in the cerebral cortex, hippocampus, striatum, septum and amygdala (Monaghan *et al.*, 1989; Monaghan *et al.*, 1983). Glu has a higher affinity for NMDA receptors, when compared to AMPA and KA receptors, and NMDA receptors are particularly Ca^{2+}-

A

Recognition site $\quad$ K$^+$ $\quad$ Glycine site

PCP site

Mg^{2+} site

P

Polyamine site

Ca^{2+} Na^{2+}

B

CNQX, NBQX

Recognition site $\quad$ K$^+$

Cyclothiazide site

Benzodiazepine site

P

Na^{2+} (Ca^{2+})

C

CNQX, NBQX, NS102

Recognition site $\quad$ K$^+$ $\quad$ Concanvalin A

Toxins

P

Na^{2+} (Ca^{2+})
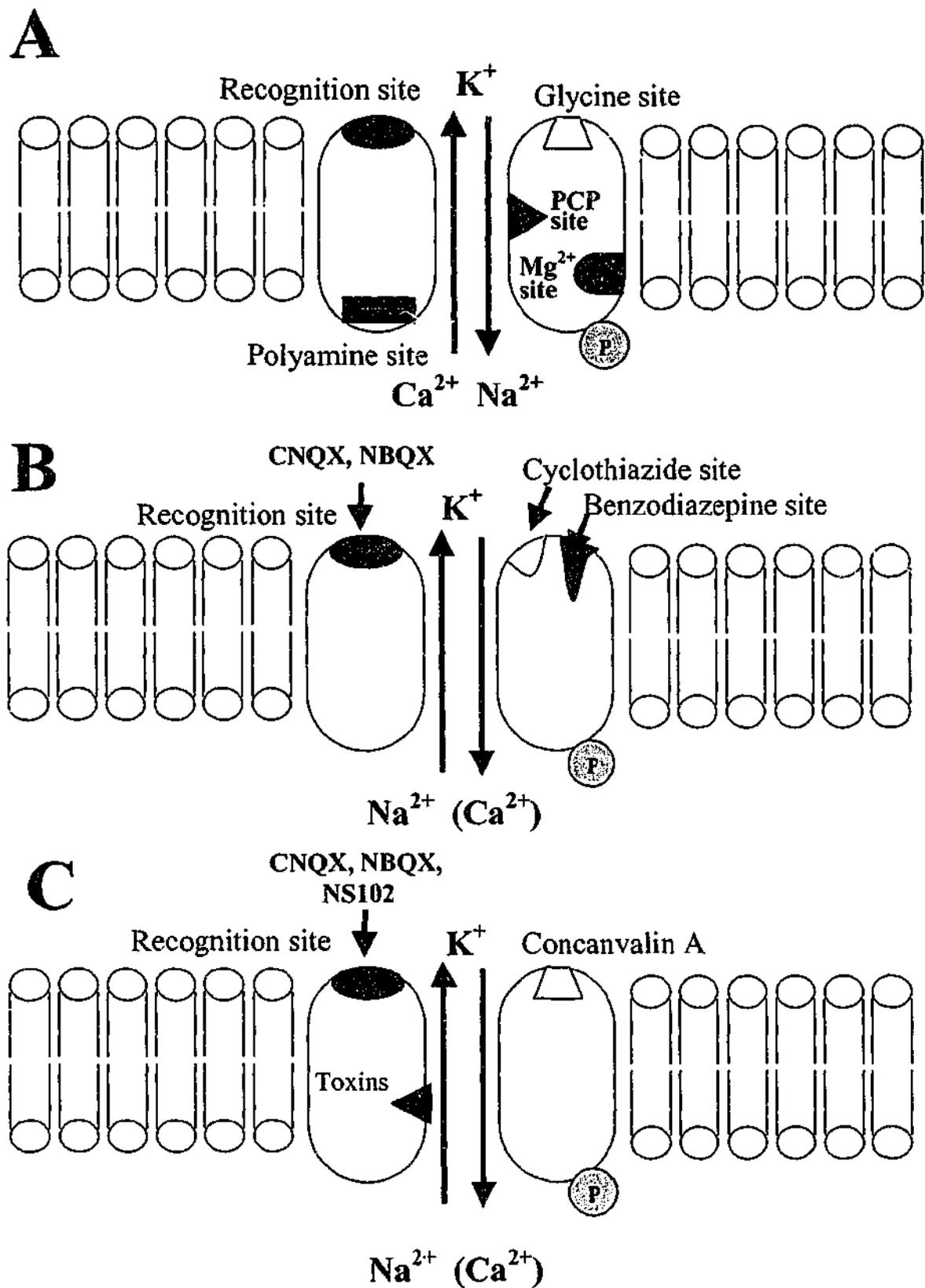
Figure 1.3 Schematic representations of iGluRs

NMDA receptor (A), AMPA receptor (B) and KA receptor (C) complexes and their binding sites.

permeable (Pumain & Heinemann, 1985). NMDA receptors play an important role in learning, memory and nociception and various studies have implicated them in a variety of neurological diseases, particularly stroke and ischaemia (Qin *et al.*, 1996; Ikonomidou & Turski, 1995; Foster *et al.*, 1988).

1.1.5.1.2 The non-NMDA receptors

Early studies revealed a second class of iGluRs, termed the non-NMDA receptors, however it was not until the early 1990's that incontestable evidence emerged to suggest that non-NMDA receptors consist of two individual receptor subtypes, now termed KA and AMPA. AMPA and KA receptors are less well defined than the NMDA receptors, mainly due to the lack of selective agents for both these receptors, and hence initially were commonly referred to collectively as the non-NMDA receptors (Johnston *et al.*, 1974; McCulloch *et al.*, 1974). The rapid receptor desensitization that occurs after KA and AMPA receptor stimulation also perplexed early attempts to characterize the non-NMDA receptors. Initial studies concluded that AMPA and KA acted on the same receptor with different potencies (Nakanishi *et al.*, 1998; Patneau *et al.*, 1992; Keinanen *et al.*, 1990). However, binding studies and the development of AMPA enabled a clearer distinction between the two non-NMDA receptors (Wong *et al.*, 1994; Patneau *et al.*, 1992; Davies & Watkins, 1981). Binding and *in situ* hybridization studies employing radiolabeled AMPA and KA revealed different regional distributions (Table 1.2) (Lunn *et al.*, 1996; Ganakas *et al.*, 1994; Monaghan *et al.*, 1989; Honore *et al.*, 1988) and different sized protein targets, 76.6kDa and 51.6kDa, for KA and AMPA, respectively (Honore *et al.*, 1986). One of the most important developments in the differentiation of AMPA and KA receptors from NMDA receptors came from the development of the two first non-NMDA receptor antagonists, CNQX and DNQX, although these two compounds are not completely

Region	AMPA Receptors				KA Receptors				
	GluR1	GluR2	GluR3	GluR4	GluR5	GluR6	GluR7	KA1	KA2
olfactory bulb				++	++	++	++		
neocortex	++	++	++	++	++		++		++
hippocampus	++	++	++	++		++			++
CA1	++	++	++	++		++			++
CA2	++	++	++			++		++	++
CA3	++	++	++			++			++
dentate gyrus	++	++	++	++		++			++
subiculum	++	++	++	++	++				
piriform cortex	++		++	++	++	.			++
caudate putamen	++	++	++	++			++		++
amygdala	++		++	++	++				
reticular thalamic nucleus	++	++	++	++					
hypothalamus	++	++			++				
pons				++	++		++		
cerebellum	++	++	++	++	++	++	++		++
Bergmann glia	++			++					
granule layer		++		++		++			
Purkinje layer	++	++	++	++	++				
spinal cord		++		++					
retina	++	++	++		++				

Table 1.2 Regional distribution of non-NMDA receptor mRNA detected by *in situ* hybridization

(Adapted from Hollmann & Heinemann, 1994).

selective for either AMPA or KA receptors (Honore *et al.*, 1988). Recently, with the use of various molecular biology techniques the AMPA receptor has been better characterized (Blaschke *et al.*, 1997; Fletcher & Lodge, 1996; Wahl *et al.*, 1996), however many studies are still hindered by the lack of selective agonists and antagonists (Donevan & Rogawski, 1993; Fletcher *et al.*, 1988). Expression of AMPA receptors in Xenopus oocytes have allowed the dissection of the characteristics of the various AMPA receptor subunits in their homomeric conformations. These studies have revealed the differences not only between the AMPA and KA receptors but also between the individual AMPA subunits (Wahl *et al.*, 1996). In particular, these techniques have been particularly useful in characterizing the selectivity of various agonists and ion permeabilities (Fletcher & Lodge, 1996; Wahl *et al.*, 1998a; Wahl *et al.*, 1996).

AMPA and KA receptor activation causes a Na^+-dependent depolarization of the cell membrane, leading to the activation of voltage-operated Ca^{2+} channels, allowing a secondary influx of Ca^{2+} ions (Gibbons *et al.*, 1993; Murphy & Miller, 1988). Molecular biological studies, especially those involving the cloning and sequencing of receptors, have provided new insights into Glu receptor function, and for example contrary to initial belief, some AMPA and KA receptor assemblies are Ca^{2+}-permeable (Lu *et al.*, 1996; Hollmann & Heinemann, 1994; Frandsen & Schousboe, 1993). In particular, homomeric channels assembled from GluR1, GluR3, or GluR4 subunits are highly Ca^{2+}-permeable, whereas assemblies containing GluR2 subunits have a low Ca^{2+} permeability (Lu *et al.*, 1996; Hollmann & Heinemann, 1994). In heteromeric receptor assemblies the Ca^{2+} permeability is largely determined by the presence of the GluR2 subunit (Cotton *et al.*, 2000).

1.1.5.1.2.1 AMPA Receptors

AMPA receptors were formerly known as QA receptors until it was discovered that QA also stimulated mGluRs (Sladeczek *et al.*, 1985) and the more selective agonist, AMPA, was developed. AMPA receptors are best characterized by rapid receptor desensitization, and like the NMDA receptor complex, contain various modulatory sites, including a phosphorylation site, a benzodiazepine site, at which these non-competitive antagonists act, a site at which cyclothiazide (CTZ) acts to inhibit receptor desensitization, and an intra-channel site where toxins act (Keinanen *et al.*, 1990) (Figure 1.3B).

AMPA receptors consist of both-homo- and heteromeric dimers formed from the receptor subunits GluR1-4 (Keinanen *et al.*, 1990). While KA can act on GluR1-4 receptor assemblies, binding studies revealed AMPA is more selective for these receptor subunits (Fletcher & Lodge, 1996; Keinanen *et al.*, 1990), although KA elicits a stronger response on the GluR1-4 receptor subunits as there is no receptor desensitization (Hollmann & Heinemann, 1994). Immunocytochemical studies have indicated GluR1-4 are post-synaptic receptors (Hampson *et al.*, 1992; Petralia & Wenthold, 1992) and native receptors occur in two isoforms termed flip and flop (Lambolez *et al.*, 1992; Sommer *et al.*, 1990). Neither receptor isomer shows a differential regional distribution in the adult brain, however in the neonate the flip isoform is more predominant (Sommer *et al.*, 1990). AMPA receptors are widely distributed throughout the rodent brain, as determined by *in situ* hybridization studies (Hollmann & Heinemann, 1994; Keinanen *et al.*, 1990). High density labeling for AMPA receptors is evident in the cortex, hippocampus, striatum, globus pallidus, ventral pallidum, subthalamic nuclei and cerebellum (Porter & Greenamyre, 1994; Hollmann & Heinemann, 1994; Albin *et al.*, 1992; Keinanen *et al.*, 1990; Monaghan *et al.*, 1984).

1.1.5.1.2.2 KA Receptors

KA was first isolated from the seaweed *Digenea simplex* and was commonly used to eliminate worms from the intestinal tract in the nineteenth century (Takemoto, 1978). As KA was structurally similar to Glu, it was postulated that KA would have excitatory actions on neurones. Studies conducted in spinal cord and cortical neurones using electrophoretic injections demonstrated that KA was indeed a potent neuroexcitant, being even more potent than Glu (Johnston *et al.*, 1974; McCulloch *et al.*, 1974; Shinozaki & Konishi, 1970). Studies soon followed demonstrating KA to be a potent neurotoxin (Olney *et al.*, 1974), and hence KA attracted much attention and many models of acute neuronal damage employed KA as a neurotoxin, including the kindling model of epilepsy (Loscher, 1998) and the neostriatal microinjection model of Huntington's chorea (Coyle *et al.*, 1978).

In 1957 domoic acid (Dom) was isolated from the seaweed *Chondria armata* and preliminary experiments indicated that Dom had similar properties to that of KA (Takemoto, 1978). Like KA, Dom is a potent and long-acting neuroexcitant (Johnston *et al.*, 1974). However Dom is more potent than KA in electrophysiological and binding studies and unlike KA demonstrates no activity at the AMPA receptor (Fletcher & Lodge, 1996; Hollmann & Heinemann, 1994) making it a useful tool for studying KA receptors, without interferences from AMPA receptor responses.

KA receptors are categorized into two subclasses termed the high and low-affinity KA receptors, identified and named after binding studies revealed high and low affinity sites (Honore *et al.*, 1986; London *et al.*, 1980). Subsequently, molecular cloning experiments and pharmacological analyses of individual KA receptor subunits confirmed this division (Hollmann & Heinemann, 1994). High affinity KA receptors include the KA1 and KA2 subunits, whilst low affinity receptor subunits are termed GluR5-7 (Hollmann & Heinemann, 1994). Unlike AMPA and NMDA receptor complexes, few modulatory sites

have been identified on the KA receptor, which include a phosphorylation site and a site whereby toxins act to inhibit KA receptor-mediated currents. The KA receptor also contains a site whereby plant lectins, including concanavalin A, act to attenuate receptor desensitization (Figure 1.3C). While the high affinity receptor subunits do not form functional homomeric receptors (Fletcher & Lodge, 1996), the GluR5-7 subunits may form either homo- or heterometric receptor assemblies (Schiffer *et al.*, 1997). Like the AMPA receptors KA receptors are widely distributed throughout the adult brain (Lunn *et al.*, 1996; Ganakas *et al.*, 1994; London *et al.*, 1980; London & Coyle, 1979), but GluR5-7 and KA1 mRNA levels are lower than that of the AMPA subunits GluR1-4 (Hollmann & Heinemann, 1994; Wisden & Seeburg, 1993).

GluR5-7 subunits share ~80% sequence homology with each other, but only ~40% with GluR1-4, while KA1 and 2 have 70% sequence homology with each other and ~40% with GluR1-4 and GluR5-7 (Hollmann & Heinemann, 1994). The GluR5 and 6 subunits are rapidly desensitized by KA (Hollmann & Heinemann, 1994) and the potency of agonists acting on the KA receptor subunits differs for each KA subunit as determined by pharmacological analyses of functionally expressed cloned Glu receptors (Table 1.3). Interestingly, stimulation of the native GluR7 subunit evokes no response, but a novel splice variant termed GluR7b, forms a receptor with low affinity for KA and Glu and is unresponsive to both AMPA and Dom (Schiffer *et al.*, 1997). Low-affinity KA receptors have been shown by receptor autoradiography to be widely distributed through the rodent and primate neuroaxis (Carroll *et al.*, 1998b; Toms *et al.*, 1997).

In addition to high and low affinity KA receptors, recent evidence suggests some KA receptors are linked, either directly or indirectly, to a pertussin-toxin sensitive G-protein which stimulates inositol-phosphate turnover (Sugiyama *et al.*, 1987). Early studies concluded that the increase in inositol phosphates was due to an increase in intracellular

Receptor Type	Gene	Order of Agonist Potency
AMPA	GluR1	QA>Dom~AMPA>Glu>KA
AMPA	GluR2,-4	QA> AMPA>Glu>KA
Low affinity KA	GluR5	Dom>KA>QA~Glu>AMPA
Low affinity KA	GluR6	Dom>KA>QA>Glu
Low affinity KA	GluR7	Dom>KA>Glu>QA>AMPA
High affinity KA	KA1,2	KA>QA>Dom>Glu>>AMPA

Table 1.3. Agonist potency orders for KA and AMPA receptor subunits
(Adapted from Fletcher and Lodge 1996; Hollmann & Heinemann 1994; Keinanen *et al.*
1990).

Ca^{2+} (Murphy & Miller, 1988; Sladeczek *et al.*, 1985), and recent electrophysiological evidence suggests that metabotropic KA receptors may act presynaptically to modulate GABA release (Rodriguez-Moreno & Lerma, 1998). The metabotropic KA receptors are thought to mediate their intracellular effects by elevating the levels of the mitogen activated protein (MAP) kinase ERK2 in a time- and concentration-dependent manner that is sensitive to PKC and PLC inhibitors (Liu *et al.*, 1999).

While selective agonists and antagonists for the NMDA receptor have been available for some time, selective drugs that can distinguish AMPA and KA receptor responses are still not readily available. Quinoxalinediones, 1,3-dihydrobenzodiazepines and LY293558 show some preferential affinity for AMPA receptors, and whilst NS-102 has been claimed to have some selective actions at KA receptors, this result has not been generally confirmed (Fletcher & Lodge, 1996). However, recent advances in this area of research have produced agonists that are more selective for the low affinity KA receptors (Bleakman & Lodge, 1998), allowing the investigation into the roles these receptors play in neuronal function and excitotoxicity (Moldrich *et al.*, 2000a; Moldrich *et al.*, 2000b; Bleakman & Lodge, 1998).

1.1.6 Glutamate and Excitotoxicity

1.1.6.1 Historical Perspective

Although Glu plays important roles in normal physiology, excessive stimulation of GluRs has been implicated in neuronal injury and death in numerous pathological conditions such as stroke, epilepsy and hypoglycaemia, and neurodegenerative diseases including Huntington's chorea, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (Leist & Nicotera, 1998; Lipton & Rosenberg, 1994; Meldrum &

Garthwaite, 1990). Evidence to suggest the involvement of Glu in various acute and chronic neurological dysfunctions has come from cytopathological evidence from both *in vivo* and *in vitro* and from direct evidence, whereby high concentrations of Glu have been recovered from damaged brain areas (Faden *et al.*, 1989). Excessive Glu exposure leading to neuronal injury has been appropriately termed excitotoxicity (Olney & Sharpe, 1969).

As circumventricular organs lie outside the blood brain barrier, they are vulnerable to dietary excitotoxins. Infants with underdeveloped blood brain barriers are particularly vulnerable to the dietary ingestion of Glu. Monosodium glutamate (MSG) is a common food additive, and studies conducted in immature animals have demonstrated neurodegeneration of cells after the oral administration of MSG (Olney, 1969). The most well studied incidence of Glu receptor-mediated neurotoxicity after oral administration was in Canada in 1987, where patients experienced confusion, seizures, amnesia, coma and some died, after the ingestion of mussels contaminated with Dom. Pathological investigations revealed neurodegeneration of the hippocampus and amygdala, but no damage was found in the spinal cord or the brain stem (Bose *et al.*, 1990; Teitelbaum *et al.*, 1990), similar to damage caused by focal injections of KA.

Pioneering studies conducted by Olney and his colleagues demonstrated that Glu and its analogues induced neurodegeneration in areas with poorly developed blood brain barriers in immature rodents, with relatively no damage in adult counterparts unless high concentrations of Glu were used (Olney, 1971; Lucas & Newhouse, 1957). Early observations classified neurotoxicity mediated by Glu into to distinct types: that in which local necrosis occurs, commonly caused by Glu, NMDA and ASP, and a second type which not only caused local necrosis but sustained limbic seizures (including KA, QA, and AMPA) (Olney, 1983). From these early studies the selectivity of neuronal injury was also established, whereby non-neuronal cells were usually unaffected by Glu, unless extremely

high concentrations were used (Olney, 1971). These early studies also noted that different populations of neurones varied in their vulnerability to excitotoxins, possible due to different Ca^{2+} buffering mechanisms and Glu receptor expression.

Recently it has been demonstrated that NMDA and non-NMDA receptor antagonists attenuate hypoxic-ischaemic injury *in vivo* (Gill & Lodge, 1997; Schousboe *et al.*, 1994). *In vivo* studies involving the direct administration of NMDA into various brain regions, such as the hippocampus and striatum, demonstrate a delayed neurotoxic effect, whereby NMDA receptor antagonists are capable of rescuing neuronal damage up to several hours after the insult (Foster *et al.*, 1988). This phenomenon may be explained in two ways: 1) NMDA exposure is causing apoptosis, which is a much slower process than necrosis or 2) NMDA causes widespread damage that results in Glu leakage and the neuroprotection observed is against the secondary damage. In addition to the neuroprotective nature of the NMDA receptor antagonists, numerous studies have demonstrated neuroprotective qualities of non-NMDA receptor antagonists after cerebral ischaemia (Buchan *et al.*, 1991; Sheardown *et al.*, 1990) and epileptic seizures (Loscher, 1998; Fletcher & Lodge, 1996).

Once Glu was identified as a neurotoxin, many studies soon followed implicating endogenous Glu in various disease states such as ischaemia, Alzheimer's disease and epilepsy. Firstly, in studies of focal and global ischaemia, high concentration of extracellular Glu were recovered from damaged brain regions (Faden *et al.*, 1989), within the same range as that found to be toxic *in vitro* (Choi *et al.*, 1987). The most persuasive evidence to implicate Glu in ischaemic damage has come from studies employing Glu receptor antagonists, particularly the NMDA receptor antagonist MK-801, where there is an attenuation in post-ischaemic neuronal damage (Bond *et al.*, 1999; Ikonomidou & Turski, 1995). Less established is the role the non-NMDA receptors play in mediating

ischaemic damage, due to the lack of selective antagonists and to conflicting evidence (Gill & Lodge, 1997; Muir & Lees, 1995; Sheardown *et al.*, 1990). Clinical trials have successfully begun in treatment of stroke using both NMDA receptor antagonists and other agents that inhibit the release of Glu, such as sodium channel blockers (Muir & Lees, 1995), however a number of non-NMDA antagonists have also been shown to reduce ischaemic stroke injury (Gill & Lodge, 1997). Like ischaemic damage, prolonged epileptic seizures share similar histopathological patterns of neuronal damage. Hence, a common pathway for acute neuronal damage has been postulated and is most likely to involve energy deficiencies and overstimulation of Glu receptors (Olney, 1983). Both KA and NMDA have been strongly implicated in seizure-mediated damage (Ben-Ari, 1981), since both neuronal damage and seizure activity are reduced by the appropriate iGluR antagonists (Dingledine *et al.*, 1990), and KA and NMDA administration result in seizure activity (Loscher, 1998; Van Den Pol *et al.*, 1996).

While Glu is implicated in acute neuronal damage it is also involved in the slow, chronic neurodegenerative diseases. ALS is characterized by selective loss of the lower motorneurones of the spinal cord and the upper motorneurones in the cerebral cortex (Mitosumoto *et al.*, 1998), and patients suffering ALS eventually die from paralysis. Evidence to implicate Glu in ALS have come from increased levels of Glu in the plasma and cerebrospinal fluid of patients suffering ALS, and there is some evidence to suggest impaired Glu uptake systems (Mitosumoto *et al.*, 1998).

Evidence to suggest Glu mediates various neurodegenerative diseases, such as Alzheimer's disease, has become stronger in the past few years due to the increase in interest in Glu uptake systems. Alzheimer's disease, a severe neurodegenerative disease, is characterized by severe memory loss and histologically by the presence of plaques and neurofibrillary tangles. A recent hypothesis has been advanced in Alzheimer's disease

implicating the deficient functioning of GluTs, leading to neurodegeneration resulting from the failure to clear excess Glu from the synaptic cleft (Masliah *et al.*, 1996). Moreover, post-mortem analyses of brains from patients suffering Alzheimer's demonstrate a marked increase in the expression of mRNA of the AMPA subunits GluR1-3 throughout the brain (Garcia-Ladona *et al.*, 1994). Exposure of human spinal neurones to Glu results in morphological changes resembling neurofibrillary tangles (De Boni & McLachlan, 1985) and in the presence of β-amyloid, a peptide associated with plaques, GluR-mediated toxicity is exacerbated (Gray & Patel, 1995).

Huntington's chorea, a hereditary condition characterised by the select degeneration of the spiny neurones of the neostriatum, can be mimicked in animal models by intrastriatal injections of KA, which induces biochemical and morphological characteristics closely paralleling this disease (Coyle *et al.*, 1978). Cerebrospinal fluid of patients with Huntington's chorea demonstrates elevated levels of Glu, and there is a depletion of the NMDA receptors in the striatum (Young *et al.*, 1988). More recently transgenic animal models of Huntington's disease have demonstrated a decrease in the expression AMPA and KA receptors, whereas no change in NMDA receptor expression is evident (Cha *et al.*, 1998). Transgenic animal models of Huntington's disease have a reduced sensitivity to KA and NMDA receptor-mediated toxicity (Morton & Leavens, 2000; Hansson *et al.*, 1999) again indicating differential GluR function and expression.

Post-mortem tissue from Parkinson's disease, a progressive neurodegenerative disease characterized by rigidity, tremor and bradykinesia, demonstrate a selective degeneration of neuromelanin-containing neurones, especially the nigral dopaminergic neurones. While the evidence to link excitotoxicity to Parkinson's disease is limited, animal models employing 1-methyl-4-phenylpyridinium (MPP^+) to selectively damage dopaminergic neurones, are protected by the NMDA receptor antagonist MK-801 (Coyle

& Puttfarcken, 1993). Recently it has been shown that non-NMDA receptor antagonists are also effective at protecting MPP⁺-induced lesions in this model of Parkinson's disease (Merino *et al.*, 1999; Klockgether *et al.*, 1991), also implicating the non-NMDA receptors in this neurodegenerative disease. Studies employing the 6-hydroxydopamine lesion model of parkinsonism have demonstrated a marked decrease in the NMDA receptor subunits NR1 and NR2B (Dunah *et al.*, 2000), but this evidence is not consistent with other findings (Ball *et al*, 1994).

1.1.6.2 Apoptosis versus Necrosis

Glu receptor-mediated toxicity, or excitotoxicity, involves two forms of neuronal cell death, necrosis and apoptosis, dependent on the intensity of the insult and mitochondrial function (Ankarcrona *et al.*, 1995). Necrosis, or accidental cell death, is characterized by a rapid swelling of the cell, caused by a rapid influx of Na^+ ions followed by a passive, secondary influx of water and Cl^- ions. These biochemical changes result in the disruption of the internal and external cellular membranes, causing lysis of the cellular contents and thereby releasing cytoplasmic material, which is often toxic to neighbouring cells (Steller, 1995). Necrosis is typically caused by osmotic, chemical and physical damage to the cell and frequently results in an inflammatory response *in vivo* (Kroemer *et al.*, 1995). Apoptosis, from the Greek term referring to the naturally occurring seasonal loss or falling leaves (Wyllie *et al.*, 1980), is a much slower process involving complex mechanisms requiring energy and protein synthesis (Dessi *et al.*, 1994a). Apoptosis is a normal physiological event that is involved in the removal of damaged cells in most major organ systems, ensuring a balance between cellular loss and proliferation. Apoptosis occurs in various cell types throughout development and ageing, and is essential for the development of various tissues, including the CNS (Kroemer *et al.*, 1995; Oppenheim,

1991). Morphological changes induced by apoptosis are characterized by cell shrinkage, nuclear condensation, oligonucleosomal fragmentation of nuclear DNA to fragments of approximately 50-300 base pairs, neurite degeneration and the extrusion of intracellular organelles and nuclear fragments, which *in vivo* are rapidly digested by macrophages or by neighbouring cells (Philpott *et al.*, 1996; Kroemer *et al.*, 1995). The rapid removal of these cells protects healthy cells from the leakage of potential harmful substances from the dead cells (Steller, 1995; Clarke, 1990). Plasma membrane integrity is still maintained during apoptosis, unlike necrotic cell death, until the cell signals its own phagocytosis through an alteration of phosphatidylserine in the plasma membrane (Savill *et al.*, 1993). Apoptosis is thought to be largely due to the intracellular overload of Ca^{2+} ions, where evidence from *in vitro* model systems have demonstrated that removal of Ca^{2+} from culture medium hinders cell death (Dessi *et al.*, 1993), and the use of various Ca^{2+} channel antagonists can attenuate Glu receptor-mediated excitotoxicity (Malcolm *et al.*, 1996).

An excessive influx of Ca^{2+} leads to elevation of free cytosolic Ca^{2+} and the resultant loss of Ca^{2+} homeostasis can subsequently influence numerous cellular mechanisms such as the activation of kinases and proteases, changes in cellular metabolism and changes in gene expression, resulting in severe cellular injury (Siesjo *et al.*, 1989) (Figure 1.4). An increase in free cytosolic Ca^{2+} can also result in the generation of free radicals through activation of various phospholipases. Furthermore, a rise in intracellular Ca^{2+} can also stimulate further release of Glu, worsening cellular damage by means of a positive feedback loop (Choi *et al.*, 1987). Phospholipases act to break down the cell membrane liberating arachidonic acid and causing lipid peroxidation with the subsequent generation of oxygen free radicals, including superoxide (O_2^-) and hydroxyl ($HO^{\cdot}$) radicals (Choi *et al.*, 1987). In addition, the enzyme nitric oxide synthase is activated by a rise in intracellular Ca^{2+}, especially through the NMDA channel, and is also involved in the
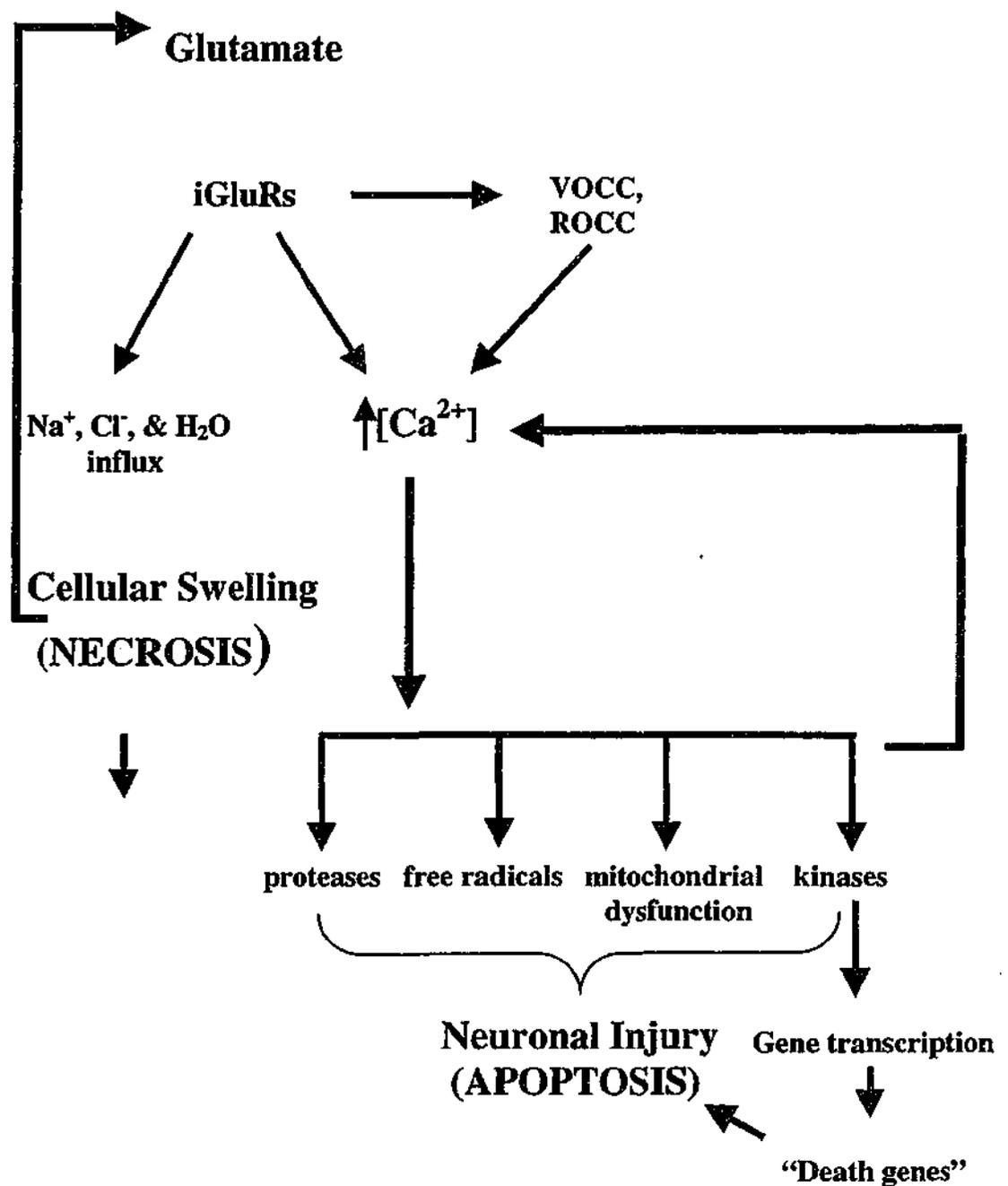
Figure 1.4 The Excitotoxic Cascade

Overstimulation of Glu receptors can cause cell death by a process known as excitotoxicity. Excitotoxicity involves two forms of cell death; necrosis and apoptosis. Necrosis involves cellular swelling and lysis of the intracellular contents. Apoptosis is a more complex mechanism of cell death, involving various kinases, proteases and the expression of various "death" genes, mediated by a rise in intracellular Ca^{2+} from the activation of receptor (ROCC) and voltage operated Ca^{2+} channels (VOCC) and Glu receptor activation. (Adapted from McDonald *et al.,* 1990).

generation of free radicals, including nitric oxide (NO·) and peroxynitrite (NO$_2^-$) (Dawson, 1995). When prolonged stimulation of Glu occurs, excessive amounts of nitric oxide and superoxides are produced (Lipton & Rosenberg, 1994). Much evidence implicating the involvement of free radical generation in Glu-induced apoptosis has come from studies whereby free radicals scavengers have attenuated Glu receptor-mediated cellular damage (Simonian & Coyle, 1996; Whittemore *et al.*, 1994) and free radicals are generated after excessive GluR stimulation (Savolainen *et al.*, 1995; Coyle & Puttfarcken, 1993). Glu-mediated Ca^{2+} influx may play an important role in mediating apoptosis. Glu mediates a rise in intracellular Ca^{2+} by stimulating release from intracellular stores (Frandsen & Schousboe, 1993) and allowing Ca^{2+} influx from the extracellular spaces. Interestingly, voltage-operated Ca^{2+} channel blockers are not protective against neurodegeneration that occurs quickly after Glu receptor stimulation (Weiss *et al.*, 1990), but are highly protective against the slower, apoptotic type of Glu-mediated cell death (Pizzi *et al.*, 1991; Weiss *et al.*, 1990).

In vitro studies have further unravelled the mechanisms involved in excitotoxic cell death, since *in vivo* investigations are often difficult to interpret and are limited by the complex nature of neuronal networks and the inherent heterogeneity of the neuronal milieu, plus contributions by phagocytes and by various Glu uptake mechanisms masking the effects of Glu. Indeed, in a classical series of studies employing neuronal cultures Choi *et al.* (1987 & 1988) drew attention to the neurotoxic and pharmacological profile of excitotoxic injury suggesting that Glu-mediated excitotoxicity was occurring by necrosis. Later studies demonstrated lower concentrations of Glu could indeed also result in apoptosis (Cheung *et al.*, 1998b; Nicotera *et al.*, 1997; Portera-Cailliau *et al.*, 1997a; Ankarcrona *et al.*, 1995). Necrosis seems to be mediated by both NMDA and non-NMDA receptors, since Glu receptor-mediated toxicity is only partially attenuated by the selective

NMDA receptor antagonist MK-801, with larger attenuation achieved by the co-addition of the non-NMDA receptor antagonist CNQX (Cheung *et al.*, 1998b; Koh & Choi, 1991). NMDA receptor agonists have been implicated, and well documented, to produce apoptotic cell death in primary neuronal cultures after short exposure times (5-30min) (Qin *et al.*, 1996). More recently non-NMDA receptors have also been implicated in excitotoxicity (Moldrich *et al.*, 2000a; John *et al.*, 1999; Larm *et al.*, 1997a; Frandsen *et al.*, 1989). However, for similar toxicity to that achieved by NMDA receptor activation, longer exposure to non-NMDA receptor agonists is required (12-48h) (John *et al.*, 1999; Larm *et al.*, 1997b; Larm *et al.*, 1996), suggesting a possible role in chronic neurodegenerative diseases. Overstimulation of the KA receptor can result in apoptosis as demonstrated by studies conducted *in vitro* (Cheung *et al.*, 1998a; Pollard *et al.*, 1994) and *in vivo* (Gillardon *et al.*, 1995, LeGal LaSalle, 1988), as does overstimulation of the AMPA receptor (John *et al.*, 1999; Larm *et al.*, 1997b; Larm *et al.*, 1996).

Apoptosis is a tightly controlled mechanism involving gene and protein synthesis (Sastry & Rao, 2000; Dessi *et al.*, 1994a). Numerous genes have been identified as "death" genes whereby activation leads to apoptosis, or as anti-apoptotic genes that protect cells from various apoptotic stimuli. Genes thought to be involved in apoptosis have been commonly identified through investigations of the processes regulating cellular number in the nematode *Caenorhabditis elegans*, where homologues of many mammalian genes have been identified (Bargmann & Kaplan, 1998). Some examples of "death" genes include *c-myc*, interleukin converting enzyme (ICE) and BAX, whereas anti-apoptotic genes include Bcl-2 and p35 (Steller, 1995; Vaux *et al.*, 1988) (see table 1.4).

Table 1.4 Examples of genes that control apoptosis.

Gene Name	Other common name	Function or Mechanism of action
PRO-APOPTOTIC		
caspase 1	ICE, CED-3	Cleaves proteins responsible for cell repair and cytoskeletal integrity
caspase 2	ICH-1, NEDD-2	
caspase 3	CPP32, Yama, apopain	
caspase 6	Mch2	
caspase 9	ICE-LAP6, Mch6	
caspase 10	FLICE2, Mch4	
Bad		Binds Bcl-2 or Bcl-X_L, blocking their anti-apoptotic effects. Act on the mitochondrial membrane
BAX		
BAK		
BID		
Bcl-Xs		
ANTI-APOPTOTIC		
Bcl-2		Binds BAX and other pro-apoptotic molecules. Controls the mitochondrial permeability transition.
Bcl-X_L		
p35		Binds caspases subsequently inhibits their activity
PI3K		Mediate transcription through the Nuclear Factor $\kappa\beta$ signaling pathway. Regulates the phosphorylation of Bad and inhibits caspase activity.
AKT	Protein kinase B	

1.1.6.3 In vivo versus In vitro Models of Excitotoxicity

In vivo models of toxicity have allowed invaluable insights into the way in which different types of cells interact with each other, for example, the way in which apoptotic cells are phagocytosed by macrophages (Fadok, 1999). However, the limitations of *in vivo* models are numerous and can be related to the difficulties encountered by investigators in many early studies. Identification of apoptotic cell bodies can be difficult *in vivo* due to the similarity in appearance of autophagotic vacuoles (Kerr, 1995) and the rapid clearance of dying cells by the immune system (Fadok, 1999). In addition, when exogenous compounds are being examined, consideration must be given to the accessibility of the target tissue, for instance the brain is protected by the blood brain barrier and hence many compounds cannot readily enter the brain. Due to this limitation various compounds may be applied directly to the brain region of interest and use of such a technique can cause physical damage to the tissue resulting in necrotic cell death. In addition, the brain contains many glial cells, which are a strong protective system for the neurones. Glial cells secrete a variety of growth factors including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), two well known neuroprotectants (Mattson & Scheff, 1994).

Early excitotoxicity studies, conducted *in vivo,* used extremely high systemic doses of Glu to produce cell death in mature animal models (McBean & Roberts, 1984). In addition to high concentrations required to cause cell death, even higher concentrations were required to penetrate through the inner layers of the brain (Garthwaite & Wilkin, 1982). Studies conducted with Glu itself have caused further problems due to the efficient uptake systems present *in vivo* (Vandenberg, 1998). Thus most early studies employed either NMDA or KA as excitotoxins, as neither of these compounds are readily taken up by GluTs, and are indeed more potent neurotoxins *in vivo* than Glu itself (Johnston *et al.*, 1979). *In vitro* models of excitotoxicity have allowed the dissection of the intracellular

pathways that mediate the cell death in a controlled milieu (Choi *et al.*, 1987). The full extent of toxicity mediated by Glu and other agonists is clearly evident in *in vitro* model systems. These systems are particularly favourable, as GluTs can be readily blocked, allowing minute quantities of agonist to produce an insult (Frandsen & Schousboe, 1990a). *In vitro* studies revealed that Glu was a much more potent neurotoxin than what was initially believed from studies conducted *in vivo* (Choi *et al.*, 1987). Not only was the concentration of Glu required to cause cell death some 1000-fold less than that required to induce injury in the adult brain *in vivo* (Frandsen & Schousboe, 1990a), the actual type of cell death was more readily examinable (Ankarcrona *et al.*, 1995). Clearly the intact nervous system, containing glial cells and GluTs, masked the potency of Glu as a neurotoxin. In addition, excitotoxicity was for the first time shown to be mediated by two forms of cell death, namely an initial necrotic cell death followed by a slower phase of cell death (Cheung *et al.*, 1998b; Portera-Cailliau *et al.*, 1997b; Ankarcrona *et al.*, 1995; Choi *et al.*, 1987), subsequently shown to be apoptosis (Cheung *et al.*, 1998b; Larm *et al.*, 1997b; Ankarcrona *et al.*, 1995). With current culture technology various specific cell types can be isolated and readily examined. Such cultures include hippocampal cells, cerebellar granule and Purkinje cells, neocortical cells, glial cells and mesencephalic neurones. Therefore, while *in vivo* studies are essential for the full understanding of mechanisms in a physiological environment, *in vitro* studies have allowed the cellular and molecular examination of excitotoxicity in a controlled milieu with less confounding influences that are found in studies conducted in the intact CNS.

While the evidence implicating non-NMDA receptors in various neurological and neurodegenerative conditions has grown considerably over the last 5 years, much less is known about the mechanistic aspects of how they mediate cell death, and in particular apoptosis. This thesis examines Glu receptor-mediated apoptosis, and in particular

neurotoxicity mediated by the KA subtype of non-NMDA receptor, and the involvement of various pathways that have been implicated in neuronal apoptosis, including the cell cycle, Bcl-2, and the MAP kinases families of proteins. The following overview will provide a background of these pathways to introduce concepts surrounding their involvement in apoptosis and in normal physiological processes.

1.2 THE BCL-2 FAMILY

Bcl-2 is a proto-oncogene first discovered as a result of a chromosomal translocation [t(14;18)] in human B cell lymphomas and leukemias (Tsujimoto *et al.*, 1984). Like other proto-oncogenes Bcl-2 overexpression results in tumor formation, however unlike most proto-oncogenes, Bcl-2 does not enhance proliferation in transfected cell lines (Nunez *et al.*, 1990), but enhances the survival of various cell types when deprived of trophic support (Nunez *et al.*, 1990; Vaux *et al.*, 1988). The gene product of Bcl-2 is a 26kDa protein that is localized in the mitochondrial, endoplasmic reticulum and perinuclear membranes (Hockenbery *et al.*, 1991; Chen-Levy *et al.*, 1989). The exact mechanism by which Bcl-2 produces its protective effects are still unknown, but is thought to involve mitochondrial Ca^{2+} (Green & Reed, 1998) and an anti-oxidant function (Skulachev, 1998). During apoptosis cytochrome c (cyto c) is released from the mitochondria, an event that is attenuated by Bcl-2 and by the inhibition of caspases. The release of cyto c from the mitochondria can lead to either necrosis, via a disruption of electron transport, or apoptosis through the activation of various downstream events, involving caspases (Green & Reed, 1998). The role caspases play in apoptosis is discussed later in this chapter. The normal physiological function of Bcl-2 remains largely unknown, despite the great detail of interest and research into this area. Upon cloning Bcl-2 it was evident that the protein products of the normal and oncogenic form were identical

(Hockenbery *et al.*, 1991; Chen-Levy *et al.*, 1989), thus its oncogenic properties comes from the overexpression of the gene, rather than a mutation causing a deregulation of its function.

Bcl-2 was the first member of a superfamily of proteins found to be involved in the apoptotic pathway. Since then 14 homologs of Bcl-2 have been described, including the pro-apoptotic proteins BAX, Bcl-X_S, Bak, Bid, Bik and Bad and the anti-apoptotic proteins Bcl-X_L, Bcl-W, Mcl-1, Nr-13, A1/Bfl-1 and CED-9 (Chao & Korsmeyer, 1998; Boise *et al.*, 1995). The common feature between these proteins is defined by two common homologous domains, termed BH1 and BH2 (Williams & Smith, 1993). The third and fourth domains, termed BH3 and BH4, are shared between some of the Bcl-2 family members, but not all (Chittenden *et al.*, 1995). The homologs of Bcl-2 have been discovered through a variety of molecular biological techniques. Bcl-X was discovered through low stringency hybridization in chickens with mouse Bcl-2 (Boise *et al.*, 1993). The Bcl-X clone hybridized to chicken, mouse and human DNA, suggesting conservation across these species. However, Bcl-X and Bcl-2 probes bind different genomic fragments suggesting they recognize separate genetic loci. Screening studies further identified two proteins encoded on the Bcl-X gene, termed Bcl-X_S and Bcl-X_L. The longer cDNA clone, Bcl-X_L, encodes a 233 amino acid protein, whereas Bcl-X_S encodes a 170 amino acid protein. Homologous regions between Bcl-X and Bcl-2 include the first 20 amino acids in the NH_2 terminal. Additional biochemical differences between the two Bcl-X splice variants have been identified, and are related to the way in which these proteins are implicated in apoptosis, where Bcl-X_S is pro-apoptotic and Bcl-X_L is anti-apoptotic (Fang *et al.*, 1994).

BAX (Bcl-2 associated X protein) is a 21kDa protein, first identified through its co-immunoprecipitation with Bcl-2 (Oltvai *et al.*, 1993). BAX does not covalently bind Bcl-2,

and shares 21% homology with Bcl-2. Overexpression of BAX in numerous paradigms has been demonstrated to cause or to accelerate cell death. In addition, the protective effects of Bcl-2 seem to be dependent on the ratio of Bcl-2 to BAX. When BAX is in excess and more BAX/BAX homodimers are formed, and cells undergo apoptosis, whereas when Bcl-2 predominates more Bcl-2/BAX heterodimers are formed and the cells are protected against apoptosis (Sedlak *et al.*, 1995; Yin *et al.*, 1995; Oltvai *et al.*, 1993).

1.2.1 Interactions Between the Bcl-2 Family of Proteins

The interactions between the Bcl-2 family of proteins are mediated via the BH1, BH2 and BH3 domains. BH1 and BH2 are responsible for mediating the anti-apoptotic function of Bcl-2 and its interactions with BAX (Yin *et al.*, 1994). Subtle changes in the amino acids sequence of these two domains prevent heterodimerization with BAX, but do not effect homodimerization (Yin *et al.*, 1994). There is evidence to suggest that BAX is a common partner for the interactions between the Bcl-2 family of proteins (Sato *et al.*, 1994). Bcl-2 interacts with Bcl-2, Bcl-X_L, MCL-1 and A1 (Sedlak *et al.*, 1995), Bad (Yang *et al.*, 1995), Bak (Chittenden *et al.*, 1995), but not Bcl-X_S, which does not contain the BH1 and BH2 motifs, indicating the importance of these domains in determining protein interactions.

1.2.2 The Bcl-2 Family and the Nervous System

Cell death plays an important role in organ homeostasis and development. Within the CNS apoptosis is a closely controlled mechanism, whereby populations of cells are closely matched to available trophic support ensuring adequate innervation of all target tissues (Oppenheim, 1991). During the development of the mammalian CNS

approximately a third of the neuronal population dies before birth (Oppenheim, 1991). Physiological cell death throughout the CNS is though to occur by apoptosis, and is therefore under genetic control (Lo *et al.*, 1995). Therefore, not surprisingly, the Bcl-2 family of genes have been implicated in shaping the CNS (Martinou *et al.*, 1994b; Merry *et al.*, 1994) and in pathophysiological neuronal cell death (Offen *et al.*, 1998; Tamatani *et al.*, 1998; Clark *et al.*, 1997; Chen *et al.*, 1995). During the development of the nervous system BAX is down-regulated directly after the widespread developmental cell death that occurs after birth, but there is no change in Bcl-X expression (Vekrellis *et al.*, 1997). During neuronal development Bcl-2 is widely expressed, but declines with age (Merry *et al.*, 1994). Transgenic mice, overexpressing Bcl-2 shows reduced neuronal cell death during the development of the nervous system (Farlie *et al.*, 1995; Martinou *et al.*, 1994b) leading to hypertrophy of the brain (Martinou *et al.*, 1994b), suggesting an inhibition of the normal developmental neuronal cell death.

1.2.3 The Bcl-2 Family and Apoptosis

Whereas the anti-apoptotic affect of Bcl-2 is generally accepted, there is evidence to suggest that Bcl-2 expression does not play a central role in apoptosis. Studies conducted in post-mortem human brain of patients with Parkinson's and Alzheimer's diseases have shown no difference in Bcl-2 expression when compared to age-matched controls (Vyas *et al.*, 1997). A study conducted in HeLa cell lines found that phosphorylation of Bcl-2 is a marker of the mitotic phase events rather than a determinant of apoptosis (Ling *et al.*, 1998).

The anti-apoptotic function of Bcl-2 is thought to be dependent on its ability to dimerize with other members of the Bcl-2 family, including BAX. In contrast to Bcl-2, BAX overexpression can promote apoptosis, and therefore an anti-apoptotic function of

Bcl-2 is to bind, and thus deregulate the actions of BAX (Sedlak *et al.*, 1995; Yin *et al.*, 1995; Yin *et al.*, 1994; Oltvai *et al.*, 1993). Another hypothesis is that Bcl-2 prevents cell death by blocking the generation of free radicals (Hockenbery *et al.*, 1993). Since Bcl-2 can block numerous causes of cell death, data collectively indicating that it plays a central role in apoptosis. There are however, some apoptotic paradigms that seem to be independent of the Bcl-2 family dependent upon the neuronal cell type (Middleton *et al.*, 2000; Allsopp *et al.*, 1993).

Bcl-2 has been shown to block apoptosis in a number of toxic paradigms including 1-methyl-4-phenylpyridinium (MPP^+) exposure (Offen *et al.*, 1998), NGF withdrawal in sympathetic neurones (Garcia *et al.*, 1992), staurosporine and ceramide-induced apoptosis (Srinivasan *et al.*, 1996), and excitotoxicity mediated by the AMPA receptor (Cheung *et al.*, 2000; Zhong *et al.*, 1993). BAX expression is up-regulated after KA-induced seizures in mouse brain (Gillardon *et al.*, 1995), during normal neuronal degeneration in development (Vekrellis *et al.*, 1997) and in cultured neurones undergoing excitotoxin-mediated apoptosis (Xiang *et al.*, 1998). Interestingly, Bcl-2 expression is decreased after exposure to neuroprotectant and neurotoxic concentrations of NMDA in cultured cerebellar granule cells, suggesting a direct interaction between Glu receptor agonists and the regulation of Bcl-2 expression (Montpied *et al.*, 1993). Moreover, transgenic mice whereby Bcl-2 is overexpressed, demonstrate decreased neuronal cell death during periods whereby natural cell death is part of shaping the development of the nervous system (Montpied *et al.*, 1993). Interestingly, BAX is down regulated during PC12 cell differentiation and postnatal development of the cortex and cerebellum (Vekrellis *et al.*, 1997). While the exact mechanism of the anti-apoptotic function of Bcl-2 is still largely unknown there is evidence to suggest that an interaction with mitochondria plays an important role (Green & Reed, 1998). Cyto c release from the mitochondria can lead to

both apoptosis and necrosis depending on the cell type and the presence of endogenous caspase inhibitors, including Bcl-2 (Ellerby *et al.*, 1997). Indeed, the mitochondrial permeability transition is suggested to be the rate limiting step in apoptosis (Kroemer *et al.*, 1998). Recently, overexpression of Bcl-2 was shown to attenuate apoptosis, but not necrosis (Cheung *et al.*, 2000), suggesting an involvement of another mechanism in the regulation of how cells respond to necrotic stresses. Interestingly, the Bcl-2 family members BAX, Bik and Bad all trigger cyto c release from the mitochondria, but for these proteins to exert this effect they must be translocated from the cytosol to the mitochondria by a mechanism still to be elucidated (Nomura *et al.*, 1999). Cyto c release from the mitochondria is essential for the activation of the effector pro-caspase-9 by apoptosis protease-activating factor 1 (Apaf-1). Cyto c release can be inhibited by the overexpression of Bcl-X$_L$ or Bcl-2 (Shimizu *et al.*, 1999; Rosse *et al.*, 1998). There is indirect evidence to suggest Bcl-X$_L$ is associated with Apaf-1, and in this state the pro-apoptotic function is inhibited, whereby activation is mediated by BAX heterodimerization resulting in the release Apaf-1 (Pettmann & Henderson, 1998). While the exact signaling mechanism that mediates the response from the cell membrane to the mitochondria is still largely unknown, it is believed that two kinase families are involved, namely the mitogen activated protein (MAP) kinases and the phosphoinositide-3-kinase (PI3K) (Scheid *et al.*, 1999; Lee *et al.*, 1998; Maundrell *et al.*, 1997) (Figure 1.5). MAP kinases are discussed in detail later in this chapter.

1.2.4 The Bcl-2 Family and Excitotoxicity

Bcl-2 is protective against various insults and excitotoxicity is no exception. Transfection of Bcl-2 into cortical neurones can rescue neurones up to 8h after Glu
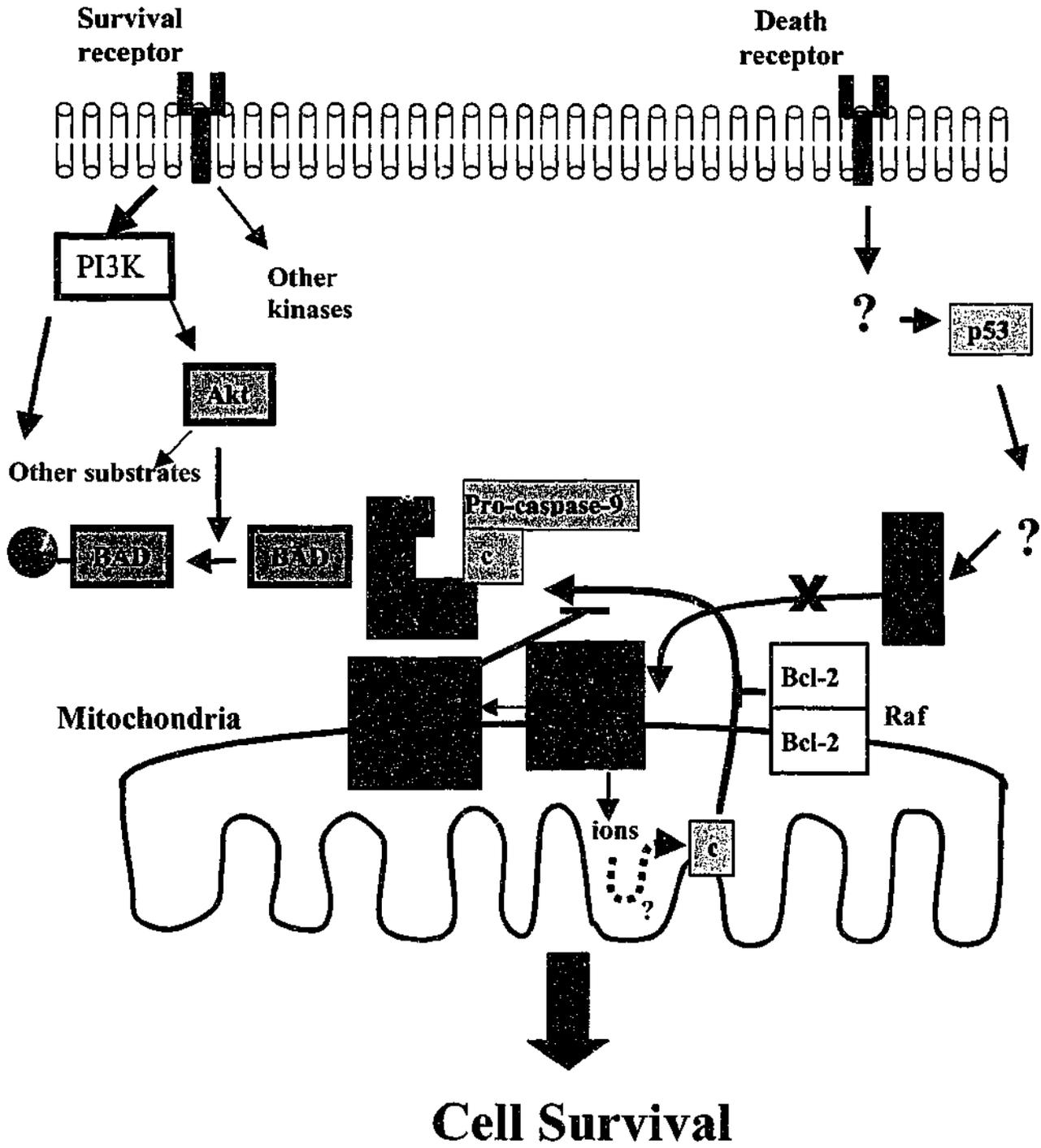
Figure 1.5 The proposed mechanisms of BCL-2

Activation of the survival receptors leads to activation of intracellular kinases, including PI3K. This leads to a cascade of events, including the activation of AKT (PKB), resulting in the inactivation of Bad, and the release of Bcl-X$_L$ allowing heterodimerization, and hence inactivation, with BAX. Cytochrome c (c) release is inhibited by Bcl-2 and Apaf-1, and in the presence of Bcl-X$_L$, cannot activate pro-caspase-9, and hence caspase activation is hindered. (Adapted from Pettmann & Henderson 1998).

exposure (Wang *et al.*, 1997). BAX expression is highly correlated with excitotoxicity (Xiang *et al.*, 1998; Miller *et al.*, 1997) and has been associated with the expression of the pro-apoptotic gene p53 (Xiang *et al.*, 1998). KA-mediated apoptosis induces an up-regulation of Bcl-2 and a down-regulation of BAX (Gillardon *et al.*, 1995), suggesting neurones are attempting to promote their own survival. AMPA receptor-mediated apoptosis is attenuated in transgenic mice overexpressing human Bcl-2, when compared to wildtype control, an effect that is not evident in Glu-mediated necrosis (Cheung *et al.*, 2000). However, transgenic models of Huntington's chorea, although resistant to NMDA receptor-mediated toxicity, often predominantly necrotic, demonstrated no altered expression in the Bcl-2 protein, suggesting another mechanism rendering them resistant to excitotoxicity (Hansson *et al.*, 1999). A study conducted in ganglion cell layer of the rat eye demonstrated an exacerbation of excitotoxicity after viral transfection of the Bcl-2 proto-oncogene (Simon *et al.*, 1999), however protection is evident in cortical cultures (Jia *et al.*, 1996), suggesting the role Bcl-2 plays in apoptosis is dependent upon the cell type.

1.3 THE ICE/CED-3 FAMILY

Apoptosis involves the precise degradation of macromolecules, carried out by enzymes called proteases (Chinnaiyan & Dixit, 1996; Patel *et al.*, 1996). Studies conducted in the nematode *Caenorhabditis elegans* demonstrated the CED-3 gene is critical for apoptosis to occur. Interleukin-converting enzyme 1β (ICE; now known as caspase-1) was soon found to be the mammalian equivalent of CED-3, demonstrating 30% sequence homology. Numerous enzymes related to ICE have since been discovered and are now collectively termed caspases (cysteine aspartic acid specific proteins) (Alnemri *et al.*, 1996). Currently 14 different caspases have been discovered and exhibit varying degrees of

sequence homology (Thornberry & Lazebnik, 1998). Caspases are normally present in the inactive form, procaspases, and converted to their active form, caspases, after apoptotic stimuli by proteolytic enzymes or by autocatalysis. Autocatalysis generally involves the removal of inhibitors or the binding of cofactors (Thornberry & Lazebnik, 1998).

1.3.1 Caspases in the Central Nervous System

CED-3/ICE protease mRNA levels have been identified in various brain regions (Marks & Berg, 1999). However, the exact role these proteases play in neuronal function is still unsure. Transgenic studies conducted in mice have demonstrated that caspase-3 is essential for normal neuronal development, and in the absence of the caspase-3 or caspase-9 gene the brain is grossly deformed and approximately 2-3 times larger than normal brains (Hakem *et al.*, 1998; Kuida *et al.*, 1996). Caspase-3 knockout animals are a lot smaller than their control counterparts and die within 1-3 weeks after birth, probably due to neuronal hyperplasias and huge deformations in brain structure (Kuida *et al.*, 1996). Caspase-9 knockout animals die perinatally with marked structural malformations and enlargement of the cerebellum (Hakem *et al.*, 1998). During neuronal development caspase-2 is highly expressed, however if procaspase-2 is deleted there is no significant difference in the development of the brain (Bergeron *et al.*, 1998). Caspase-1 and -11 knockout animal models show no changes in the neuronal ultrastructure, but rather demonstrate immunological changes (Li *et al.*, 1995).

1.3.2 The ICE Family and Apoptosis

Caspases are responsible for cleaving specific proteins including interleukin 1β (IL-β), DNA fragmentation factor, poly-(ADP-ribose)-polymerase (PARP), and caspase-

activated DNase, which may also mediate the pro-apoptotic activity of caspases. Whereas, many apoptotic paradigms seem to be caspase-dependent, there are studies that demonstrate apoptosis can be caspase-independent. Studies conducted in primary cultures of cerebellar granule cells from BAX knockout animals (Miller *et al.*, 1997) and radiation-induced apoptosis in hippocampal cultures (Johnson *et al.*, 1998) have demonstrated a caspase-independent mechanism of apoptosis, suggesting an alternative pathway by which programmed cell death occurs.

Studies conducted on the amino acid sequences of caspases have allowed the development of caspase inhibitors, which are effective in attenuating apoptosis, but not necrosis (Armstrong *et al.*, 1997). Initially it was found that inhibition of caspases with p35 and Crm A, two viral genes which potently inhibit CED-3/ICE proteases (Xue & Horvitz, 1995; Ray *et al.*, 1992), attenuated neuronal apoptosis (Gagliardini *et al.*, 1994; Rabizadeh *et al.*, 1993). Selective inhibitors of ICE/CED-3 caspases have since been discovered to attenuate apoptosis in motor neurones deprived of trophic support (Milligan *et al.*, 1995) and low K$^+$-induced apoptosis in cerebellar granule cells (Armstrong *et al.*, 1997), but not necrotic cell death caused by high concentrations of Glu (Ankarcrona *et al.*, 1995), NMDA, KA or oxygen-glucose deprivation (Gottron *et al.*, 1997). However, caspase-3 activation seems essential for excitotoxic neuronal cell death in cultured neurones (Ishikawa *et al.*, 1999; Du *et al.*, 1997).

Deshmukh *et al.* (1996) demonstrated that although the non-specific caspase inhibitor bocaspartyl (0-Me)-fluoromethyl ketone inhibited apoptosis after NGF withdrawal in sympathetic neurones, it did not prevent the induction of the immediate early genes *c-jun* and *c-fos*, two common mediators of apoptosis (Cheung *et al.*, 1998a; Guegan *et al.*, 1997; Anderson *et al.*, 1996; Gillardon *et al.*, 1994). Caspase-2 has been implicated in trophic factor withdrawal-mediated apoptosis in PC12 cells (Haviv *et al.*, 1998) and

caspase-1 in apoptosis induced by reactive oxygen-treated in hippocampal neurones (Ishikawa *et al.*, 1999). However, radiation-induced apoptosis in cultured hippocampal neurones is completely independent of caspases, but involves BAX and p53 (Johnson *et al.*, 1998). Interestingly, in post-mortem brain tissue from Alzheimer's patients the activity of caspases is no different from age matched controls (Kitamura *et al.*, 1998), however caspase-3 is responsible for the cleavage of the β-amyloid protein (Gervais *et al.*, 1999). Miller *et al.* (1997) found no significant change in the cleavage of substrates for caspases-2 and -3 in transgenic BAX knockout animals, although there was a marked increase in *c-jun* expression. The role Bcl-2 plays in inhibiting caspases is controversial. Interestingly, caspases seem to cleave Bcl-2 after some apoptotic stimuli, not only rendering them inactive as was initially believed, but promoters of apoptosis rather than inhibitors (Chen *et al.*, 1995; Xue & Horvitz, 1995). Serum withdrawal, ceramide and staurosporine-induced apoptosis in the neuronal cell line GT1-7 overexpressing Bcl-2 does not involve the rapid induction of ICE/CED-3-like caspases (Srinivasan *et al.*, 1996) and low K^+-induced apoptosis in cerebellar granule cells is dependent upon BAX, and only marginally dependent upon caspase-3 (Miller *et al.*, 1997). Pro-caspase-9 is thought to play an essential role in initiating the Bcl-2/caspase pathway, and is therefore commonly referred to as the effector caspase. Pro-caspase-9 is thought to autocatalyse when complexed with Apaf-1 and cyto c and generate caspase-9. The generation of caspase-9 is enhanced by BAX and BAX is thought to inhibit the sequestration of Apaf-1 by Bcl-X_L and to directly promote the release of cytochrome c (cyto c) from the mitochondria to the cytoplasm. Pro-caspase-3 is a substrate for caspase-9 and as discussed above caspase-3 is essential for a variety of apoptotic paradigms (Figure 1.6).
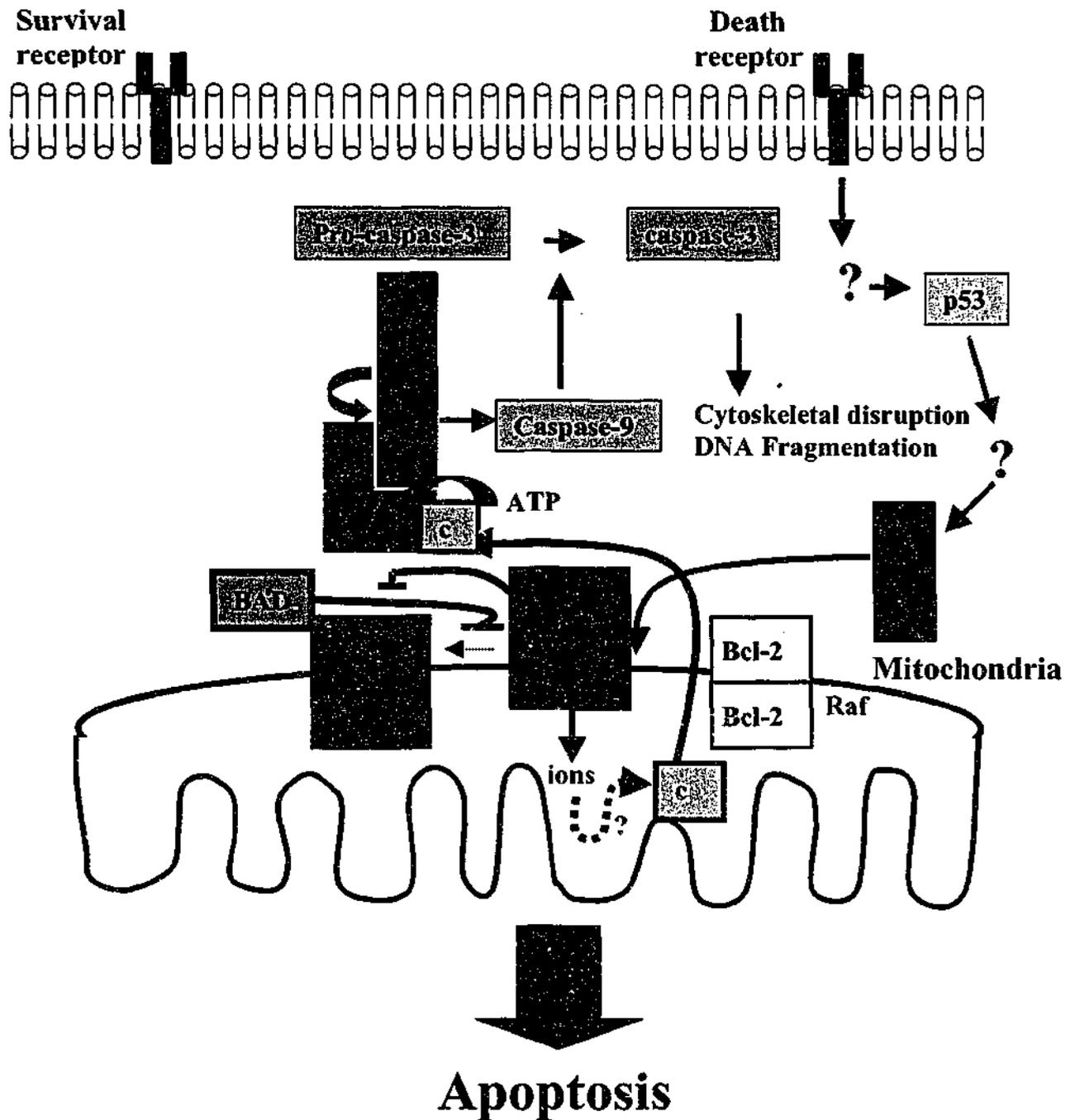
Figure 1.6 The proposed mechanisms of Caspases.
Apoptosis seems to be mediated by caspases, enzymes that degrade various structural proteins and DNA. Most apoptotic paradigms involve the activation of caspase-3. This occurs through a cascade of events, initiated most likely by activation of p53. Pro-caspase-3 is activated by caspase-9. Pro-caspase-9 undergoes autocatalysis once complexed with Apaf-1, cyto c and ATP. This complex is enhanced by translocation of BAX from the cytoplasm to the mitochondria, which inhibits the anti-apoptotic members of the Bcl-2 family, including Bcl-X$_L$ and Bcl-2. c = cytochrome c. (Adapted from Pettmann & Henderson, 1998).

1.3.3 Excitotoxicity and Caspases

Whether excitotoxic neuronal injury is caspase-dependent and/or independent is far from clear. There is some evidence to suggest caspase-3 is required for apoptosis in cultured cerebellar granule cells exposed to low concentrations of Glu, but interestingly, does not require the synthesis of new RNA or protein (Du *et al.*, 1997). The activation of caspase-3, but not caspase-1, by Glu, also closely parallels the time-course for the commitment to neuronal apoptosis (Du *et al.*, 1997). In addition, caspase activity has been associated with various insults *in vivo*, that mediate toxicity through the endogenous release of Glu, including ischaemia (Tamatani *et al.*, 1998), Huntington's chorea (Petersen *et al.*, 1999), and Alzheimer's disease (Masliah *et al.*, 1998). Caspase-3 expression, but not caspase-1 increases in mice after traumatic brain injury (Yakovlev *et al.*, 1997), and in post-mortem brains of Alzheimer's patients (Gervais *et al.*, 1999). Furthermore, the ALS transgenic mouse model demonstrates a greater survival rate when crossed with mutant ICE transgenic mice, indicating that caspases play an important role in the neuronal degeneration of motor neurones (Friedlander *et al.*, 1997), a disease that has been associated with neurotoxicity mediated by the non-NMDA receptors (Couratier *et al.*, 1993). *In vivo* direct administration of the non-selective caspase inhibitor z-VAD.fmk can reduce neurotoxicity mediated by AMPA and to a lesser extent NMDA in the middle artery occlusion model of stroke (Hara *et al.*, 1997). Therefore, the importance of capases is evident in excitotoxic neuronal cell death, however there are caspase-independent mechanisms by which cells can still undergo excitotoxic, apoptotic cell death by a process still to be elucidated, but likely to involve the cell cycle and the Bcl-2 families.

1.4 THE CELL CYCLE GENES

Mitosis is a closely controlled and regulated cellular mechanism. Classically, during embryogenesis, a full complement of neurones is present in the mammalian brain, but are considered to be incapable of mitotic division (Oppenheim, 1991), although neuronal proliferation has been reported on the basis of new evidence in certain subpopulations of neurones (Luo & Miller, 1998; Eriksson *et al.*, 1998; Bengzon *et al.*, 1997; Gould & Tanapat, 1997). Recently it has been hypothesized that genes controlling the cell cycle may be involved in neuronal apoptosis. Direct evidence for this role of cell cycle genes is sparse, however with increased interest in this area the body of supportive findings is growing rapidly (Ross, 1996). Some cellular characteristics are similar in apoptosis and mitosis, including cell rounding, chromatin condensation and the breakdown of the nuclear envelope (Wyllie *et al.*, 1980). Other evidence comes from molecular biology studies whereby numerous genes that control the cell cycle have been reported to be stimulated during neuronal apoptosis, and in some cases can directly cause apoptosis (Ross, 1996). Therefore, neuronal apoptosis has been postulated to be the result of abnormal and/or inappropriate cell cycle entry, and since various genes and proteins required for cell cycle progression are unavailable, due to the post-mitotic state of neurones, the cell may signal itself to die (Ross, 1996).

Somatic cell division is characterized by two phases, mitosis and interphase, and mitosis is further subdivided into four stages: prophase, metaphase, anaphase and telophase. Completion of mitotic division involves chromosome replication and the production of two daughter cells. Cellular division lasts approximately 24 hours and consists of two major phases: the S phase (DNA replication), which lasts approximately 6 hours, and the M phase (mitosis) which lasts about 30 minutes. The S and M phases are

segregated by two gap phases: G1 and G2, which last 12 and 6 hours respectively (Figure 1.7).

1.4.1 Cyclins and Cyclin-Dependent Kinases

Cell cycle progression is controlled by various protein complexes composed of cyclins and cyclin dependent kinases (CDKs), whereby cyclins act as the regulatory unit and the CDKs as the catalytic unit (Prosperi *et al.*, 1997; Nurse, 1990). Cyclins and CDKs regulate the progression through the cell cycle via phosphorylation of various substrates including the retinoblastoma protein (pRb). The cyclin-CDK complex is in turn modulated by various tumour suppressors, such as pRb and p53, and CDK inhibitors including p16, p21 and p27 (Cordon-Cardo, 1995).

Multiple cyclins have been isolated and characterized relative to their expression during various times in the cell cycle. Five major groups of cyclins have been isolated, termed cyclin A-H. Cyclins D1-3, C and E are expressed during the G1 phase of the cell cycle and regulate the transition from G1 to the S phase. Cyclins A and B1,2 reach peak expression during the S and G2 phases of the cycle, and regulate the transition into the M phase (Johnson & Walker, 1999; Ross, 1996). Multiple CDKs have been isolated and their cyclin partners and expression patterns been described. Complexes formed by cyclin D1 and CDK4 are thought to modulate G1 progression, whereas cyclin E and CDK2 modulate entry into the S phase. Cyclin A and CDK2 regulate S phase progression and cyclin B and CDK1 controls entry into the M phase (Lewin, 1990; Nurse, 1990). CDK5 has not been shown to be associated nor activated by any cyclins, however the brain specific protein p35 has been shown to regulate CDK5 (Tsai *et al.*, 1994) and CDK5 has been implicated in various forms of neuronal apoptosis (Pei *et al.*, 1998; Henchcliffe & Burke, 1997; Zhang *et al.*, 1997). From studies involving cancer cell lines, two major checkpoints have been
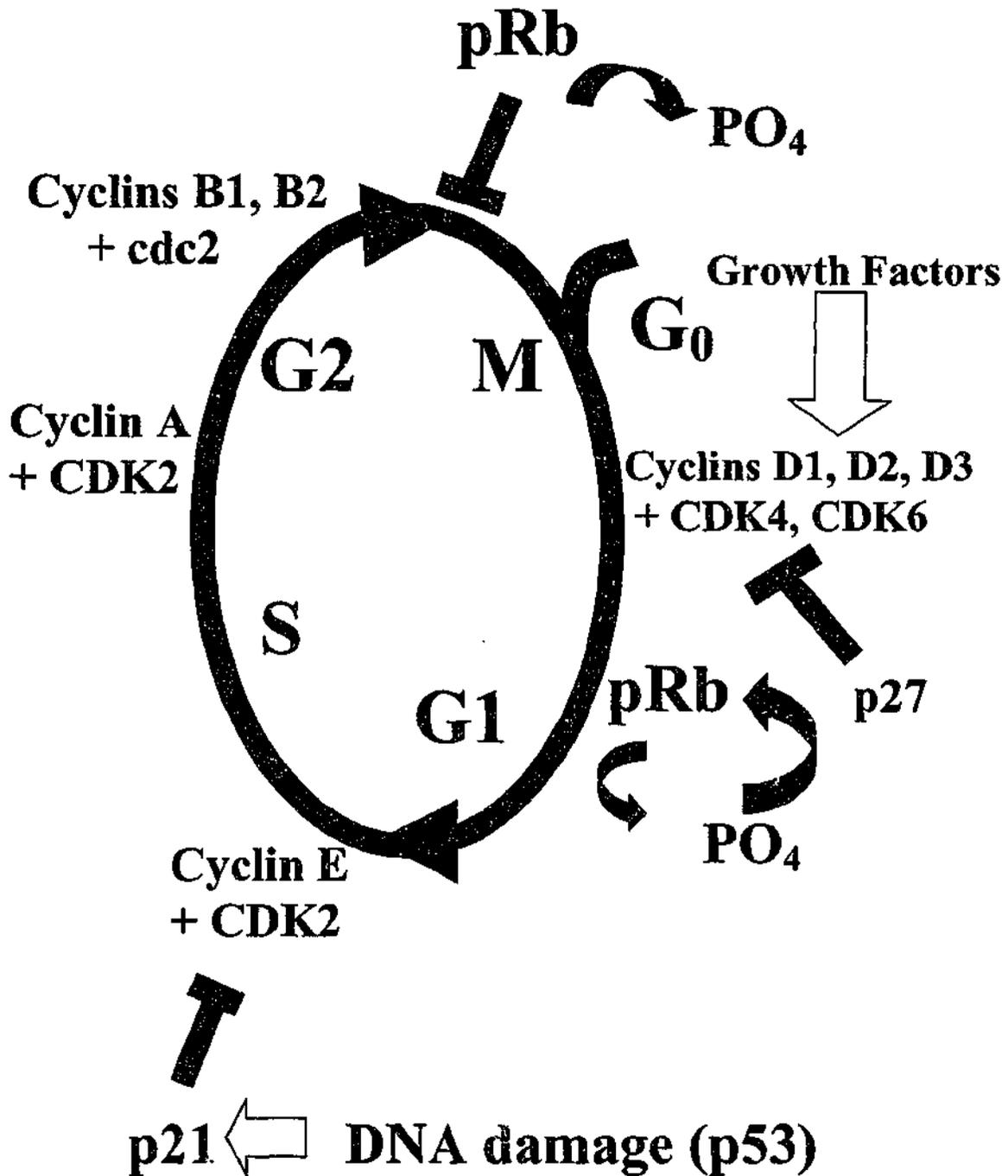
Figure 1.7 The Cell Cycle

The major cycle regulatory proteins, the cyclins and cyclin dependent kinases (CDK) are shown in relation to the cell cycle phases: mitosis (M), first gap (G1), DNA synthesis (S) and second gap (G2) phases. Various factors influence the transition through the cell cycle and include various growth factors and proteins, such as the retinoblastoma protein (pRb), p53 and p21. (Adapted from Ross *et al.* 1996).

identified that ensure the accuracy of cellular division in terms of genetic information and cellular structure, and these occur during the mid to late G1 and G2 phases during mitosis, respectively (Lin *et al.*, 1992; Stewart *et al.*, 1995).

Regulation of cell cycle progression by CDKs is primarily mediated by the inhibition of phosphorylation of pRb, which is a substrate for both CDK4 and CDK6 (Kato *et al.*, 1993). The amount of pRb is relatively stable throughout the cell cycle, however the phosphorylation state of this protein varies dependent on the stage in the cycle. pRb becomes highly phosphorylated during the late G1 and early S phases, remaining in this state until G2. The hypo-phosphorylated state of pRb is the active form of this compound and is responsible for progression through the cell cycle by regulating gene expression through complex formations with DNA binding proteins during G_0 and G1 (Cordon-Cardo, 1995). pRb inhibits E2F-like transcription factors, which bind various promoters such as thymidine kinase, c-myc, DNA polymerase-α and dihydrofolate reductase, which are required for the progression into the S phase (Nevins, 1992). Phosphorylation of pRb by cyclin D- and cyclin E-associated CDKs releases the transcription factors and allows proliferation to advance (Meikrantz & Schlegel, 1995). Endogenous inhibitors of the cyclin D-CDK4 complex, such as p27, are expressed throughout the cell cycle and it is thought that cyclin D must overcome a threshold level to overcome this inhibition before advancement into G1. Cyclin E-CDK2 regulation is also under direct inhibition by p21, which is modulated by DNA damage via the oncogene p53 (Polyak *et al.*, 1994).

p53 is thought to control a cell cycle checkpoint that is responsible for ensuring the fidelity of the genome (Cordon-Cardo, 1995; Stewart *et al.*, 1995; Lin *et al.*, 1992). In addition, it seems that p53 is involved in transcriptional control, possessing a sequence-specific DNA-binding domain (Fields & Jang, 1990). Knockout animals where the p53 and pRb gene have been removed, are predisposed to cancers, emphasising the importance of

these genes in regulating normal cell proliferation (Cordon-Cardo, 1995; Picksley & Lane, 1994). p53 is responsible for monitoring the restriction point that requires damaged DNA to be repaired before entering the S phase, or the cell enters the apoptotic pathway (Cox *et al.*, 1995; Kaufmann *et al*, 1995). Evidence for an apoptotic function of p53 comes from knockout animals, where DNA-damaging agents fail to cause apoptosis (Clarke *et al.*, 1993). p53 acts to modulate cell cycle dynamics via control and regulation of CDK activation and inactivation. p53-dependent G1 arrest induces p21, which subsequently inhibits cyclin D and E activity, preventing the phosphorylation of pRb by CDK activating kinase (CAK) (Dulic *et al.*, 1994; Figure 1.8). Tumour cells containing wild type p53 demonstrate induction of p21 and these cells undergo apoptosis after γ radiation, but in p53 null mutant cells, p21 induction is absent, as is apoptosis (el-Deiry *et al.*, 1994). Thus, the induction of p21 and the consequent inhibition of CDKs, may be an initial step in p53-dependent apoptosis.

1.4.2 Cyclins and Apoptosis

Historically neurones of the adult CNS were believed to be permanently in the G_0 phase of the cell cycle, or to be resting cells unable to re-enter the cell cycle. However, there are now several lines of evidence to suggest that neurones are capable of re-entering the cell cycle, and depending on cellular signaling, becoming committed to die (Ross, 1996), or as some recent studies have demonstrated, to proliferate (Bengzon *et al.*, 1997; Gould & Tanapat, 1997). Many genes that are involved in controlling the cell cycle have been implicated in apoptosis, and/or are activated in apoptotic cells. These genes are expressed in different phases of the cell cycle, as determined by their patterns of temporal expression. However, it generally appears that for apoptosis to occur the cell must pass into

Hypophosphorylated

Phosphorylated

**CYCLIN
D1/CDK**

(−)

Release

p21 ⟵ p53 ⟵ ⌐ **DNA
damage**

APOPTOSIS

S Phase   c-myc
DNA polymerase α
thymidine kinase
dihydrofolate reductase   **Transcription**
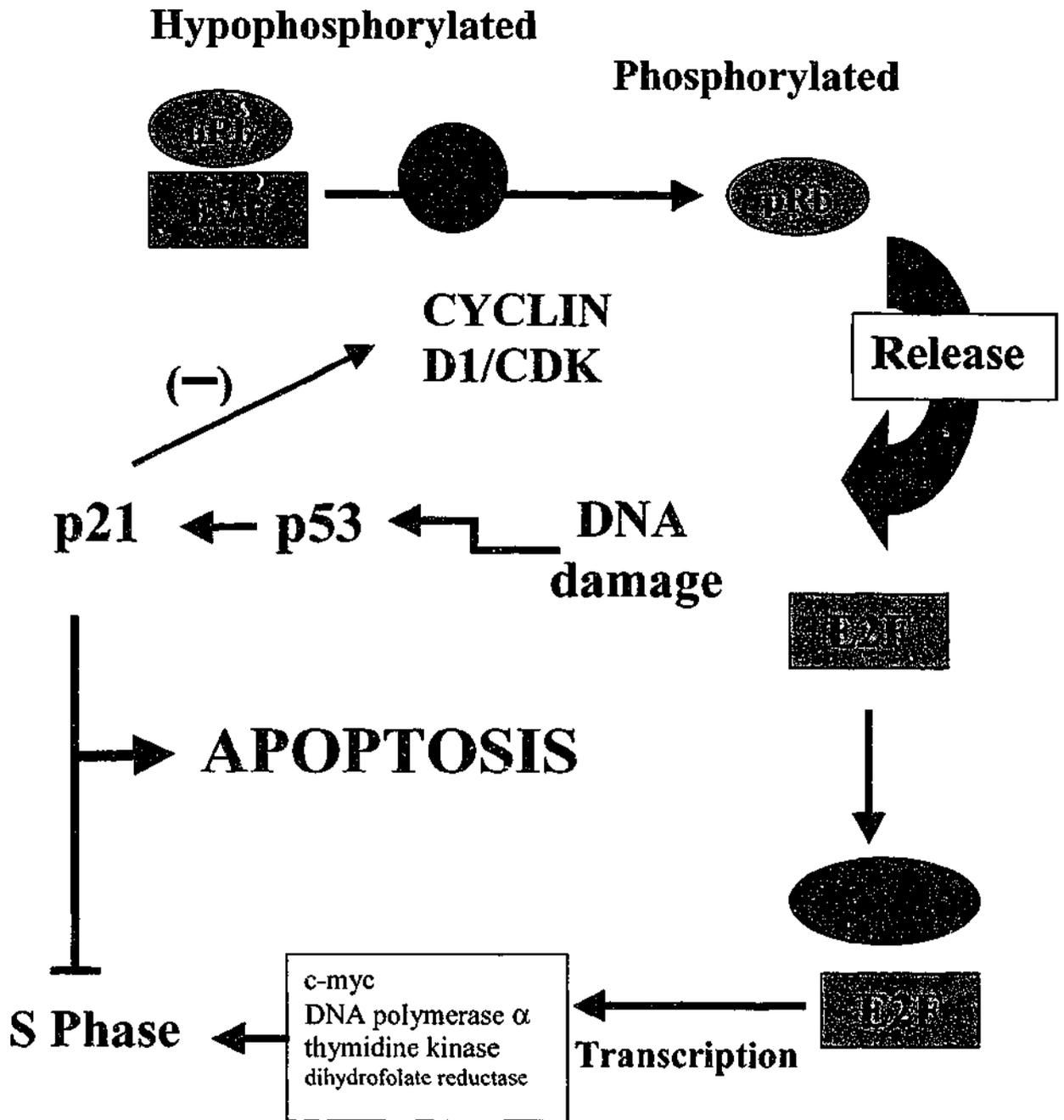
Figure 1.8 Progression into S phase

Hypophosphorylated pRb binds and thus inactivates E2F, a transcription factor. During G1, cyclin D-CDK4/6 levels progressively increase and phosphorylate pRb, and hence inactivate it. The subsequent release of E2F results in the expression of various genes required to enter the S phase. p53 may inhibit S phase entry and hence mediate apoptosis, if for example there is any DNA damage, by the activation of p21. p21 inhibits S phase entry by binding cyclin D1-CDK complexes, and inhibiting their activity. (Adapted from Cordan-Cardo, 1995).

late G1, since arrest prior to G1 blocks apoptosis, whereas arrest after this stage promotes apoptosis (Park *et al.*, 1997; Meikrantz & Schlegel, 1995).

Initial evidence suggesting the involvement of cell cycle regulating proteins in apoptosis came from lymphoma cells undergoing apoptosis mediated by cytotoxic granules from T lymphocytes, where there was a notable activation of the serine-threonine kinase, CDC2 (Shi *et al.*, 1994). Other studies demonstrated cyclin A-dependent kinases were required for apoptosis mediated by the immediate early gene *myc* (Hoang *et al.*, 1994) and for apoptosis in S phase arrested cells (Meikrantz *et al.*, 1994). In addition, cyclin B- and E-dependent kinases seem to involved in DNA damage-induced apoptosis in the human promyelocytic leukemia cell line HL-60 (Shimizu *et al.*, 1995) and cyclin B seems to be involved in NGF withdrawal-induced apoptosis in PC12 cells (Gao & Zelenka, 1995). Furthermore, overexpression of Bcl-2, a well-known anti-apoptotic protein, suppresses levels of CDK2 and attenuates apoptosis in HeLa cell lines (Meikrantz *et al.*, 1994). Cell lines incubated with agents that arrest the cell cycle are resistant to apoptosis, whereas cells arrested in G1/S are sensitized to cell death (Farinelli & Greene, 1996; Meikrantz & Schlegel, 1995), and agents that act to arrest cells in G2 induce apoptosis (Ishida *et al.*, 1992). Inhibition of neuronal apoptosis by hindering the cell cycle seems specific to G1/S cell cycle blockers, since inhibiting the S/G2 phase does not attenuate apoptosis (Farinelli & Greene, 1996; Park *et al.*, 1997). Recently cyclin D1 has been specifically implicated in neuronal cell death (Kranenburg *et al.*, 1996; Freeman *et al.*, 1994;) suggesting cell cycle genes are not only involved in apoptotic pathways in mitotic cells, but also in post-mitotic neurones.

The most convincing evidence to implicate the cell cycle in neuronal apoptosis has come from the use of chemopreventative agents. While some studies have demonstrated that many of these agents are indeed neurotoxins (Park *et al.*, 1998b; Enokido *et al.*, 1996;

Morris & Geller, 1996), some selective CDK inhibitors have been shown to be protective after various apoptotic paradigms in sympathetic neurones (Park *et al.*, 1998a; Farinelli & Greene, 1996), in PC12 cells (Park *et al.*, 1997; Farinelli & Greene, 1996; Park *et al.*, 1996a) and in peripheral neurones (Maas *et al.*, 1998). While these inhibitors are not selective for any one target and therefore the analyses of these studies are difficult, there seems to be a direct correlation between neuronal apoptosis and the cell cycle.

1.4.3 D-Type Cyclins and Apoptosis

As quiescent cells enter the cell cycle D- type cyclins are synthesized. This class of cyclin is rate limiting for S phase entry and are synthesized in different combinations and therefore it is not likely that any particular D- form is essential for G1 progression. The D-type cyclins assemble with CDK4 and 6 acquire catalytic activity by phosphorylation by CAK. The existence of the D-type cyclins is short-lived with half lives of less that 25 minutes, and failure of various growth factors to stimulate their synthesis beyond a threshold level required to overcome inhibition prevents progression passed the restriction point and mitosis in hindered (Sherr, 1995; Sherr & Roberts, 1995). The phosphorylation of pRb by cyclin D-dependent kinases, releases various transcription factors from pRb constraint, enabling the activation of various genes required for S phase entry. Cyclin D-dependent kinase complexes are inhibited by various factors, including p16, p21 and p27 (Figure 1.9). p21 and p27 are universal inhibitors of CDKs, acting to prevent the phosphorylation of cyclin-CDK complexes by CAK (Aprelikova *et al.*, 1995).

Cyclin D1 levels have been found to be selectively elevated in postmitotic neurones undergoing apoptosis (Freeman *et al.*, 1994) and overexpression of the CDK inhibitors, p16 and p27, attenuates apoptosis (Park *et al.*, 1998a). Further evidence suggesting a role
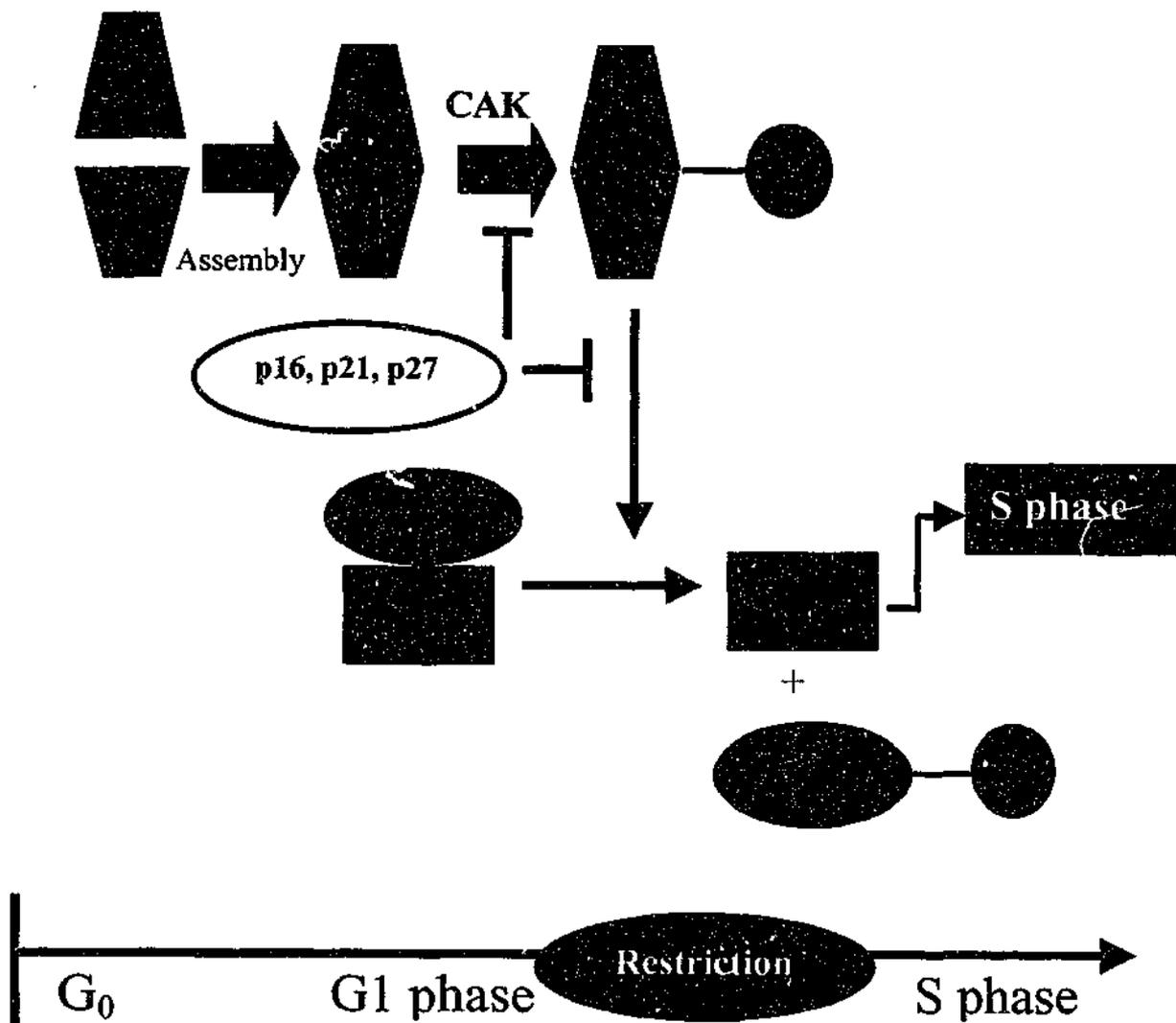
Figure 1.9 Regulators of G1 progression

Early in G1 D-type cyclins (represented by D) are produced and assemble with CDK 4 and 6 (represented by C). This complex requires phosphorylation by CDK activating kinase (CAK) to become active. D-type cyclins phosphorylate pRb and hence release the bound transcription factor E2F, which activates genes required for S phase entry. p16, p21 and p27 inhibit S phase entry by interfering with CDK and cyclin D assembly, or at a later stage to inhibit CAK function. (Adapted from Cordan-Cardo, 1995).

of CDKs in neuronal apoptosis has come from the overexpression of the CDK substrate pRb in PC12 cells, which also prevented apoptosis (Kranenburg *et al.*, 1996). However, whether cyclin D1 is actively involved in the apoptotic pathway is unclear, as increased expression of cyclin D1 stimulates expression of CDKs which may be responsible for cellular injury (Kranenburg *et al.*, 1996). As cyclin D1 mediates the progression through the G1 phase, it may be postulated that neuronal apoptosis results from a failed attempt of re-entry into the cell cycle.

From studies conducted by Freeman *et al.* (1994), cyclin D1 expression was found to be specifically activated in sympathetic neurones undergoing apoptosis mediated by NGF withdrawal. This study compared the activation of various cell cycle genes, including cyclins A, B, C, D1, D2, D3, E, and CDK4 and CDK5. Cyclin A levels were undetectable after NGF withdrawal, as was CDK2 expression. Cyclins B, C, D3 and E and CDK4 and CDK5 expression decreased after NGF withdrawal, however, there was a specific and dramatic increase in cyclin D1 expression. The peak expression of cyclin D1 occurred approximately at the same time that these cells were committed to die by apoptosis (Freeman *et al.*, 1994); that time being when cell death is no longer irreversible by the protein synthesis inhibitor cycloheximide (Wyllie *et al.*, 1984). An interesting point to note from this study is the decline in the expression of both CDK4 and CDK5, both substrates for cyclin D1. These proteins are much more stable than cyclin D1 and therefore, despite the decline in expression, there still may be ample amounts for their interaction with cyclin D1. Studies, where cyclin D1 expression is elevated in cells undergoing apoptosis, have been confirmed by numerous other studies conducted *in vivo* including excitotoxic neuronal cell death in ischaemic models (Guegan *et al.*, 1997) and KA-induced seizures (Liu *et al.*, 1996). *In vitro* studies have also demonstrated a selective upregulation of cyclin D1 in differentiated PC12 cells (Davis *et al.*, 1997). In contrast to these findings, studies

conducted in postmortem brain tissue from human patients with Alzheimer's disease revealed that cyclin Dl expression was not elevated in comparison to controls, but rather cyclin B was elevated in the dentate gyrus and CA1 region of the hippocampus (Nagy *et al.*, 1997; Vincent *et al.*, 1997). Moreover, primary neuronal cultures demonstrate a decrease in the expression of cyclin D1 after exposure to apoptotic and necrotic stimuli (Small *et al.*, 1999).

Thus the exact role of the cell cycle genes and neuronal apoptosis is still unclear, with many controversial and diverse results. Differential expression of the various cell cycle genes may reflect various cellular differences between neuronal cell populations. The fact that several cell cycle genes have been identified in post-mitotic neurones undergoing an apoptotic cell death, may suggest that apoptosis is a result of an aborted cell cycle.

1.4.4. Excitotoxicity and the Cell Cycle Genes

Limited studies have been conducted examining the relationship of excitotoxic cell death to the activation of various cell cycle genes, with few studies conducted *in vivo* (Conejero *et al.*, 1999; Kharlamov *et al.*, 1996; Sakhi *et al.*, 1996; Xiang *et al.*, 1996; Sakhi *et al.*, 1994) and *in vitro* (Small *et al.*, 1999; Uberti *et al.*, 1998; Xiang *et al.*, 1998). While, it is well established that excitotoxins stimulate p53 *in vivo* (Kharlamov *et al.*, 1996; Sakhi *et al.*, 1996; Xiang *et al.*, 1996) and *in vitro* (Uberti *et al.*, 1998; Xiang *et al.*, 1998), and indeed p53 seems to be an essential mediator of Glu receptor-mediated apoptosis (Conejero *et al.*, 1999; Sakhi *et al.*, 1994), many of the other proteins involved in cell cycle progression have been largely ignored. Cyclin D1 expression, as discussed above, has been associated with excitotoxic neuronal injury *in vivo* including ischaemic models (Guegan *et al.*, 1997) and KA-induced seizures (Liu *et al.*, 1996). Further involvement can only be regarded as speculative, as the full extent of role that Glu plays in various

neurodegenerative diseases is still largely unknown. However, postmortem tissue from patients suffering Alzheimer's disease demonstrate a marked increase in the expression of cyclin B1 (Vincent *et al.*, 1997), and cyclin E (Smith *et al.*, 1999), and strong p16 immunoreactivity exists in the neurofibrillary tangles from post-mortem brains (Arendt *et al.*, 1996). Recently CDK5 has been suggested to be one of the key kinases responsible for abnormally hyperphosphorylating the tau protein in Alzheimer's disease, and is indeed upregulated in brains of Alzheimer's patients (Pei *et al.*, 1998).

1.5 THE MAP KINASE PATHWAYS

The families of mitogen-activated protein (MAP) kinase are cytoplasmic serine kinases that play important roles in mediating extracellular stimuli to intracellular responses. The MAP kinase family is subdivided into three groups: the p42 and p44 MAPKs, also known as the extracellular signal-regulated kinases (ERKs) 2 and 1 respectively, the c-Jun N-terminal kinases (JNKs) and the p38 kinases, also known collectively as the stress-activated protein kinases (SAPKs). These protein kinases are activated by multiple mechanisms, including receptor activation, stress, growth factors, heat shock and cytokines, and control many physiological functions including cellular proliferation and apoptosis (Tibbles & Woodgett, 1999), mediating their effects through regulating transcription. MAP kinases are activated by dual phosphorylation by MAP kinase kinases (MAPKK or MEK) and MAP kinase kinase kinases (MAPKKKor MEKK). Each individual MAPK is activated by a specific kinase, the ERK subfamily is phosphorylated by MEK1 and MEK2, the p38 MAPK group is activated by MEK3, MEK4 and MEK7, and the kinases that activate the JNK subfamily include MEK4 and MEK7 (Davis *et al.*, 1999).

1.5.1 JNK MAP Kinases

Three JNKs have been identified, termed JNK1-3, encoding alternatively spliced transcripts of 46 and 55kDa, and are activated by the phosphorylation of tyrosine and threonine residues. No activity differences between the splice variants have been identified, however there is altered substrate specificity, suggesting different functional roles for the isoforms (Gupta *et al.*, 1996). JNKs are activated by environmental stress and cytokines (Nishina *et al.*, 1997) whereby they subsequently phosphorylate various transcriptions factors including *c-jun* (Sanchez *et al.*, 1994) (Figure 1.10), activating transcription factor 2 (ATF-2), Elk-1 and possibly p53, strongly augmenting their transcriptional activity (Whitmarsh & Davis, 1996). These transcription factors in turn bind to the activating protein 1 (AP-1) site in the promoters of many genes, including *c-jun* (Whitmarsh & Davis, 1996). The function of JNKs *in vivo* is poorly understood, however knockout models have demonstrated that AP-1 transcription activity is dependent upon the JNK signalling pathway (Yang *et al.*, 1997). As a role for AP-1 is strongly implicated in apoptosis, in particular neuronal apoptosis (Xia *et al.*, 1995), JNKs are also likely to be involved in the apoptotic pathway.

JNK1 and 2 double knockout mice die at embryonic day 11 (E11) from what is thought due to the ineffective closure of the neural tube. In addition, these animals have reduced developmental cell death in the hindbrain at E9, but increased cell death in the same area one day later (Sabapathy *et al.*, 1999). This model demonstrates the complex involvement of JNK1 and 2, which seem to play both pro- and anti-apoptotic roles in the development of the CNS.
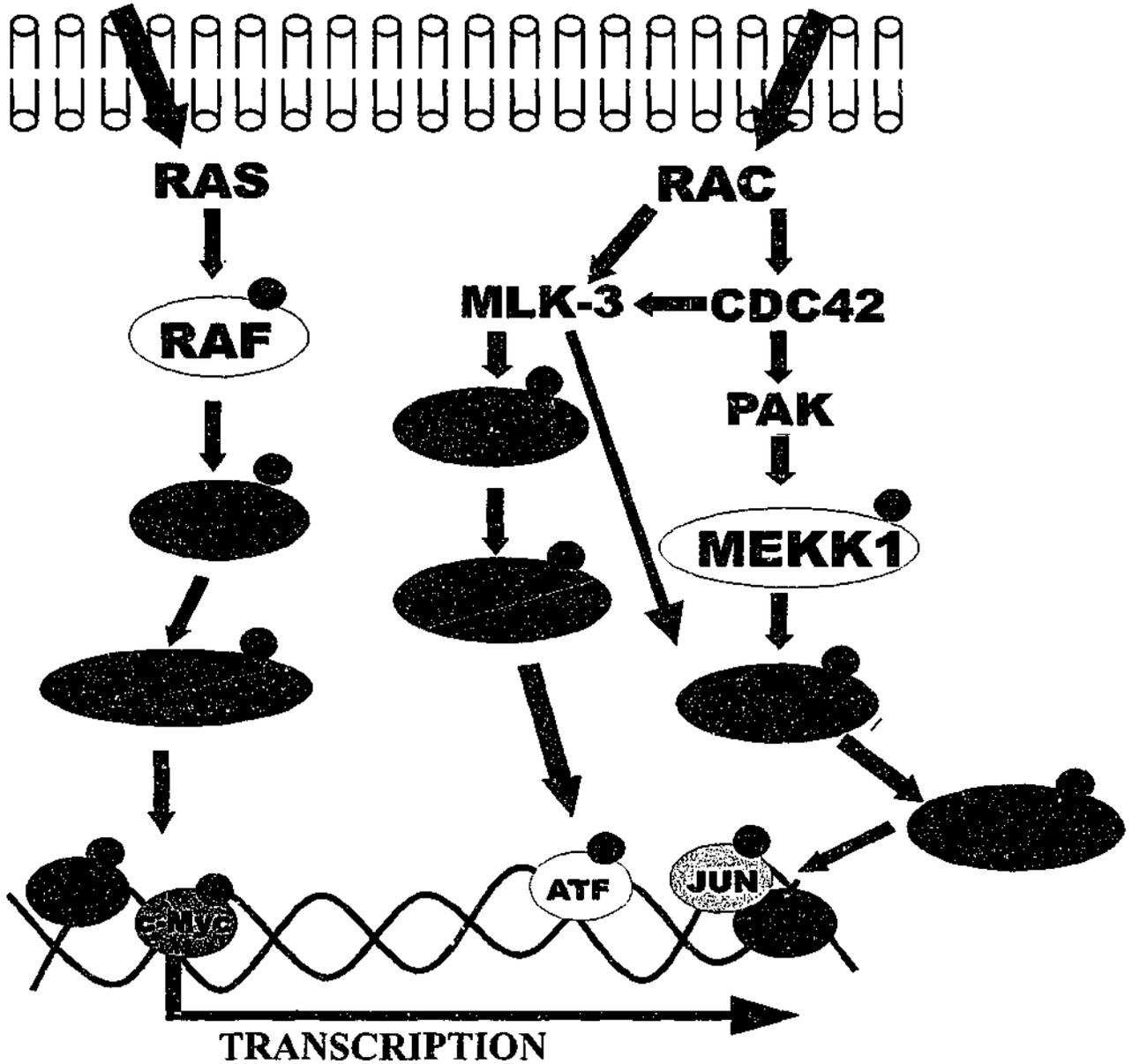
Figure 1.10 The MAP kinase pathways

Activation of the ERK pathway (left) occurs through activation of the small GTP binding protein Ras, leading to the activation of MEK1 and 2, which in turn phosphorylate the ERK kinases. The ERK kinases phosphorylate various proteins including the transcription factors CREB and c-myc. Activation of the SAPK pathway (right) occurs through the activation of the small Rho GTPases (Rac and cdc42), which in turn stimulates p21 activating kinase (PAK). PAK in turn activates various MEKKs that in turn phosphorylate JNK and p38, which subsequently activate *c-jun* and ATF-2, Sap and stimulation gene transcription.

1.5.2 p38 MAP Kinases

The p38 kinases consist of p38α, p38β, p38γ and p38δ transcripts and are activated by a variety of stimuli including heat shock, osmotic shock, DNA damage and pro-inflammatory stimuli (Cohen, 1997). Like the other sub-families of MAP kinases, the p38 kinases are activated by phosphorylation on threonine and tyrosine residues in the T-loop (Galcheva-Gargova *et al.*, 1994) mediated by MEK3 and MEK6. The p38 kinase cascade is activated by the Rho family of GTPases and seems to involve p21-activated kinase (PAK) (Zhang *et al.*, 1995). Substrates of p38 kinase include MAP-kinase-activated protein kinase-2 (MAPKAP kinase-2), MAPKAP kinase-3, MAP-kinase-interacting kinase (Mnk) and the transcription factors activating transcription factor-2 (ATF-2), SFR accessory protein 1 (Sap1) and the growth arrest and DNA damage transcription factor CHOP (Ono & Han, 2000) (Figure 1.10). In addition p53 is phosphorylated by p38α and therefore may play a role in mediating p53-dependent transcription (Huang *et al.*, 1999). A variety of genes that are transcriptionally regulated by p38 have been implicated in apoptosis, including *c-fos* and *c-jun*, tumor-necrosis factor (TNF) and interleukin-1, 6 and 8 (Ono & Han, 2000). In addition, the p38 MAP kinases play an important role in cell cycle progression, where it is thought that they are involved in spindle assembly and some studies conducted in cell lines suggest that they mediate G1 arrest (Molnar *et al.*, 1997).

The p38 kinases are upregulated in cells exposed to oxidative stress (New & Han, 1998), in PC12 cells exposed to hydrogen peroxide (Guyton *et al.*, 1996) and in cerebellar granule cells exposed to Glu (Kawasaki *et al.*, 1997). NGF withdrawal-mediated apoptosis is dependent upon p38 kinase (Kummer *et al.*, 1997), and consistent with a pro-apoptotic role for p38 kinases, neurotrophic factors down-regulate this kinase (Heidenreich & Kummer, 1996).

1.5.3 p44 and p42 MAP Kinases

The p44 and p42 kinases are the best characterized members of the MAP kinase family and are largely activated by growth factors. The p42 and kinases were discovered after they became phosphorylated after insulin stimulation (Ray & Sturgill, 1988). They can be activated by stimulation of tyrosine kinase receptors or G-protein coupled receptors which autophosphorylate after activation, including receptors for platelet derived growth factor, angiotensin II, thrombin, acetylcholine and noradrenaline (Denhardt, 1996). Activation of these receptors initiates a cascade of events ultimately resulting in the phosphorylation of MAP kinase kinase (MEK1) by Raf-1. MEK1 in turn phosphorylates and activates p42 and p44 MAPK, allowing the promotion of gene transcription mediated by the phosphorylation of other transcription factors such as ETS-like protein 1 (Elk-1) (Marshall, 1994), c-myc (Treisman, 1995), and *c-fos* Treisman, 1995) (Figure 1.10).

The ERK pathway is activated after sublethal doses of ischaemia and neuronal cells are more tolerant to any subsequent ischaemic insults, indicating a role in preconditioned neuroprotection (Shamloo *et al.*, 1999). However, numerous studies have indicated a detrimental effect after activation of ERK in hippocampal slices (Runden *et al.*, 1998) and after excitotoxic cell death in PC12 cells (Xia *et al.*, 1995). The ERK pathway can be activated by both ras-dependent (Boglari *et al.*, 1998) and -independent mechanisms (Barrie *et al.*, 1997), depending upon the cell type and the receptor activated.

1.5.4 MAP Kinases and the Central Nervous System

The MAP kinases are activated by various neurotransmitters, growth factors and G-coupled receptors in the nervous system. The p42 and p44 kinases are developmentally regulated in the nervous system (Fukunaga & Miyamoto, 1998). While it is thought that

the MAP kinases may play an important role in neuronal differentiation, the mechanisms of how this occurs are still largely unknown. However, it is known that many growth factors that are essential for neuronal survival activate the MAP kinase pathways (Tibbles & Woodgett, 1999; Fukunaga & Miyamoto, 1998). The transcription factor Pax6 is required for the normal development of the nervous system (Warren *et al.*, 1999) and is phosphorylated by both ERK and p38 MAP kinases, but not JNK (Mikkola *et al.*, 1999), directly implicating MAP kinases in the development of the CNS.

1.5.5 MAP Kinases and Apoptosis

Recently it has been suggested that apoptosis is mediated by all MAP kinases, the p38 kinases and JNK being involved in the "death pathway" whilst the ERK kinases in cell survival (Xia *et al.*, 1995). While the MAP kinases have been implicated in cell death, and in particular neuronal cell death (Mackay & Mochly-Rosen, 1999; Maas *et al.*, 1998; Walton *et al.*, 1998; Kummer *et al.*, 1997; Johnson *et al.*, 1996; Xia *et al.*, 1995). The exact mechanism by which MAP kinases are involved in neuronal apoptosis is still unknown, partially due to the lack of knowledge regarding their regulation of various genes. One such gene, that is regulated by JNK, and has been implicated in apoptosis in a variety of models is *c-jun* (Araki *et al.*, 1998; Cheung *et al.*, 1998a; Guegan *et al.*, 1997; Anderson *et al.*, 1996). Findings from a number of apoptotic paradigms have demonstrated activation and/or phosphorylation of the MAP kinases including: NGF withdrawal in PC12 cells (Xia *et al.*, 1995), global forebrain ischaemia (Walton *et al.*, 1998), and hydrogen peroxide-induced apoptosis in oligodendrocyte cell lines (Bhat *et al.*, 1999). In particular, Xia *et al.*, (1995) demonstrated activation of both JNK and p38 kinases after NGF withdrawal in PC12 cells, where the p38 MAP kinase pathway was essential for cell death. In contrast, Eilers *et al.* (1998) found that JNK was activated in sympathetic neurones after NGF-

withdrawal, as was *c-jun*, but p38 MAP kinase levels were unchanged. Furthermore, this study demonstrated that cell death could be attenuated by co-expressing the dominant negative mutant of SEK1, an activator of JNK, and that apoptosis could be initiated directly by overexpression of MEKK1, an activator of JNK.

The effects that the MAP kinases have on apoptosis are most likely due to their regulatory mechanisms on transcription. The varying effects of these kinases on apoptosis seem to be dependent upon the cell type and the proteins already present in cell, and the ability of MAPK to phosphorylate them. For instance ERK has been shown to phosphorylate the protein tau in Alzheimer's disease (James *et al.*, 1996) and the Bcl-2 family member Bad (Scheid *et al.*, 1999). p38 MAP and p42/p44 MAP kinases are associated with both the negative and positive regulation of cyclin D1, respectively, in cultured fibroblasts (Lavoie *et al.*, 1996). Moreover, p42/p44 is required for passage through the G1 phase of the cell cycle, an event thought to be mediated by the phosphorylation of the CDKs (Pages *et al.*, 1993). Cells expressing dominant-negative p44 MAPK show not only inhibition of the expression of cyclin D1, but also CDK2 and E2F expression. These phenomena may be the mechanisms by which the ERK and SAPK pathways mediate their anti-apoptotic and pro-apoptotic signals, respectively, since cyclin D1 is strongly implicated in neuronal apoptosis as described earlier (Kraneburg *et al.*, 1996; Freeman *et al.*, 1994).

1.5.6 MAP kinases and Excitotoxicity

The association between neuronal apoptosis and MAP kinases has only recently gained recognition, so there is limited evidence to suggest an involvement in excitotoxicity, with studies thus far producing confounding and inconclusive results. KA and AMPA receptor stimulation results in the marked activation of the p42 MAP kinases in

oligodendrocytes by a Ca^{2+}-dependent mechanism (Liu *et al.*, 1999). JNK3, which is selectively expressed in the nervous system, is a major mediator of excitotoxic cell death, as determined by mutating the gene encoding this kinase and rendering the cells resist to KA-mediated cell death (Yang *et al.*, 1997). JNK3 knockout animals demonstrate a resistance to excitotoxicity-induced apoptosis in the hippocampus of adult mice, findings indicating an essential role of JNK3 in excitotoxicity and/or GluR expression (Yang *et al.*, 1997). Transgenic animals, whereby *c-jun* phosphorylation by a mutant isoform of JNK is not possible, demonstrate normal development of nervous system, although, these animals are resistant to apoptosis by KA-induced seizures (Behrens *et al.*, 1999a). Interestingly, JNK1-/-JNK2+/- transgenic animals are not resistant to this form of neuronal trauma (Sabapathy *et al.*, 1999). In contrast cerebellar granule cells exposed to apoptosis-inducing levels of Glu did not demonstrate any change in JNK or p38 MAP kinase activity (Gunnmoore & Tavare, 1998), although many studies have demonstrated a marked increase in *c-jun* after excitotoxic neuronal injury (Beer *et al.*, 1998; Cheung *et al.*, 1998a), suggestive of an upstream activation of JNK. Axotomy-mediated apoptosis is attenuated by inhibitors of p38 MAP kinases (Castagne & Clarke, 1999) and neuronal apoptosis induced by seizure activity is attenuated by p44/42 MAP kinase inhibitors (Murray *et al.*, 1998). This line of evidence contradicts the general belief that JNK and p38 MAP kinases are mediators of apoptosis, whereas p44/42 MAP kinases have a negative effect on the apoptotic cascade (Xia *et al.*, 1995). Ischaemic models have also demonstrated the activation of p38 MAP kinases in microglia over a period of 4 days after the infarct, with no change in the expression of JNK or ERK (Walton *et al.*, 1998). The mechanisms by which MAP kinases are activated by Glu are most likely to occur by a rise in intracellular Ca^{2+} (Kamata & Hirata, 1999; Fukunaga & Miyamoto, 1998), as this key cellular effect is

readily achieved by stimulation of iGluRs (Lu *et al.*, 1996) MAP kinases are likely to play

a central role in excitotoxicity.

CHAPTER TWO

DEVELOPMENT AND CHARACTERIZATION OF

CEREBELLAR GRANULE CELL CULTURES

2.1 INTRODUCTION

Excessive stimulation of the iGluRs leading to neuronal cell death, termed excitotoxicity, has been implicated in a variety of neurodegenerative and neurological diseases (Fonnum, 1998; Ikonomidou & Turski, 1995; Lipton & Rosenberg, 1994). While neurotoxicity mediated by the NMDA receptor is well established (Qin *et al.*, 1996; Bruce *et al.*, 1995; Koh & Choi, 1991), the lack of selective drugs acting on the non-NMDA receptors has hindered past attempts to characterize excitotoxicity mediated by the KA or AMPA receptors. Recently, with increased interest in the roles Glu plays in a variety of neuropathologies non-NMDA selective compounds have been developed (Table 2.1). While the relative selectivity of these compounds is controversial (Moldrich *et al.*, 2000b; Larm *et al.*, 1997a; Larm *et al.*, 1996; Paternain *et al.*, 1995), they have allowed more insight into the roles of AMPA and KA receptor-mediated toxicities in neuropathologies, without interference from NMDA receptor-mediated responses. Furthermore, recombinant receptor techniques, including receptor expression in cell lines, have allowed insight into the characteristics of the different iGluR receptor subtypes and revealed, for example, contrary to initial belief, that some AMPA and KA receptor assemblies were actually Ca^{2+}-permeable (Savidge & Bristow, 1997; Lu *et al.*, 1996; Bennett *et al.*, 1996; Geiger *et al.*, 1995; Jonas *et al.*, 1994; Hollmann & Heinemann, 1994). While these studies have aided in the development of novel selective compounds for the non-NMDA receptors, it is still important to evaluate these compounds at native receptors, a point later discussed in some detail in Chapter three.

Neurotoxicity mediated by iGluRs can occur by two different mechanisms, apoptosis and necrosis. Necrosis occurs when a rise in intracellular water level, attributed to a rapid influx of Na^+ ions followed by a passive influx of Cl^- and water, results in

Compound	Action at AMPA Receptors	Action at KA Receptors
Glu	agonist	agonist
AMPA	agonist	weak agonist
KA	agonist	agonist
DOM	agonist	agonist
QA	agonist	agonist
IW	agonist	agonist
FW	agonist	agonist
NMDA	inactive	inactive
ATPA	agonist	agonist
LY339434	unknown	agonist
4-MG	unknown	agonist
GYKI 53655	antagonist	antagonist
GYKI 52466	antagonist	antagonist
CNQX	antagonist	antagonist
DNQX	antagonist	antagonist
NBQX	antagonist	antagonist
NS-102	antagonist	antagonist

Table 2.1 Activity of non-NMDA receptor compounds at the KA and AMPA receptors.

Abbreviations: DOM, Domoate; QA, quisqualate; IW, (*S*)-iodowillardiine; FW, (*S*)-5-fluorowillardiine; ATPA, (*RS*)-2-amino-3-(hydroxy-5-*tert*-butylisoxazol-4yl)propanoic acid; LY339434, (2*S*,4*R*,6*E*)-2-amino-4-carboxy-7-(2-naphthyl)hept-6-enoic acid; 4-MG, (2*S*,4*R*)-4-methylglutamate; GYKI 53655, 1-(4-aminophenyl)-4-methylcarbamyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benz-odiazepine HCl; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine HCl; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*f*)quin-oxaline; NS-102, 5-nitro-6,7,8,9-tetrahydrobenzoy[g]indole-2,3-dione-3-oxime. (adapted from Bettler & Mulle, 1995).

cellular swelling and subsequent lysis of the cellular contents. Apoptosis is a slower process, dependent on an increase in intracellular Ca^{2+}, and requires energy and protein synthesis. Apoptosis is characterized morphologically by cellular shrinkage, neurite blebbing and chromatin condensation (Clarke, 1990). NMDA receptor activation can cause apoptosis or necrosis (Portera-Cailliau *et al.*, 1997a; van Lookeren Campagne *et al.*, 1995; Bonfoco *et al.*, 1995) dependent upon the intensity of the insult. NMDA receptors can also play a neuroprotective role in cultures of cerebellar granule cells (CGCs), an action mediated by the stimulation of various growth factors, including brain-derived growth factor, (Favaron *et al.*, 1993; Balazs *et al.*, 1990; Balazs *et al.*, 1989) depending upon the age of the cultures and the culture conditions. Toxicity mediated by the AMPA receptor is generally apoptotic in nature (John *et al.*, 1999; Larm *et al.*, 1997b), and in some cases requires cyclothiazide (CTZ), an inhibitor of the rapid receptor desensitization (Brorson *et al.*, 1995; May & Robison, 1993; Partin *et al.*, 1993), to unmask the toxicity (Cowen & Beart, 1998; Hoyt *et al.*, 1995; May & Robison, 1993). KA receptor-mediated toxicity, like NMDA, can elicit apoptosis or necrosis (Cheung *et al.*, 1998b; van Lookeren Campagne *et al.*, 1995) and KA is a common tool to induce toxicity in *in vivo* studies, including kindling models of epilepsy (Meldrum *et al.*, 1999; Loscher, 1998) and the neostriatal injection model of Huntinton's chorea (Coyle *et al.*, 1978).

Studies conducted *in vivo* have demonstrated KA to be a potent neurotoxin (Garthwaite & Wilkin, 1982; Coyle *et al.*, 1981; Coyle *et al.*, 1978; Olney *et al.*, 1974). However, KA also acts on AMPA (GluR1-4) receptors (Keinanen *et al.*, 1990) and therefore the exact mechanisms underlying neurotoxicity has been difficult to establish. Many studies conducted *in vivo* have employed KA administration as a model system for epilepsy, since systemic administration of KA cause seizures similar to epileptic seizures. Interestingly, this model demonstrates a change in the expression of the AMPA receptor

subunits in the hippocampus, implying KA is also interacting with these receptors (Pollard *et al.*, 1993). Functional studies conducted *in vitro* and the development of novel and selective compounds have further distinguished between KA and AMPA receptor-mediated responses. While there are more selective compounds available for the study of AMPA and KA receptors, to completely isolate the responses *in vivo* has proven difficult. No compound is yet completely selective for either the AMPA or KA receptors, and because of the widespread distribution of the AMPA receptors, KA is likely to also act upon these receptors *in vivo* (Ohno *et al.*, 1997). Therefore, various studies conducted in cultured cells *in vitro* have provided great insight into the non-NMDA receptors, as the receptor content of the cultures can readily be examined by immunocytochemistry or *in situ* hybridization, and recombinant receptor technology is more readily applied.

Primary neuronal cell culture provides a valuable model system for *in vitro* neurotoxicity studies (Schousboe *et al.*, 1985). Primary neuronal cultures permit controlled experiments, whereby known concentrations of agonists can be applied directly to neurones without confounding influences from other major organ systems, such as the immune and central nervous systems (Kroemer *et al.*, 1995). Moreover, primary neuronal cultures, unlike cell lines, are likely to express appropriate biochemical and genetic materials similar to that found in their *in vivo* counterparts. Culture provides the unique opportunity to observe cell death in a variety of cell types, such as neocortical, hippocampal, and cerebellar granule and Purkinje cells, in essentially pure neuronal populations. In addition, cell culture allows the easy manipulation of various molecular characteristics through techniques such as transfection of genes, and the downregulation of genes and proteins through the use of antisense oligonucleotides and neutralizing antibodies, respectively (Shafron *et al.*, 1998). The morphological changes of neurones exposed to various insults, including excitotoxins, can be readily examined *in vitro* as the

cells are generally grown in a monolayer allowing easy visualization of the morphology of individual cells.

Although neurones in culture may behave differently to those *in vivo*, various issues can be more readily examined in culture, but not in brain slices or in whole animal experiments. Pure neuronal cultures are advantageous in that they contain negligible glia populations, which often confound results since they are a rich source of neuronal growth factors and uptake mechanisms (Cotter & al-Rubeai, 1995). Culture also enables close morphological changes to be observed, without interference from the immune system, where *in vivo* macrophages often rapidly engulf dying cells (Kroemer *et al.*, 1995). Such observations and insights from molecular investigations of cell death *in vitro* often represent excellent guidelines for further experimentation in the more complicated *in vivo* models.

Cellular viability assays, for the quantification of cell death, are an invaluable tool in studying neurotoxicity. One such assay employs the tetrazolium dye MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), which is a measure of mitochondrial activity, which diminishes in dying neurones (Mosmann, 1983). MTT is a tetrazolium salt that readily enters the cell membrane. Viable cells cleave the tetrazolium ring by the mitochondrial dehydrogenase enzymes, converting the tetrazolium salt to a coloured, impermeable formazan product, which can be spectrophotometrically measured (Liu *et al.*, 1997; Mosmann, 1983). Recently a number of fluorescent dyes have been developed which bind the DNA, including propidium iodide (PI; Frey, 1995; Vitale *et al.*, 1993) and 4,6-diamidino-2-phenylidole (DAPI; Hotz *et al.*, 1994). Their use enables cell death mechanisms to be evaluated and thus in living cells, with damaged cell membranes, the dye is readily incorporated and the condensed chromatin, indicative of apoptosis, can be visualised by employing fluorescent microscopy (Cheung *et al.*, 1998b; Ankarcrona *et*

al., 1995). Apoptotic and necrotic cellular membrane damage occurs at different times after the insult, necrosis occurs 1-4h after the insult, whereas apoptotic membrane damage after 8-24h. This differing time frame can be manipulated, and hence PI can be used to examine necrosis (1-4h after insult; Moldrich *et al.,* 2000a) or apoptosis (8-24h after insult; Vitale *et al.,* 1993), dependent upon the time the dye is applied to the cells. Trypan blue exclusion is yet another means of determining cell death, where healthy cells actively exclude trypan blue, whereas dying cells are unable to exclude the dye and appear blue under bright-field microscopy (Dickson, 1970). However, analysing results from this procedure, unlike the fluorescent dyes, cannot be automated and is therefore extremely laborious and time-consuming.

A valuable tool for studying apoptosis is the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick end labeling (TUNEL) technique (Gavrieli *et al.,* 1992). This method labels DNA fragments *in situ* which are characteristic of apoptosis. DNA fragmentation is caused by DNA polymerase, an enzyme activated shortly after the cell signals its own death (Chen *et al.,* 1997; Prosperi, 1997). DNA fragmentation can also be evaluated by employing DNA gel electrophoresis, by which "DNA laddering", or smaller fragmented DNA, can be separated from genomic DNA (Peitsch *et al.,* 1993), considered to be a "hallmark" of apoptosis.

The current study involved the development of a primary culture of cerebellar granule cells, which is a convenient system to study the activation of genes after apoptotic stimuli, since it is an essentially homogenous cell preparation. These cells are essentially glutamatergic (Van der Valk *et al.,* 1991; Schousboe *et al.,* 1985; Fonnum, 1984), and contain negligible glia when grown in media that is preferable for neurones and/or treated with appropriate mitotic inhibitors (Miller & Johnson, 1996; Dessi *et al.,* 1995). Development of the cerebellum occurs largely postnatally, where extensive cell formation

and neurogenesis occurs in the first four weeks after birth (Altman, 1972). Granule cells migrate and proliferate during the first 3 weeks after birth and cell counts of cells from the cerebellum at postnatal day 6 indicates a predominance of granule cells (Cohen *et al.*, 1978). Hence, cells taken from cerebellum at this time are mainly granule cells, allowing the generation of an essentially homogenous cell preparation (Schousboe *et al.*, 1989; Messer, 1977). Unlike other neuronal cell cultures, CGCs require maintenance in depolarizing levels of K^+ (20mM) (Schulz *et al.*, 1996; Yan *et al.*, 1994; Hack *et al.*, 1993) or they do not survive. While the exact mechanism high levels of K^+ protect CGCs from dying by apoptosis is unknown, it is thought that depolarizing levels of K^+ mimics the innervation from the glutamatergic mossy fibres observed *in vivo* (Balazs *et al.*, 1988) and has also been shown to increase growth factor expression (Marini *et al.*, 1998; Zhang *et al.*, 1998).

The current studies were designed to examine the development of CGCs in culture and to determine the receptor expression via cell viability assays on various days *in vitro*. The Glu agonists and antagonists used in the present study to determine receptor expression and neurotoxicity are given in Figure 2.1. NMDA receptors in CGCs have been reported from *div* 4-6 (Resink *et al.*, 1994), whereas AMPA receptors appear to be present from *div* 14 (Hack *et al.*, 1995). Due to the likely effects of numerous experimental variables, including serum treatment and culture media, on the expression of KA and AMPA receptors (Resink *et al.*, 1994), the present experiments were conducted from *div* 1-16 to determine optimal times for analyses of neurotoxicity.
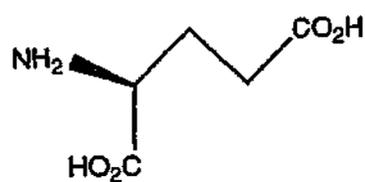
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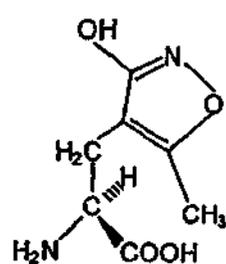
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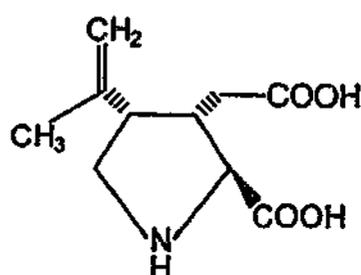
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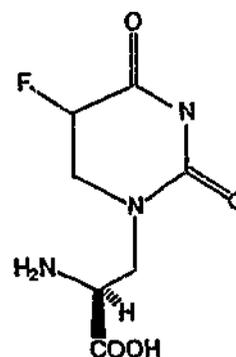
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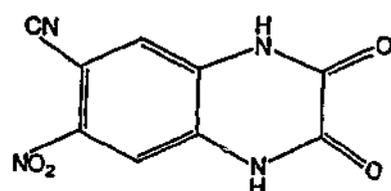
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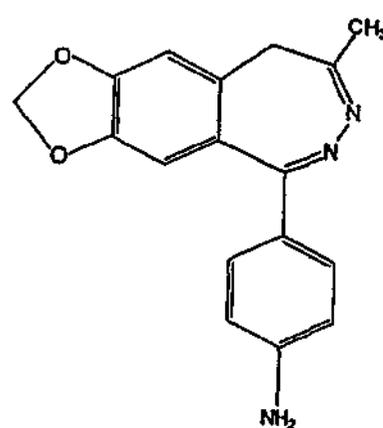
GYKI 52466

Figure 2.1 Chemical structures of non-NMDA receptor agents used in the present study

2.2 METHODS

2.2.1 Materials

Neurobasal Medium™, B27 and N2 supplements, Hank's Balanced Salt Solution (HBSS), Glu, Gln, normal goat serum and penicillin-streptomycin were obtained from Gibco BRL Life Technologies (Melbourne, Australia). 1-(4-Aminophenyl)-4-methyl-7,8-methylendioxy-5H-2,3-benzodiazepine (GYKI 52466), CTZ and MK-801 were purchased from Research Biochemicals International (MA, USA), and (S)-AMPA, CNQX, FW, NMDA, were purchased from Tocris Cookson (Bristol, UK). Rabbit anti-GFAP and mouse anti-MAP2 were purchased from Dupont-NEN (MA, USA) and Boehringer Mannheim (Germany), respectively. Tissue culture plates were obtained from Nunclon (Denmark). Terminal transferase, dATP, digoxigenin-11-dUTP, and anti-digoxigenin-AP were all purchased from Boehringer Mannheim (Germany). KA, poly-D-lysine, trypsin, soy bean trypsin inhibitor (SBTI), deoxyribonuclease (DNase I), dialyzed foetal calf serum, MTT, RPMI 1640 medium, trypan blue, dimethyl formamide, sodium dodecyl sulphate and all other reagents were from Sigma-Aldrich (Sydney, Australia) and were of cell culture or molecular biology grade.

2.2.2 Cell Culture

2.2.2.1 Animals

All animals were from Monash University Central Animal Services, housed at the Pharmacology Animal House and fed *ad libitum*. Swiss-White mice (6-8 days old) were

used throughout all experimentation to obtain cerebella for neuronal preparations. Animals were killed by decapitation.

All experiments were performed in accordance with the ethical code of the National Health and Medical Research Council (Australia) with permission from the Standing Committee for Ethics in Animal Experimentation (Monash University) and the Animal Experimentation Committee (Department of Pharmacology, Monash University).

2.2.2.2 Isolation of Cerebellar Granule Cells

Primary cultures were obtained by a modification of a previously described method (Schousboe *et al.*, 1989). Swiss-White mice (6-8 days old) were sterilized in 70% ethanol in a sterile enclosure, and decapitated with a sharp pair of scissors. The heads were immediately placed in sterile, ice-cold HBSS solution (Appendix I) for approximately 5min to remove excess blood. Heads were transferred to a sterile petri-dish containing fresh HBSS and the whole brain removed, using scissors to cut through the skull and curved forceps to remove the brain from the head. The whole brain was transferred to a sterile petri-dish containing fresh, cold HBSS solution, and the cerebellum was micro-dissected under a dissecting microscope with a light source (Industrial and Scientific Supply Company, Australia). The cerebellum was gently detached by gently teasing the cerebellum free from the remainder of the brain with forceps and the meninges were carefully removed. Typically cerebella were removed from 8-12 mice and transferred to a petri-dish containing fresh HBSS solution and gently minced by drawing the cerebella up into a sterile 1ml pipette (x⁴) immediately before placing the suspension into a sterile 50ml centrifuge tube. The cerebellar suspension was centrifuged (Bench Top centrifuge with a swing bucket rotor, Heraeus, Germany) at room temperature 1000 x g for 1min. The supernatant was removed with a sterile Pasteur pipette and the pellet was subsequently

resuspended in 20ml HBSS solution containing 0.2mg/ml trypsin, 80µg/ml DNase, 3mg/ml BSA and 1.16mM $MgSO_4$ (37°C). Tissue digestion was undertaken in a shaking water bath at 37°C for 30min at speed 7 (Gallenkamp, UK). After this time, the digestion was terminated by the addition of 20ml HBSS solution containing 90µg/ml SBTI, 13.6µg/ml DNase, 3mg/ml BSA and 1.7mM $MgSO_4$ (room temperature), and the suspension was centrifuged at room temperature at 1200 x g for 3min. The supernatant was aspirated and the pellet was resuspended in 20ml HBSS solution containing 0.52mg/ml SBTI, 80µg/ml DNase, 3mg/ml BSA and 3mM $MgSO_4$ (room temperature). The tissue was mechanically dissociated using a 1ml pipette, 10 strokes, flush to the base of the centrifuge tube. This procedure was repeated using a 20ml sterile syringe and 24-gauge needle (10 strokes). The resultant suspension was allowed to settle for 1min so that any undigested tissue would precipitate to the bottom. Using a sterile Pasteur pipette 15-18ml of the cell suspension was removed from the top and placed into a centrifuge tube, carefully avoiding any visible clumps. This suspension was subsequently centrifuged at 1200 x g for 3min (room temperature), the supernatant removed and the pellet resuspended in 200ml of Neurobasal™ supplemented medium (37°C) (Brewer *et al.*, 1993) containing 25.4mM K^+, 100U/ml penicillin, 100µg/ml streptomycin, 0.5mM Gln, and 3% B27 components (Appendix II). Cultures were exposed to 10% dialysed foetal calf serum (10,000 MW cut off) for 24h on day 1 *in vitro* (*div* 1) and were subsequently maintained under serum-free conditions in a humidified incubator (5% CO_2, 8% O_2 and N_2 at 37°C).

2.2.2.3 Coating of Culture Plates

Poly-D-lysine coating was optimized to achieve even cell distribution with negligible cell death. Numerous conditions were examined including length of exposure time of plates to poly-D-lysine (1-24h), incubation temperature (room temperature and

37°C), and washing with MilliQ water (0-3 times). Optimal conditions involved coating plates the day immediately prior to culturing in 50µg/ml poly-D-lysine aqueous solution (500µl) and incubated at 37°C for 24h with no washing step. Culture plates were allowed to dry down at room temperature for 2-3h prior to plating of cells.

2.2.2.4 Cerebellar Granule Cell Culture

Cell density of the cell suspension was determined by counting cells using a Neubauer haemocytometer (Weber Scientific, Sheffield, UK). Approximately 10-14 x 10^6 cerebellar cells/animal were obtained. Cells were seeded using an eppendorf multi-pipette at a density of 0.4 x 10^6 cells/well for 24 well plates, 2.0 x 10^6 cells/well for 6 well plates and 0.1 x 10^6 cells/well for 96 well plates, adjusted for the surface area of the well. Dialyzed fetal calf serum contains no Glu, which can be highly toxic to cultured cells (Freeman *et al.*, 1994), and was therefore used instead of conventional serum. From *div* 1 cultures were maintained under serum-free conditions in the presence of 2µg/ml aphidicolin, a mitotic inhibitor, to prevent any non-neuronal cell proliferation. Cultures were observed under phase-contrast microscopy with an inverted microscope (Olympus IMT-2).

2.2.3 Colorimetric Immunocytochemical Characterization of Cultures

Cultures were characterized by immunocytochemical staining for microtubule associated protein 2 (MAP2), a marker specific for neurones (Whittaker *et al.*, 1993), and glial fibrillary acidic protein (GFAP), a marker specific for astrocytes (Raff *et al.*, 1979). Cultures were grown on 24 well plates and immunocytochemistry was performed on various days after seeding (1-15 *div*).

Culture medium was aspirated and cells immediately fixed with 500μl of 4% paraformaldehyde (w/v), prepared with phosphate buffered saline (PBS; 4.5mM NaH_2PO_4, 19mM $NaHPO_4$, 150mM NaCl, pH 7.4) for 15min at room temperature and subsequently washed with Tris buffered saline (TBS; 50mM Tris, 0.9% NaCl; pH 7.4). Endogenous peroxidase activity was quenched by exposure to 300μl of Immunopure Peroxidase Suppressor (Pierce, Rockford, IL, USA) for 5min at room temperature and non-specific binding was blocked with 500μl of 10% normal goat serum (NGS), 0.1% Triton X-100 (TX-100), in solution with TBS for 1hr, 4°C. Primary antibodies were incubated overnight at 4°C in solution with 2% NGS, 0.1% TX-100 and TBS (200μl) with mouse monoclonal MAP2 (1:500 dilution) or rabbit polyclonal GFAP (1:5 dilution). Cultures were washed 3 times in 500μl of TBS and incubated with the secondary antibodies conjugated to horse radish peroxidase (HRP), in the presence of 2% NGS, 0.1% TX-100 in TBS, for 3h at room temperature: anti-rabbit (GFAP) or anti-mouse (MAP2) at a dilution of 1:500 (200μl). Any unbound antibodies were removed by washing 3 times in TBS (500μl). The signal was visualized with Peroxidase Substrate Solution (Pierce), containing 3,3'-diaminobenzidine (DAB) in solution with Immunopure peroxidase suppressor according to manufacturers instructions. Colour was allowed to develop at room temperature (approximately 30min) before the reaction being terminated by washing 3 times with TBS (500μl). Immunopositive staining was visualized by bright field microscopy and were photographed on Kodak Gold™ 100 ASA film.

2.2.4 Fluorescent Immunocytochemical Characterization of Cultures

Fluorescent staining of cultures was carried essentially as described above for the colorimetric technique except for the omission of the quenching step. Secondary antibodies

were conjugated to either fluorescein-5-isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) for anti-mouse and anti-rabbit, respectively. Immunopositive staining was observed using fluorescent microscopy with wavelengths of: excitation 535nm, emission 617nm and excitation 504nm, emission 523nm for TRITC and FITC, respectively, and photographed on Kodak Ektachrome P1600 slide film, using an exposure control unit (PM20, Olympus).

2.2.5 Determination of Cellular Proliferation

To ensure negligible proliferation of any non-neuronal cell types was occurring within the cultures 5-bromo-2'-deoxy-uridine (BrdU) incorporation was determined using a BrdU detection kit (Boehringer and Mannheim, Germany) and performed as per manufacturers instructions in cultures plated on 24 well plates. BrdU is incorporated into the DNA of dividing cells in place of the nucleotide thymidine. BrdU ($10\mu M$) in $200\mu l$ of NBM was incubated with the cultures for 1h and subsequently washed 3 times in $500\mu l$ of PBS. Cultures were subsequently fixed in 70% ethanol in 50mM glycine buffer, pH 2, for 20min at $-20°C$. Cells were washed 3 times in PBS and incubated with anti-BrdU (1:20 dilution) in solution with 66mM Tris, 0.66mM $MgCl_2$, and 1mM mercaptoethanol, for 30min at $37°C$. Cultures were subsequently washed and the signal was detected using anti-mouse-Ig-fluoroscein (1:20 dilution) in PBS and visualized using fluorescence microscopy, and photographed on colour polaroid film (Polapan) using the minicam polaroid camera (Polaroid, UK).

2.2.6 Exposure of Cultures to Glutamate Receptor Agonists

CGCs were exposed to varying concentrations of AMPA, NMDA, FW and KA (10-1000μM) on various *div* (1-16) in 96 and 24 microwell plates. Glu receptor-mediated toxicity was examined on *div* 8, the day maximum toxicity was found for KA, to evaluate the non-NMDA receptor component of Glu neurotoxicity. NMDA, FW, AMPA and KA were dissolved in 10% of the final volume of 1M NaOH, made up to volume with water to give a concentration of 60mM, and stored at -20°C until required. Glu (Gibco Life Technologies, Australia) was diluted in water to final concentration of 13.6mM according to manufacturer's instructions. CTZ, CNQX and GYKI 52466 were dissolved in DMSO and further diluted in NBM. All drug exposures and relevant vehicle controls were conducted in N2 media (Appendix III) for 24h. Drugs were pre-warmed and equilibrated in N2 media for 2h at 37°C before addition to cultures. Culture media were aspirated and replaced with N2 media containing drugs and left overnight in a humidified incubator (5% CO_2, 8% O_2) at 37°C.

2.2.7 Determination of Cellular Viability

2.2.7.1 Trypan Blue Assay

Cells were exposed to 500μl of 2% Trypan Blue in PBS (37°C) for 5min at room temperature. The trypan blue solution was subsequently aspirated and the cultures were fixed in 500μl of 4% paraformaldehyde solution in PBS for 15min. The paraformaldehyde solution was removed under vacuum suction and the cultures were washed 3 times in an excess of PBS. Random, yet representative fields (n = 2) were photographed under bright field microscopy using Kodak 100 ASA print film.

2.2.7.2 MTT assay

Cell viability was determined by spectrophotometrically measuring the formazan production by the reduction of a tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells. A stock solution of MTT (5mg/ml) in RPMI 1640 medium was prepared and added to cultures to give a final concentration of 0.5mg/ml and was incubated for 30min at 37°C. The media containing the MTT was subsequently aspirated and cells were lysed and the formazan product dissolved by the addition of 500μl of 40% dimethylformamide, 20% sodium dodecyl sulphate (SDS), pH 4.4, overnight at room temperature. The coloured product was analysed using a spectrophotometer at 570nm on a Ceres UV900C microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Results were standardized against control (0% cell death) and cultures maintained in low K^+ or treated with 500μM Glu (100% cell death).

2.2.7.3 In situ Labeling of DNA Fragments (TUNEL)

The involvement of apoptosis as a mechanism of neuronal injury in non-NMDA receptor mediated toxicity was analysed by the TUNEL method. Apoptosis, analysed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin (DIG) nick end labeling (TUNEL) was performed as previously described (Cheung *et al.*, 1998a). Cultures were fixed overnight in 4% paraformaldehyde (4°C), and subsequently washed three times in 500μl of TBS. Cultures were then permeabilized with 2% TX-100 in TBS and non-specific labelling was inhibited by the addition of 500μl of 10% normal goat serum and 0.1% TX-100 in TBS (room temperature). Cultures were equilibrated with TdT buffer (200μl; 30mM Tris-HCl, 140mM sodium cacodylate, 1mM cobalt chloride, pH 7.2) for 15min at room temperature and incubated with TdT reaction mixture containing 34mU/ml TdT, 280pmol dATP, 90pmol digoxigenin-11-dUTP in solution with TdT buffer (200μl;

3h, 37°C). DIG labeled dUTP was detected by anti-DIG alkaline phosphatase (AP; 1:1000 dilution) in solution with 2% normal goat serum and 0.1% TX-100 and TBS. TUNEL-positive cells were detected using AP substrate solution (170mM 5-bromo-4-chloro-3-indolyl-phosphate; 60mM nitroblue tetrazolium chloride, 100mM Tris-HCl, 0.5mM $MgCl_2$; pH 9) and colour was allowed to develop for up to 4h at 4°C. Control cultures included the above treatment with the omission of TDT. Cells were visualized under bright field microscopy and random fields photographed.

2.2.7.4 Propidium Labeling of Necrotic Cells

Necrotic cell death was analysed by the propidium iodide (PI), a DNA binding dye, labeling of living cells with damaged membranes (Moldrich *et al.*, 2000a; Ankarcrona *et al.*, 1995). PI only penetrates cells with compromised cell membranes and if employed at early time points after an insult is a reliable technique for assessing necrosis. PI (1µg/ml) was incubated in culture medium 1-2h after NMDA, KA or Glu exposure for 30min at 37°C. Cultures were subsequently viewed using fluorescent microscopy (excitation: 535nm, emission: 617nm) and random, yet representative fields photographed with Kodak Ektachrome P1600 film.

2.2.8 Data Analysis

All data are expressed as mean ± S.E.M. Statistical analysis used a two-way ANOVA and a post-hoc Newman-Keuls test and $p < 0.05$ was considered statistically significant. All analyses were performed using computer-assisted curve fitting program (PRISM version 3.0, Graphpad software, San Diego, CA, USA). Data were representative of experiments from 4-6 independent cultures.

2.3 RESULTS

2.3.1 Development of Culture Conditions

Primary cultures of CGCs are a particular useful system for studying neurobiological mechanisms, especially various genes and proteins, because of their cellular homogeneity (Schousboe *et al.*, 1985). Although cultures of CGCs are a common and well established culture system, a few methodological changes were required to optimize this culture for the particular studies presented here. Firstly, unlike the current model system, many studies use serum throughout the period of culture. Serum contains numerous growth factors and nutrients that commonly promote the proliferation of non-neuronal cells and has led to the subsequent use of various neurotoxic mitotic inhibitors such as cytosine arabinoside (Dessi *et al.*, 1995). Therefore, in the present study serum was only used for the first 24h *in vitro*, whereby after that time cultures were maintained under a serum-free environment. Nutrients found within the serum were substituted with the chemically defined media, B27-supplemented Neurobasal medium, which is rich in nutrients and antioxidants (Brewer *et al.*, 1993). B27 supplements promote the survival of neurones, rather than non-neuronal cells, and hence this medium is an excellent one to selectively grow pure neuronal cell types (Brewer *et al.*, 1993).

Cultures were maintained at 8% O_2, which more closely matches the partial pressure of O_2 in the cerebrospinal fluid (Brewer & Cotman, 1989), rather than 95% air, which contains approximately 20% O_2. High O_2 has been shown to cause cell death via oxidative stress, or the generation of free radicals (Coyle & Puttfarcken, 1993), and therefore cultures were maintained in an environment which more closely mimics that of the *in vivo* environment.

Cell density was found to be crucial for the maintenance of neuronal survival. Cell density when too low ($< 0.1 \times 10^6$ cells/cm^2) resulted in the majority of cells dying within the first 24h. Cell density that was too high ($> 0.5 \times 10^6$ cells/cm^2) resulted in excellent cell survival, but toxicity to the Glu agonists was negligible, most likely due to the chronic acidosis caused by cellular metabolism (Leahy *et al.*, 1994) and the significant amount of trophic factors released from the cells (Ohga *et al.*, 1996). Mild, chronic acidosis has been shown to induce a form of neuroprotection in this culture system, thought to be mediated by down-regulation of the NMDA receptors (Leahy *et al.*, 1994). Optimal cell density was found to be at 0.4-0.5 $\times$ 10^6 cells/cm^2, whereby the survival rate was high and the vulnerability of the cultures to the Glu agonists was optimal and reproducible.

2.3.2 Morphology of Cultures During Development

The development of the cultures is indicated in Figure 2.2. After plating (3-4h) the cells appear round with few neurites (Figure 2.2A). Neuritogenesis was evident up to approximately *div* 6 (Figure 2.2C), with some cells extending neurites as early as *div* 1 (Figure 2.2B). By *div* 8 neurites vary in length and thickness, resulting a complex network of outgrowths (Figure 2.2D). The background cell death was negligible during the first few days *in vitro*, accounting for approximately 5% of the total populations of cells as determined by trypan blue staining (Figure 2.3).

2.3.3 Immunocytochemical Characterization of the Cultures

Immunocytochemistry was employed to determine the contamination of the cultures by non-neuronal cells. Control staining, whereby the primary or secondary
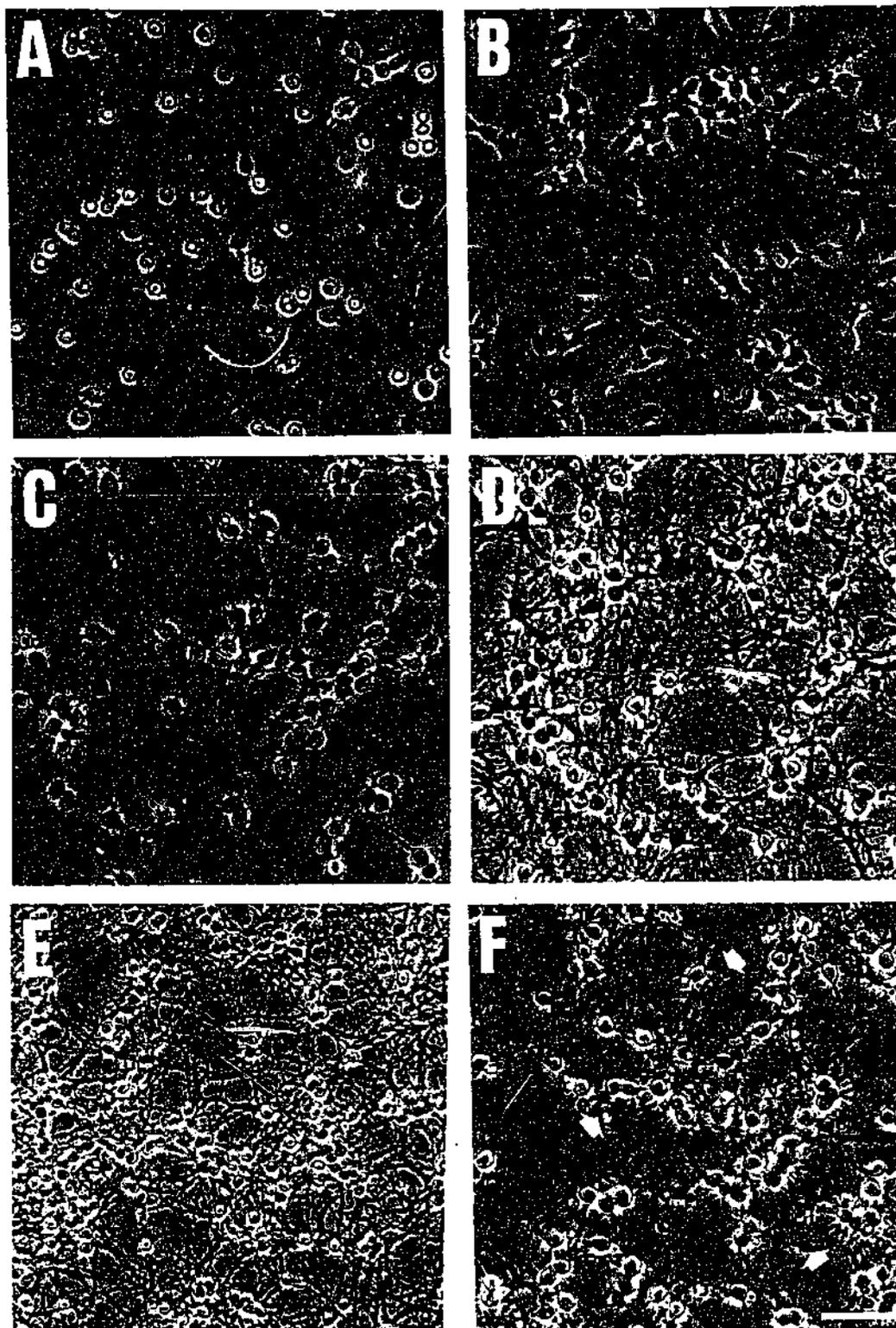
Figure 2.2 Morphology of cerebellar granule cell cultures throughout development.
Phase contrast micrographs from representative fields taken at *div* 0 (A), *div* 1 (B), *div* 6
(C), *div* 8 (D), *div* 10 (E), *div* 14 (F). Neuritogenesis is evident from *div* 1 and completely
established by *div* 6, with extensive neuritic networks evident. Some cell death is present in
older cultures and is indicated (arrows; F). Scale bar is representative of 10μm.
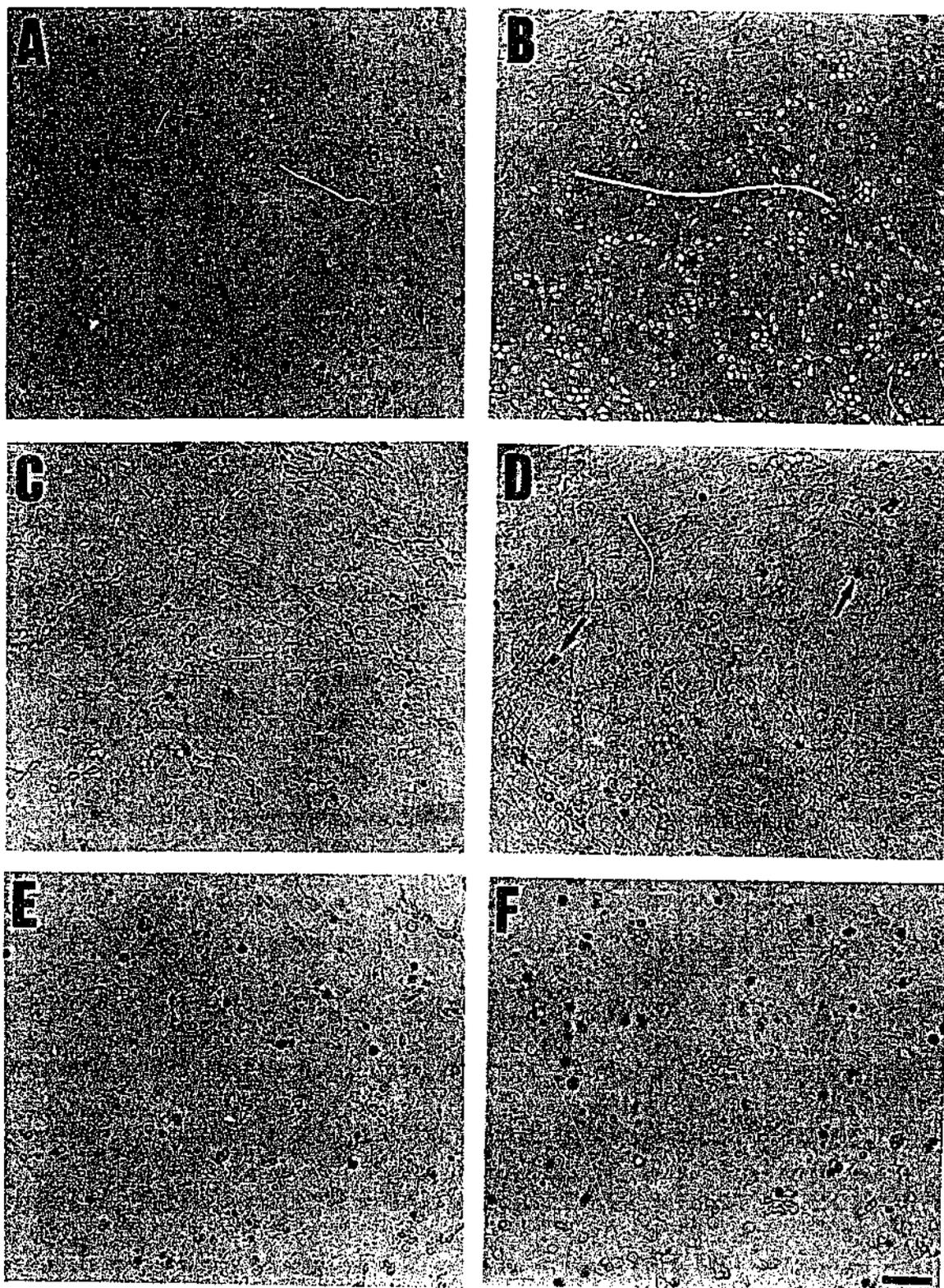
Figure 2.3 Characterisation of background cell death using trypan blue exclusion.
Bright field micrographs exhibit negligible staining on *div* 1 (A), *div* 3 (B), *div* 6 (C) or *div* 8 (D), with an increase in positive staining evident on *div* 10 (E) and *div* 14 (F). Arrows indicate trypan blue positive labelled, or dead, cells. Scale bar = 15μm.

antibody was excluded, demonstrated the absence of non-specific binding. At *div* 8 the cultures were shown to be $95 \pm 2.3\%$ neuronal by immunocytochemistry with antisera to MAP2 (Figure 2.4A) and GFAP (Figure 2.4B), where 750-1000 cells were counted. In the absence of the DNA polymerase α inhibitor aphidicolin, the number of glial cells is greatly increased, particularly in the presence of serum (Figure 2.4C), demonstrating the importance of inhibiting cell proliferation.

BrdU labelling indicated negligible staining on *div* 8 when treated with $2\mu g/ml$ aphidicolin (Figure 2.5C), when compared to untreated sister cultures (Figure 2.5A). Due to the sensitivity of the techniques used in molecular biological studies it is important to identify any cell proliferation. Any contamination by non-neuronal cells may significantly alter results and therefore lead to misinterpretation (Freeman *et al.*, 1994).

2.3.4 Neurotoxicity Studies

KA and AMPA receptor-mediated toxicity was examined over a period of 15 *div*. Although Glu receptor-mediated toxicity is well documented in culture, very little has been done on receptor characterization in cultured systems. Cells exposed to KA on various *div* were found to respond differently depending on the age of the culture. The extent of neurotoxicity mediated by KA (Figure 2.6), was maximal at *div* 8 ($EC_{50} = 69\mu M \pm 0.3$) compared to *div* 1 ($EC_{50} = 135\mu M \pm 3.1$), *div* 3 ($EC_{50} = 72\mu M \pm 0.6$), *div* 6 ($EC_{50} = 156\mu M \pm 0.35$), *div* 10 ($EC_{50} = 286\mu M \pm 0.43$) and *div* 15 ($EC_{50} = 68\mu M \pm 0.72$). KA-receptor mediated toxicity was concentration-dependent on *div* 8 $[F(4,120) = 21.60, p < 0.0001]$ and attenuated by the non-NMDA receptor antagonists GYKI 52466 ($20\mu M$; $[F(1,100) = 24.45, p < 0.0001]$) and CNQX ($50\mu M$; $[F(1,90) = 23.79, p < 0.0001]$), but not the NMDA receptor antagonist MK-801 ($10\mu M$; $[F(1,69) = 2.62, p = 0.578]$), suggesting a KA-
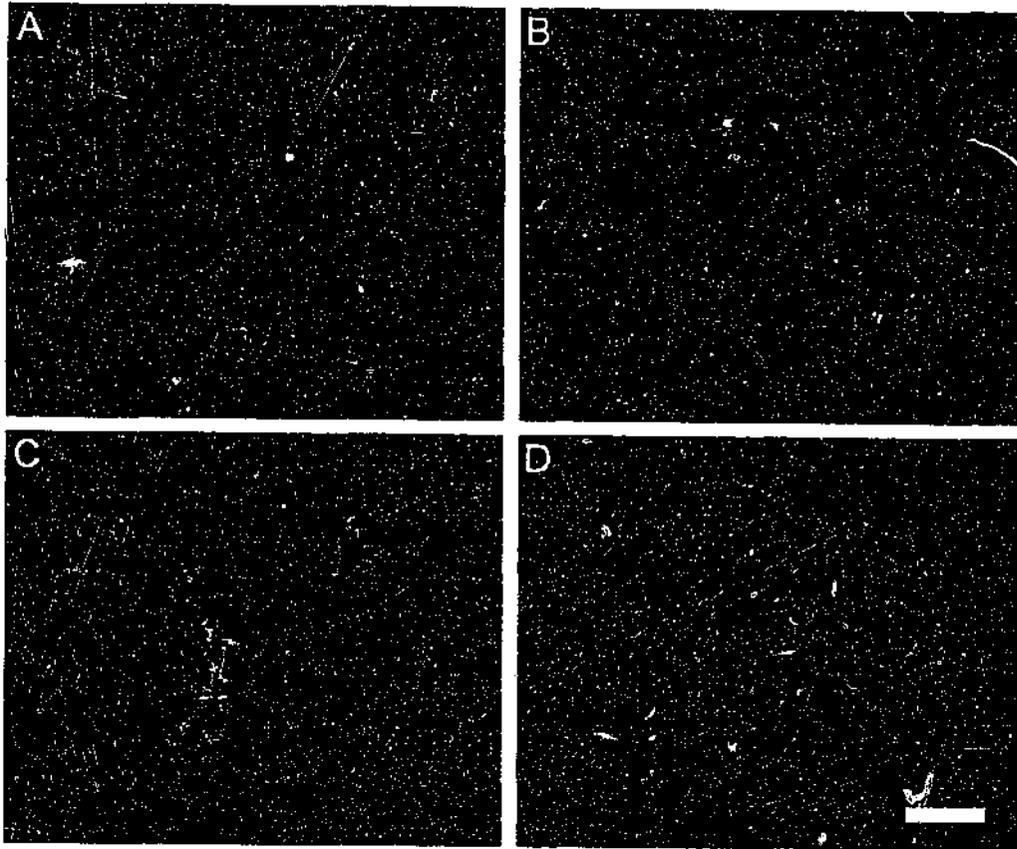
Figure 2.4 Immunocytochemical characterization of cerebellar granule cells

Fluorescent photomicrographs at 8 *div* of cultures following immunostaining for (A) microtubule associated protein 2 (MAP2) under normal culture conditions, (B) glial fibrillary acidic protein (GFAP) with cells grown in serum for 24h in the presence of the mitotic inhibitor aphidicolin (2μg/ml), (C) GFAP in cultures grown with serum, and (D) immunostaining for GFAP in cultures grown in serum and aphidicolin. Scale bar represents 10μm.
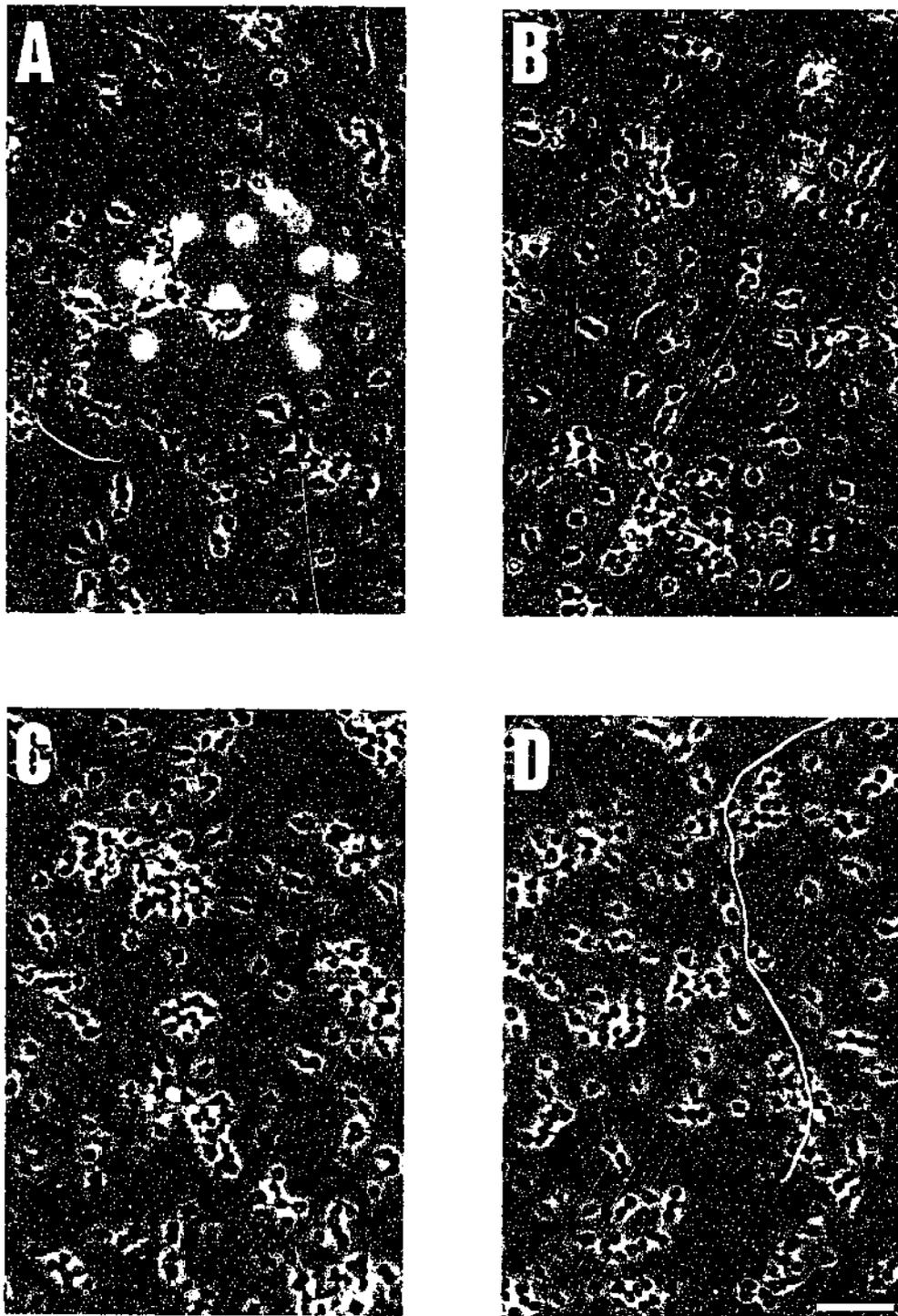
Figure 2.5 Immunocytochemical characterisation of cell division in cultures

Fluorescent/phase contrast micrographs at 8 *div* of cells following immunostaining for (A) bromodeoxyuridine (BrdU) in the absence of aphidicolin, (B) BrdU with cells in the presence of 1μg/ml aphidicolin, (C) 2μg/ml aphidicolin and (D) 3μg/ml aphidicolin. Scale bar represents 15μm.
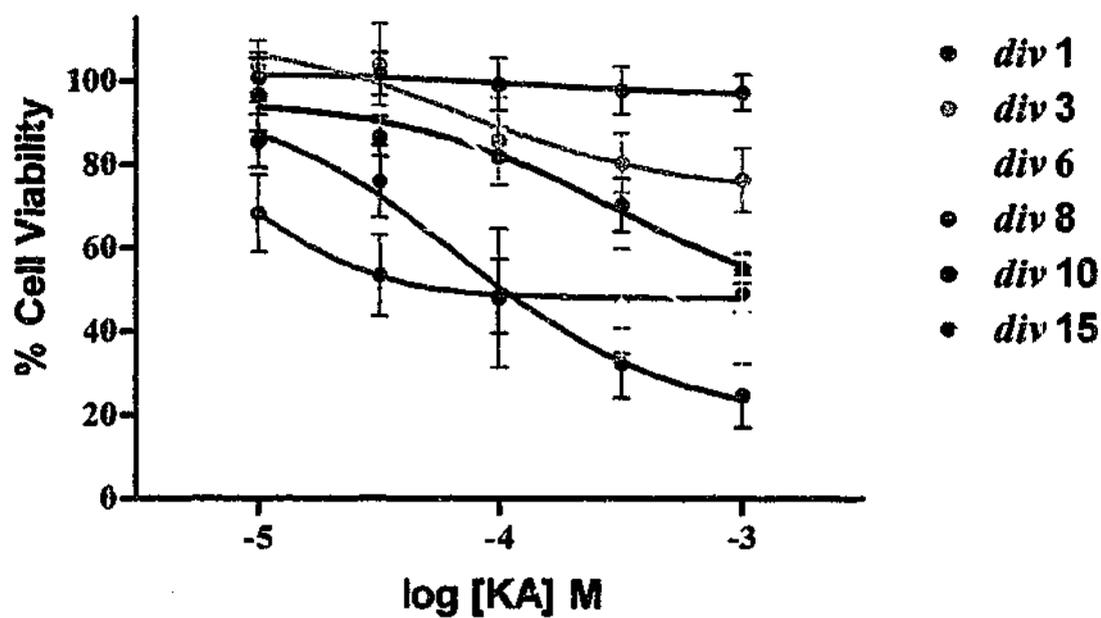
Figure 2.6 Neurotoxic profile of KA during development of cerebellar granule cells

Neuronal viability was assessed by MTT assay. Each point represents the means of 4-6 experiments across 4-6 different cultures (mean ± SEM). The MTT viability assay determined a significant difference in the way the cultures reacted to KA exposure (10-1000µM) dependent on the *div* ($F[5,157] = 36.53$; $p < 0.0001$).

receptor mediated response (Figure 2.7). CNQX more effectively attenuated KA receptor-mediated toxicity than did GYKI 52466. GYKI 52466 was effective at inhibiting cell death mediated by KA at concentrations $> 100\mu M$, however, some 15-20% of cells failed to respond to GYKI 52466 treatment at concentrations $< 100\mu M$. CNQX failed to completely attenuate cells treated with 1mM KA with approximately 5% cell death still evident, but at lower concentrations cell death was completely abolished. MK-801 produced a trend towards a minor decrease in KA-induced neurotoxicity, however this was not statistically significant.

Negligible toxicity was observed for AMPA (10-1000μM; Figure 2.8) over the entire examined time period (*div* 6-16; $[F(4,193) = 0.96, p = 0.737]$). Even in the presence of CTZ (100μM; $[F(9,193) = 1.83, p = 0.923]$), which attenuates the desensitization of AMPA receptors (Funk *et al.*, 1995; Partin *et al.*, 1994), neurotoxicity failed to occur. FW, a potent AMPA receptor agonist, also exerted negligible toxicity at *div* 8 $[F(4,25) = 10.06, p = 0.08]$, the day at which KA-mediated toxicity was maximal, even in the presence of CTZ (100μM; Figure 2.9: $[F(1,25) = 14.31, p = 14.31]$). Since KA does have agonist activity at AMPA receptors (Lomeli *et al.*, 1992), these data on the lack of effect of AMPA and FW, confirm that KA is acting on KA receptors in this culture system.

Toxicity mediated by the NMDA receptor was more complicated than that mediated by the non-NMDA receptors. At earlier days *in vitro* (div 1-3), NMDA produced a trend towards a neuroprotective response that was significantly attenuated by 10μM MK-801, $[F(1,43) = 22.68, p < 0.001]$, whereas days 6, 8 and 10 *in vitro* NMDA produced a concentration-dependent toxicity (Figure 2.10A) with EC_{50} values of 313$\mu M \pm 3.2$, 119$\mu M \pm 0.19$ and 37$\mu M \pm 0.52$, for days 6, 8 and 10, respectively. NMDA-mediated toxicity was concentration-dependent $[F(4,35) = 31.78, p < 0.0001]$ and significantly attenuated by 10μM MK-801 on *div* 8 (Figure 2.10B; $[F(1,35) = 27.86, p < 0.0001]$).
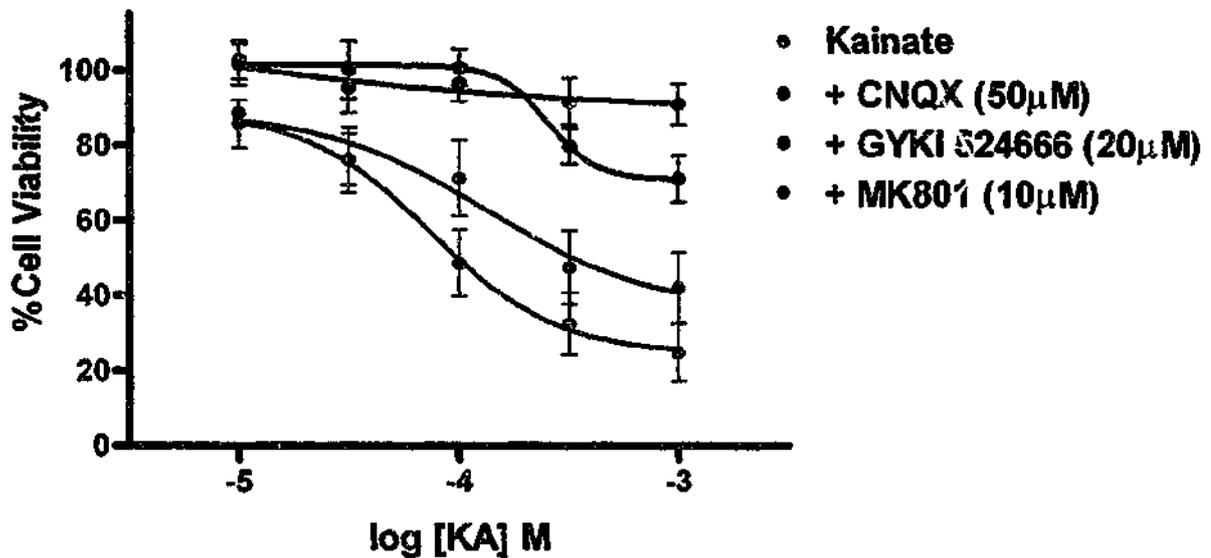
Figure 2.7 Neurotoxic profile of KA

Neuronal viability was assessed by MTT assay on *div* 8. Each point represents the mean of 4-6 experiments across 4-6 different cultures (mean ± SEM). Data from MTT experiments demonstrated a concentration-dependent neurotoxicity caused by KA [$F(4,120) = 21.60$, $p < 0.0001$], that was significantly attenuated by CNQX [$F(1,100) = 24.45$, $p < 0.0001$] and GYKI 52466 [$F(1,90) = 23.79$, $p < 0.0001$], but not MK-801 [$F(1,69) = 2.62$, $p = 0.578$].
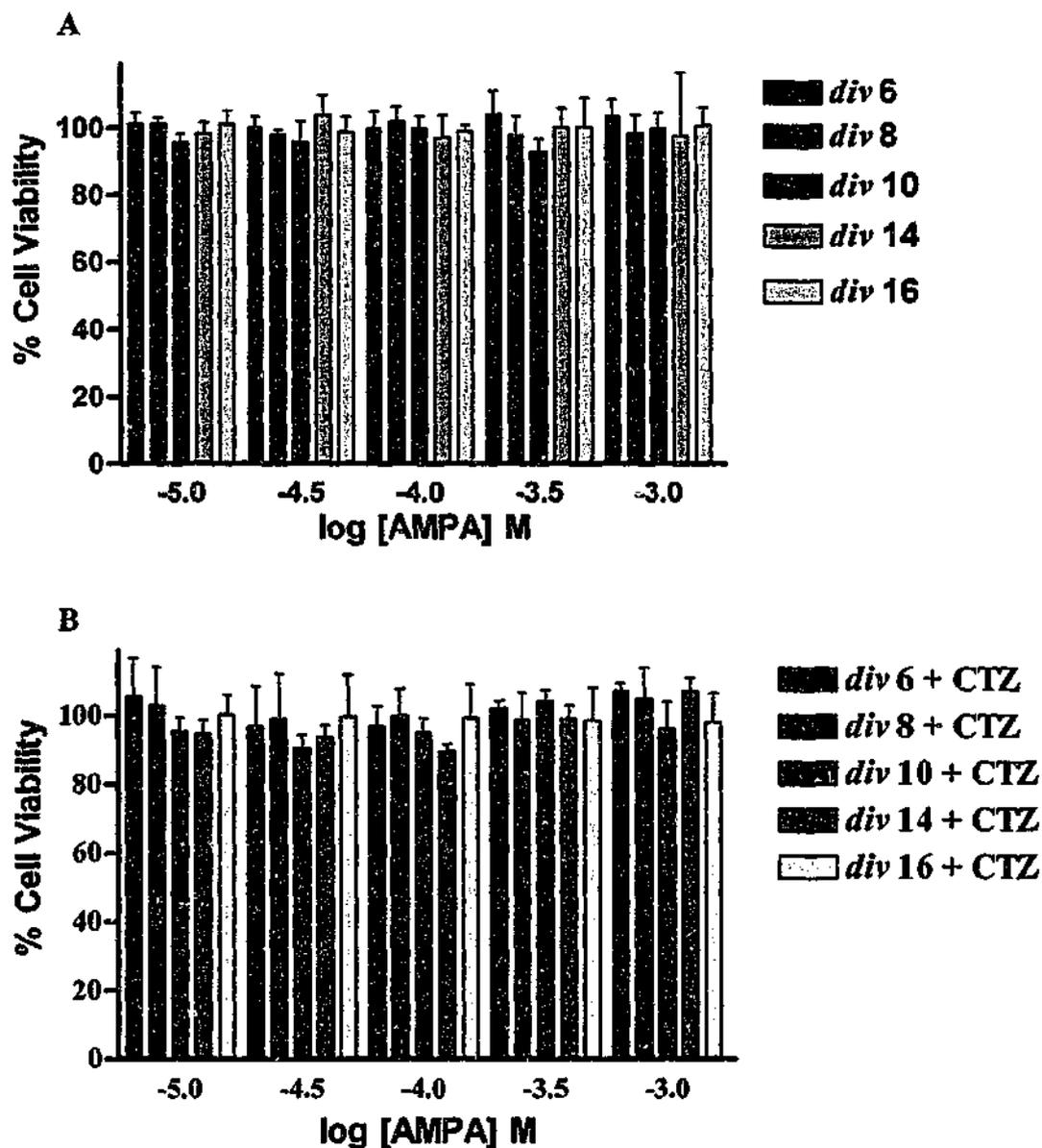
Figure 2.8 Neurotoxic profile of AMPA during development of c iltures

Neuronal viability was assessed by MTT assay. Each point repr ents the mean of 4-6 experiments across 4-6 different cultures (mean ± SEM). Data f)m MTT experiments demonstrated no significant differences in the way the cultures reacted to AMPA exposure dependent on the *div* [$F_{(4,193)}$ = 0.96, p = 0.737] relative to vehicle control treated cultures (A), even in the presence of CTZ (100μM) to attenuate the fast desensitization of the AMPA receptor [$F_{(9,193)}$ = 1.83, p = 0.923] (B).
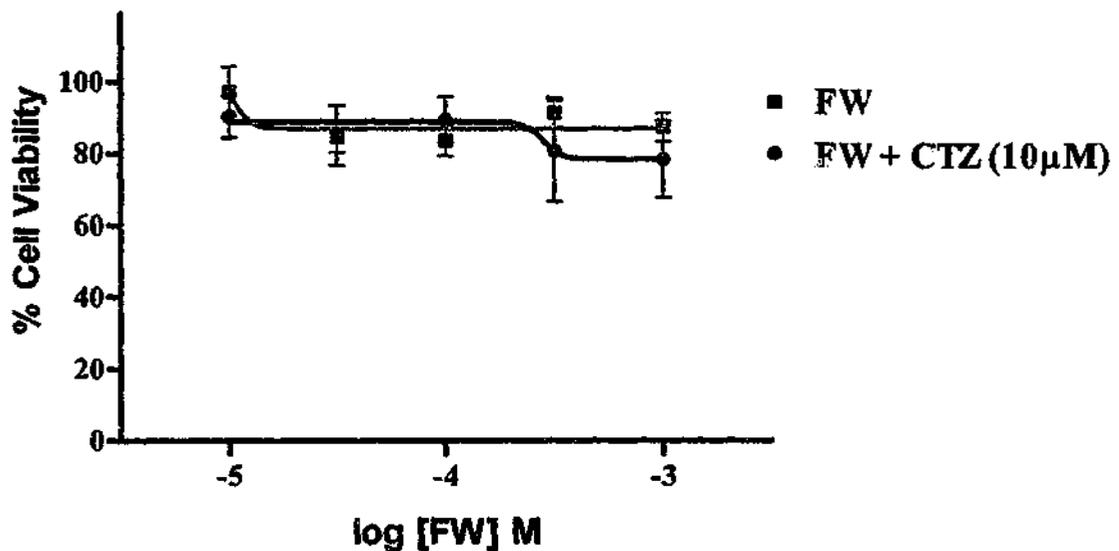
Figure 2.9 Neurotoxic profile of FW in cultured cerebellar granule cells

Neuronal viability was assessed by MTT assay. Each point represents the means of 3-6 experiments across 3-6 different cultures (mean ± SEM). MTT data indicated no significant difference in the way the cultures reacted to FW exposure dependent on *div* 8 [$F(4,25) = 10.06$, p = 0.08] relative to vehicle treated control cultures, even in the presence of CTZ (100μM) to attenuate the fast desensitization of the receptors [$F(1,25) = 14.31, p = 0.39$].

A

B
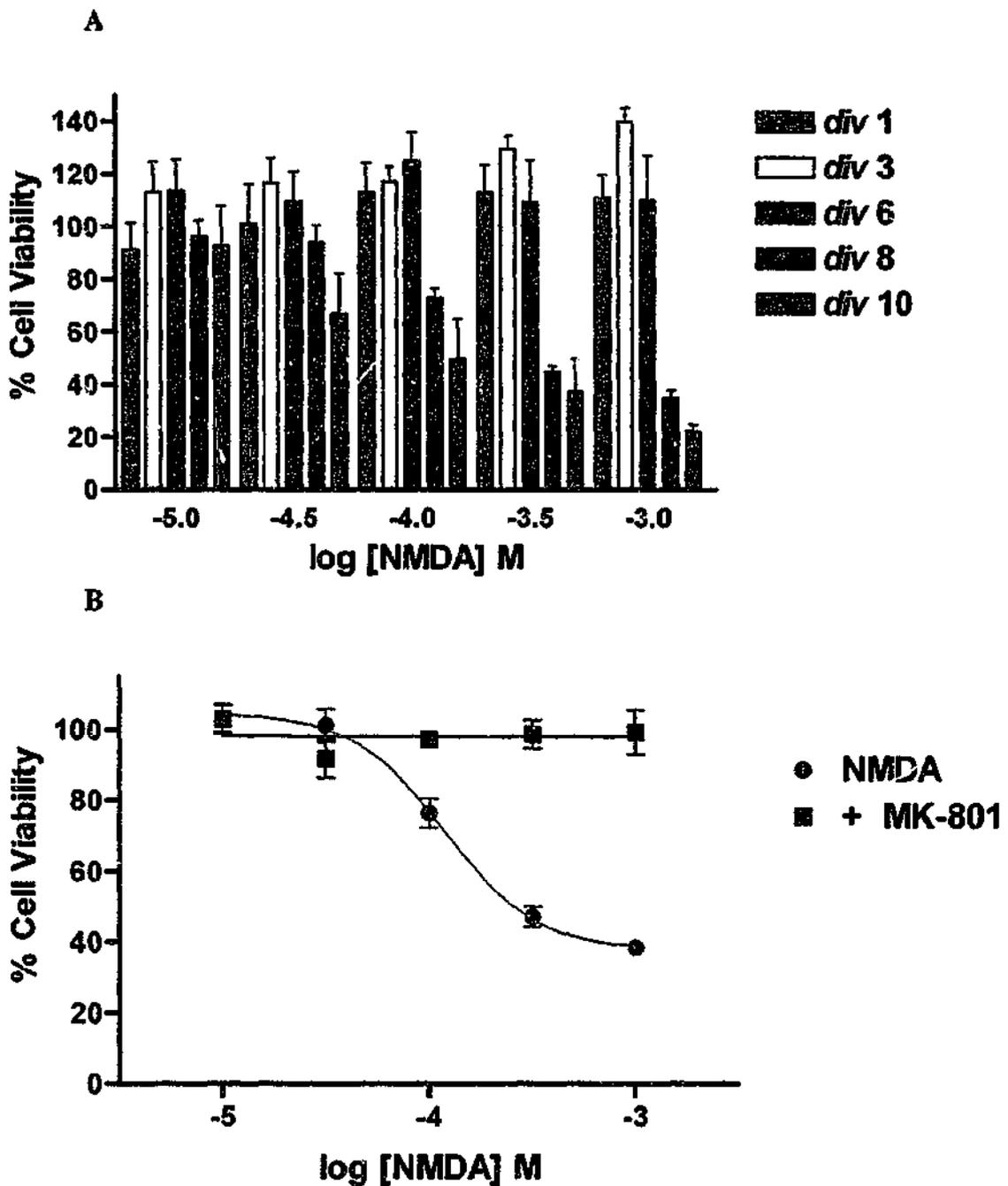
Figure 2.10 Neurotoxic profile of NMDA mediated toxicity

Neuronal viability was assessed by MTT assay. Each point represents the mean of 4-6 experiments across 4-6 independent cultures (mean ± SEM). MTT data revealed a significantly different response to NMDA on various *div* [$F_{(4,103)} = 51.06$, $p < 0.0001$] (A), and NMDA toxicity was significantly attenuated by MK-801 (10μM) on *div* 8 (B) [$F_{(1,35} = 27.86, p < 0.0001$].

Glu-mediated toxicity was examined at day 8 *in vitro*, the day at which KA produced its maximum toxicity. Glu (0.01-1000μM) produced a concentration-dependent cell death ([F(9,100) = 67.04, $p < 0.0001$]; EC_{50} = 2.3μM ± 0.3) that was not completely attenuated by any of the iGluR receptor antagonists alone (Figure 2.11). The non-NMDA receptor antagonists had little effect on Glu mediated toxicity [F(2,75) = 0.99, $p = 0.097$], whereas MK-801 inhibited up to 50% of cell loss [F(1,50) = 23.98, $p < 0.0001$], indicating a large portion of Glu-mediated neurotoxicity occurred by the overstimulation of the NMDA receptors.

2.3.5 *Morphology of Cultures Exposed to iGluR Agonists*

Morphological examination of the cultures treated with the various Glu agonists revealed a complex mechanism by which excitotoxic cell death was mediated, involving both necrosis and/or apoptosis, dependent upon the age of the cultures and the intensity of the insult. However, vehicle treated control cultures demonstrated no morphological change when compared to untreated cultures. Glu produced an intense insult at concentrations as low as 10μM (Figure 2.12B), resulting in some cellular shrinkage, indicative of apoptosis, but mostly cellular swelling, indicative of necrosis, particularly at higher concentrations (300μM; Figure 2.12C). Cellular debris and a reduced cellular density were evident across the whole range of Glu concentrations (0.01-1000μM), indicating that necrosis was the predominant mode of cell death. Glu-induced injury, although not attenuated by 50μM CNQX (Figure 2.12D) or 20μM GYKI 4266 (Figure 2.12E), was partially attenuated by 10μM MK-801 (Figure 2.12F).

Cells treated with KA at *div* 3 (Figure 2.13B) exhibited no morphological differences when compared to vehicle treated controls (Figure 2.13A), even at
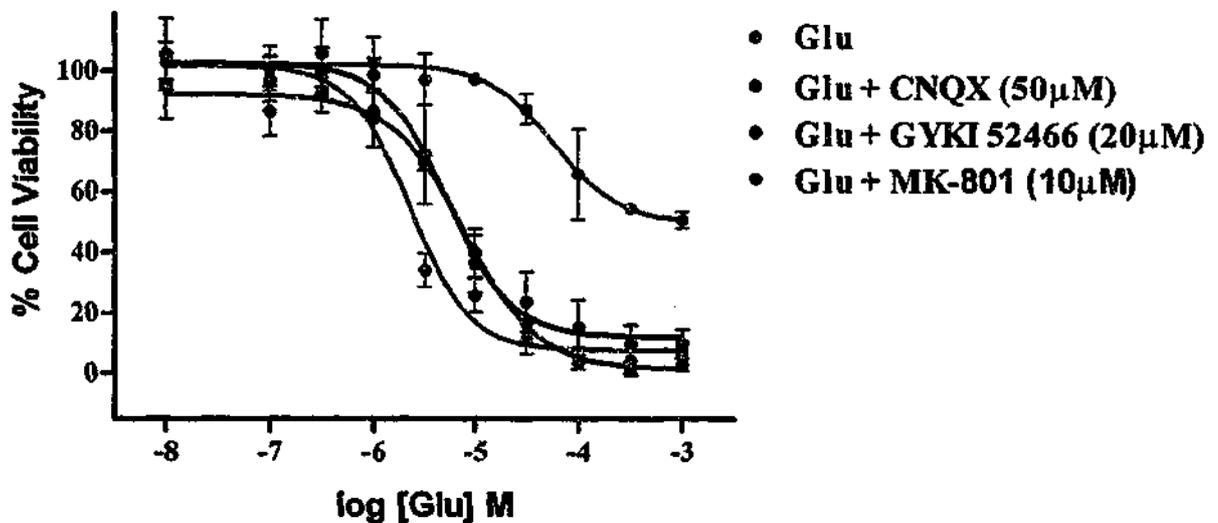
Figure 2.11 Neurotoxic profile of Glu mediated toxicity

Neuronal viability was assessed by MTT assay. Each point represents the means of 4-6 experiments across 4-6 different cultures (mean ± SEM). MTT data indicated a significant concentration-dependent response to Glu [$F(3,100)$ = 14.49, p < 0.0001], and was significantly attenuated by MK-801 (10μM) [$F(1,50)$ = 23.98, p < 0.0001], but not by CNQX (50μM) or GYKI52466 (20μM) [$F(2,75)$ = 0.99, p = 0.097].
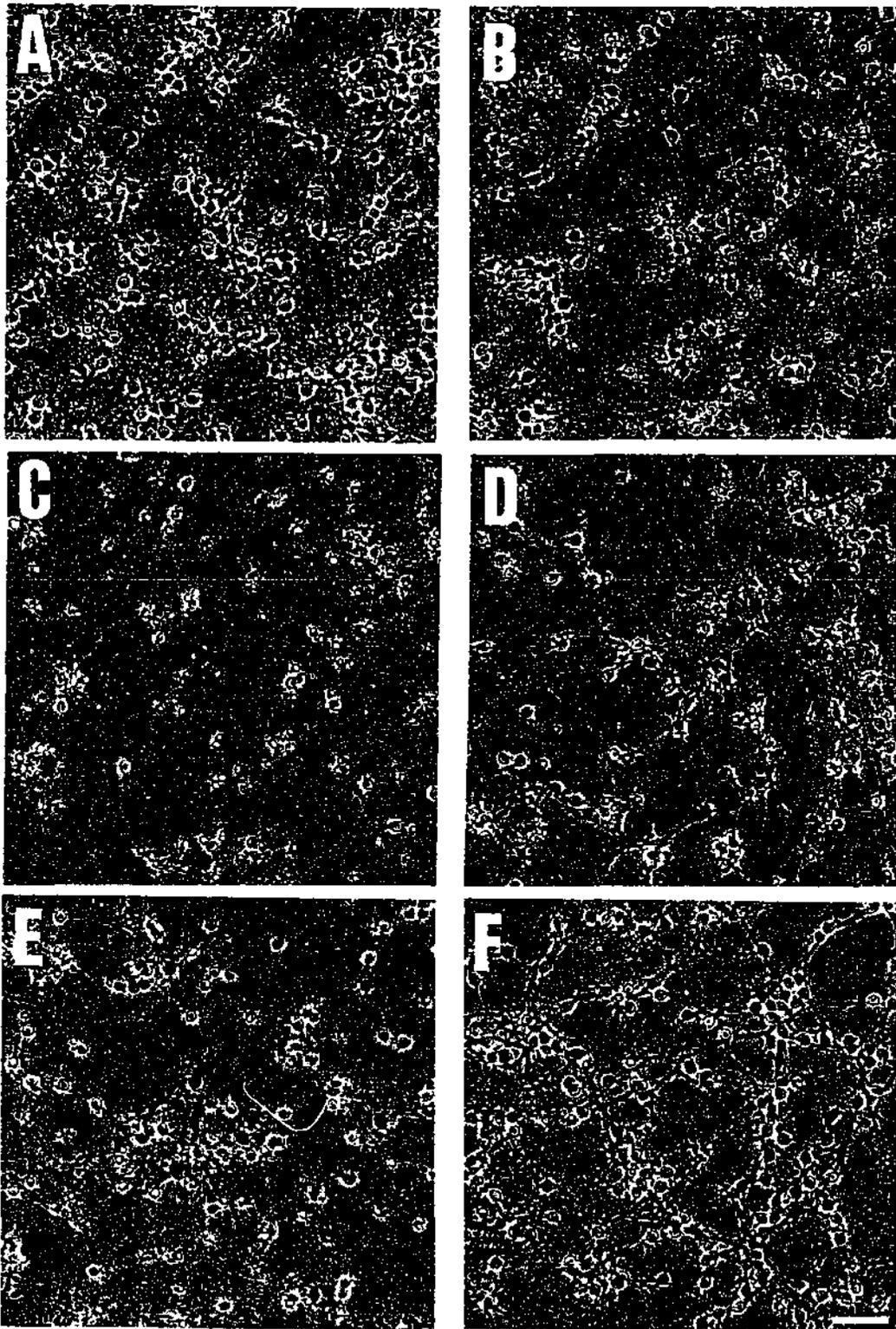
Figure 2.12 Morphology of Glu-mediated cellular injury

Neuronal viability was assessed by phase contrast microscopy 24h after Glu exposure. Glu exposure on *div* 8 resulted in some cellular shrinkage at lower concentrations (10μM, B), but at higher concentrations cellular swelling, indicative of necrosis is evident (300μM, C). Morphological changes caused by exposure to 100μM Glu were not attenuated by CNQX (50μM, D) or GYKI 52466 (20μM, E) and only partially by MK801 (10μM, F). Scale bar represents 10μm.
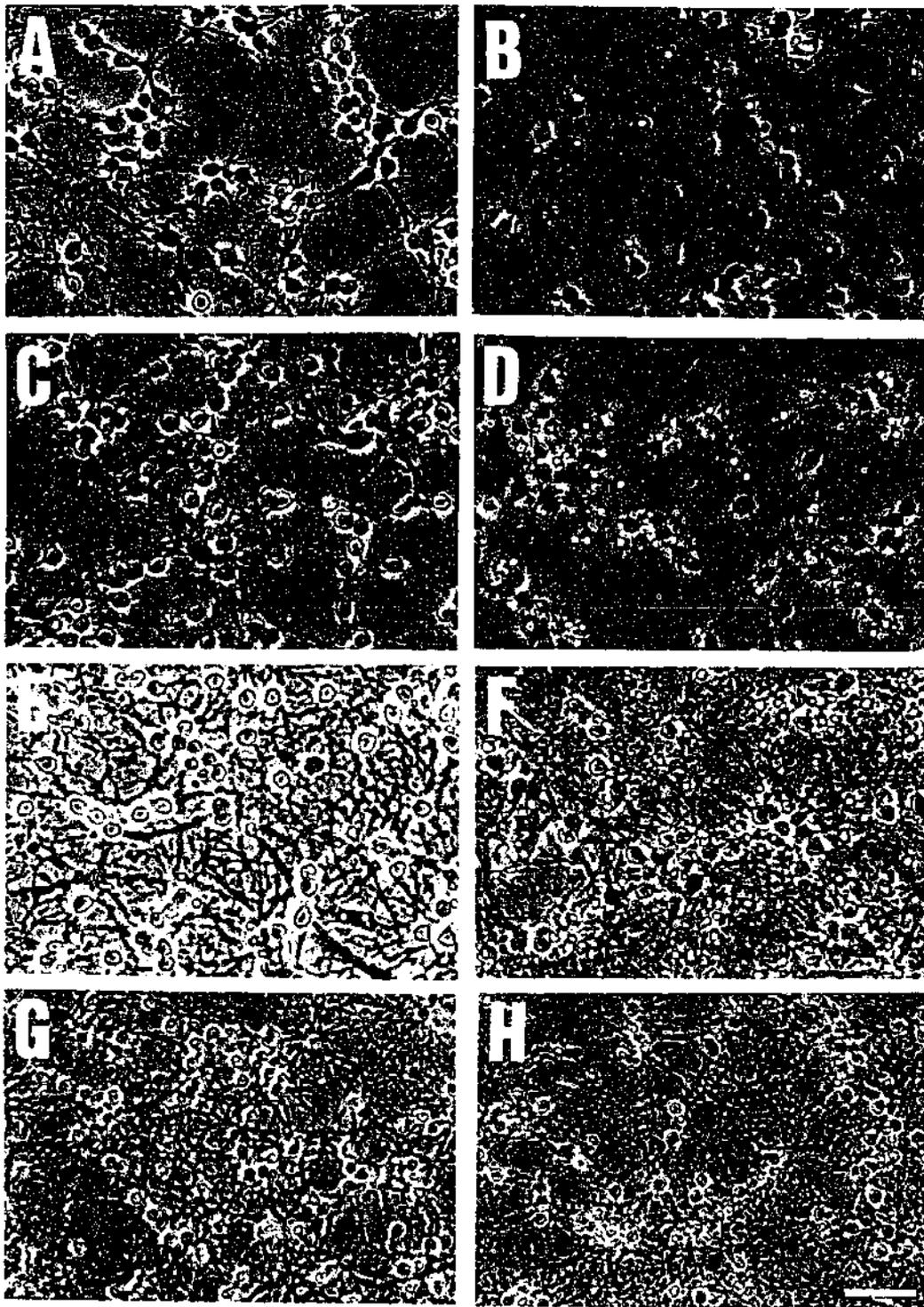
Figure 2.13 Morphology of KA-mediated cellular injury

Phase contrast micrographs taken after 24h exposure to KA on various days *in vitro*. KA exposure on *div* 3 (100µM; B) resulted in no morphological change compared to control sister cultures (A). KA-mediated some toxicity at *div* 6 (100µM; D), with some cellular shrinkage and neurite blebbing evident, compared to *div* 6 control cultures (C). By *div* 8 the majority of cells respond to KA exposure and morphological characteristics of apoptosis are evident (F), with control cultures demonstrating intact cell bodies and neurites (E). Cultures exposed to KA at *div* 10 demonstrate reduced sensitivity to KA (100µM; H) (*div* 10 control; G). Scale bar represents 10µm.

concentrations as high as 1000μM (data not shown). However, cultures treated at *div* 6 (Figure 2.13D), *div* 8 (Figure 2.13F) and *div* 10 (Figure 2.13H) all demonstrated cellular shrinkage and neurite blebbing, indicative of apoptosis. Changes in morphology mediated by KA were attenuated by the non-NMDA receptor antagonists CNQX (50μM; Figure 2.14B) and GYKI (20μM; Figure 2.14C), but not the NMDA receptor MK-801 (10μM; Figure 2.14D) on *div* 8, indicating a KA-receptor mediated mechanism. While the morphological changes induced by KA suggested apoptosis, some higher concentrations of KA caused a little cellular swelling 1-2h after KA exposure, indicative of necrotic cell death, however, the morphology of the cells largely implied apoptosis as the predominant mechanism by which KA mediates cell death.

Exposure of cultures to AMPA or FW (10-1000μM) on various *div* (6-16) produced no significant morphological changes (Figure 2.15B and Figure 2.15E), even when the fast desensitization of the AMPA receptors was attenuated using CTZ (Figure 2.15C and Figure 2.15F). These findings indicate that even if AMPA receptors are present in primary cultures of CGCs cultured under the present serum-free conditions, they do not effect excitotoxic injury.

The morphology of NMDA-mediated toxicity, like Glu, was complex, with cellular shrinkage and neurite blebbing at low concentrations ($\leq 30\mu M$), and cellular swelling leading to a decrease in cellular density was evident at higher concentrations. Toxicity was evident from *div* 6 (Figure 2.16B), with negligible morphological change in cultures treated before this time (Figure 2.16A), with the majority of cells responding to NMDA at *div* 8 (Figure 2.16C). Toxicity was completely attenuated by the NMDA receptor antagonist MK-801 (10μM; Figure 2.17D), but not CNQX (50μM; Figure 2.17C) or GYKI 52466 (20μM; data not shown), corresponding to the MTT cell viability assay.
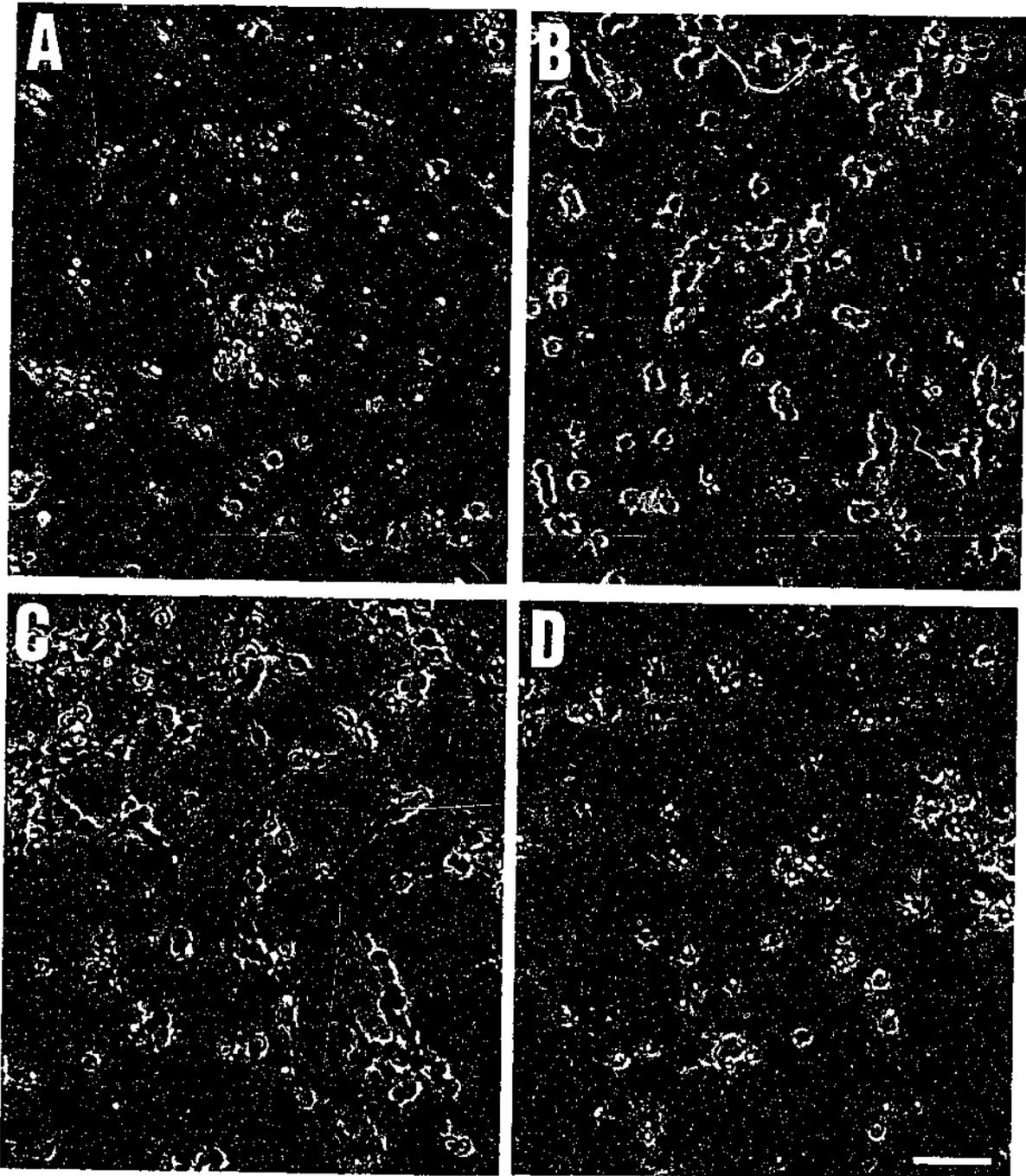
Figure 2.14 Morphology of KA-mediated cellular injury

Phase contrast micrographs taken 24h after exposure to KA in the presence and absence of iGluR antagonists. KA exposure on *div* 8 (100µM; A) resulted in morphological changes consistent with apoptosis, including cellular shrinkage and neurite blebbing that was attenuated by the non-NMDA receptor antagonists CNQX (50µM; B) and GYKI (20µM; C), but not MK-801 (10µM; D). Scale bar represents 10µm.
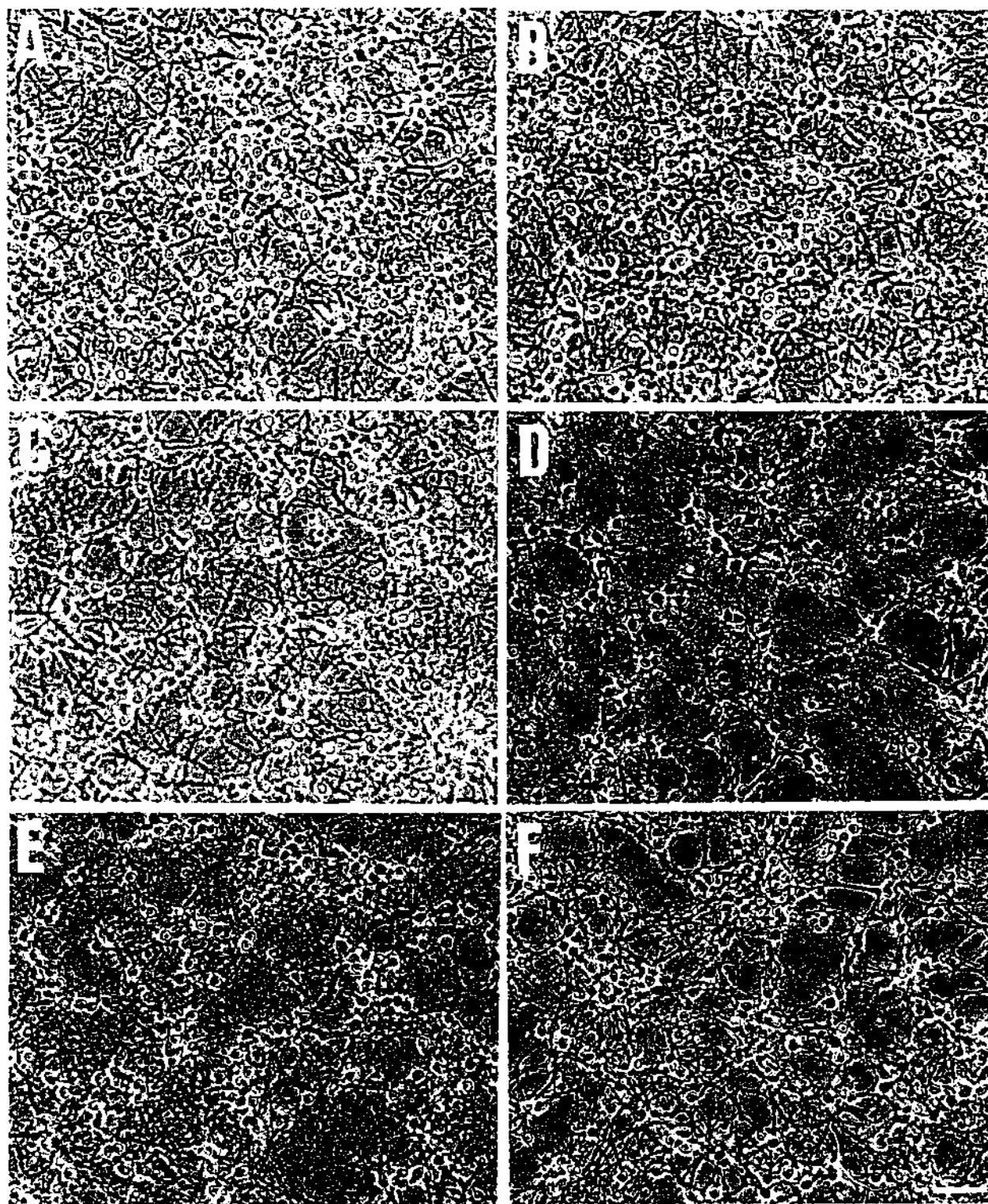
Figure 2.15 Morphology of cells after AMPA and FW exposure

Phase contrast micrographs taken 24h after exposure to AMPA and FW in the presence and absence of CTZ (100µM), which produced no morphological change on *div* 8. AMPA exposure on *div* 8 (1000µM; B) resulted in no morphological changes when compared to control (A), even in the presence of CTZ (C). FW (1000µM), a potent AMPA receptor agonist also failed to cause cellular injury on *div* 8 (E) compared to the CTZ vehicle control (D), even in the presence of CTZ (F). Scale bar represents 10µm.
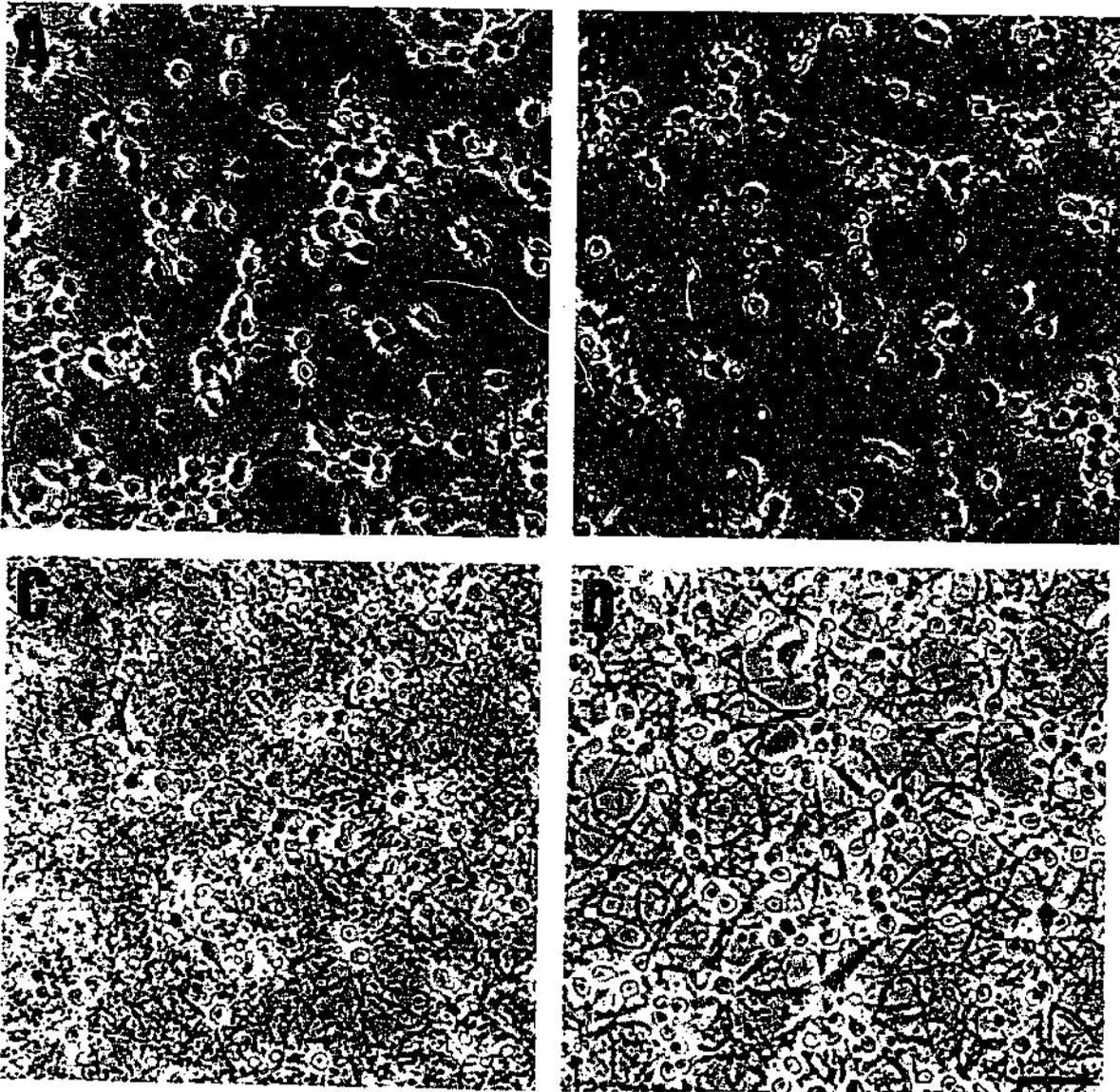
Figure 2.16 Morphology of cells after NMDA exposure on various days *in vitro*

Phase contrast micrographs taken 24h after exposure to 100μM NMDA on *div* 3 (A) caused no significant neurotoxicity. Treatment on *div* 6 resulted in some neurotoxicity (100μM; B). The majority of cells treated with NMDA at *div* 8 (100μM; C) demonstrated cellular injury consistent with necrosis, but some shrunken cell bodies are also present, an injury that is attenuated with MK-801 (D). Scale bar represents 10μm.
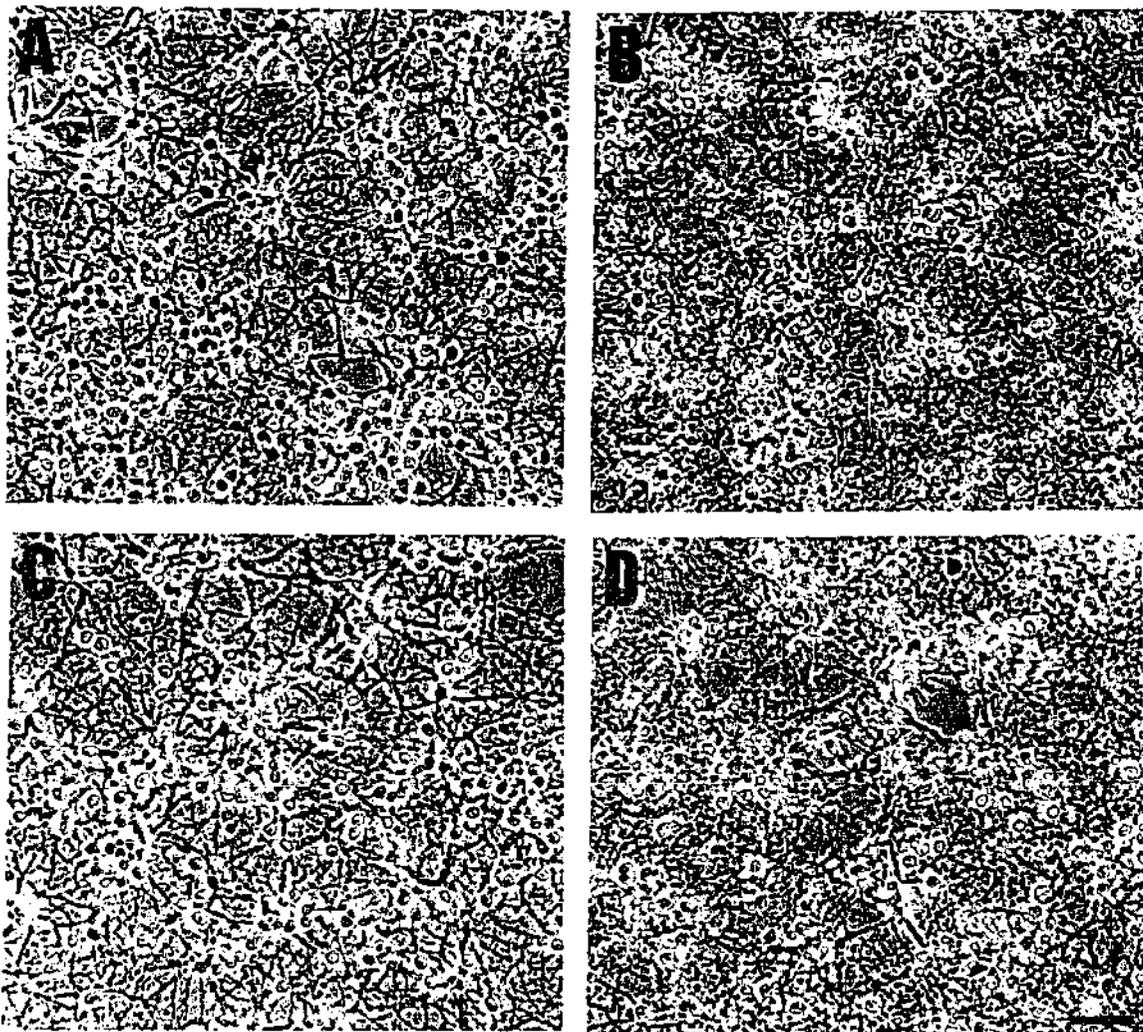
Figure 2.17 Morphological profile of NMDA-induced toxicity

Phase contrast photomicrographs taken 24h after exposure to NMDA in the presence of iGluR antagonists on *div* 8. NMDA exposure resulted in wide spread neuronal damage consistent with necrosis (B), compared to vehicle treated cells (A). MK-801 (10μM; C) attenuated injury induced by NMDA exposure, but not CNQX (50μM). Scale bar represents 10μm.

2.3.6 In situ Labeling of Necrotic Cells with Propidium Iodide

The extent of necrotic cell death mediated by the Glu receptor agonists was examined by PI incorporation 1-2h after exposure. PI is incorporated into cells with compromised cellular membranes, and therefore healthy cells, or apoptotic cells, will not incorporate the dye at early time points. Control cultures demonstrated negligible staining with PI (Figure 2.18A), however Glu (100µM) resulted in the majority of cells incorporating the dye (Figure 2.18B), an effect that was partially attenuated by MK801 (10µM Figure 2.18C), but not the non-NMDA receptor antagonists (data not shown). NMDA exposure (100µM) resulted in an increase in PI labeling (data not shown), that was attenuated by MK-801. KA-induced toxicity only resulted in some necrotic labeling at high concentrations (>300µM; Figure 2.18E), with negligible staining at lower concentrations (30µM; Figure 2.18D), and was attenuated by 50µM CNQX (Figure 2.18F) but not 10µM MK801 (data not shown).

2.3.7 In situ Labeling of Fragmented DNA (TUNEL)

As this thesis primarily concentrates on the KA subtype of iGluR, the mode of cell death produced by KA was further examined by the TUNEL technique. KA-mediated toxicity was determined to be predominantly apoptotic by analysing the extent of DNA fragmentation by TUNEL. The TUNEL technique involves labeling of 3' nick end DNA fragments with digoxigenin which are then detected with immunocytochemistry employing alkaline phosphatase. Many TUNEL-positive profiles were noted in KA treated cells (Figure 2.19B), with negligible staining in control sister cultures (Figure 2.19A). CNQX (50µM) attenuated TUNEL-positive staining (Figure 2.19E), but MK-801 (10µM) did not
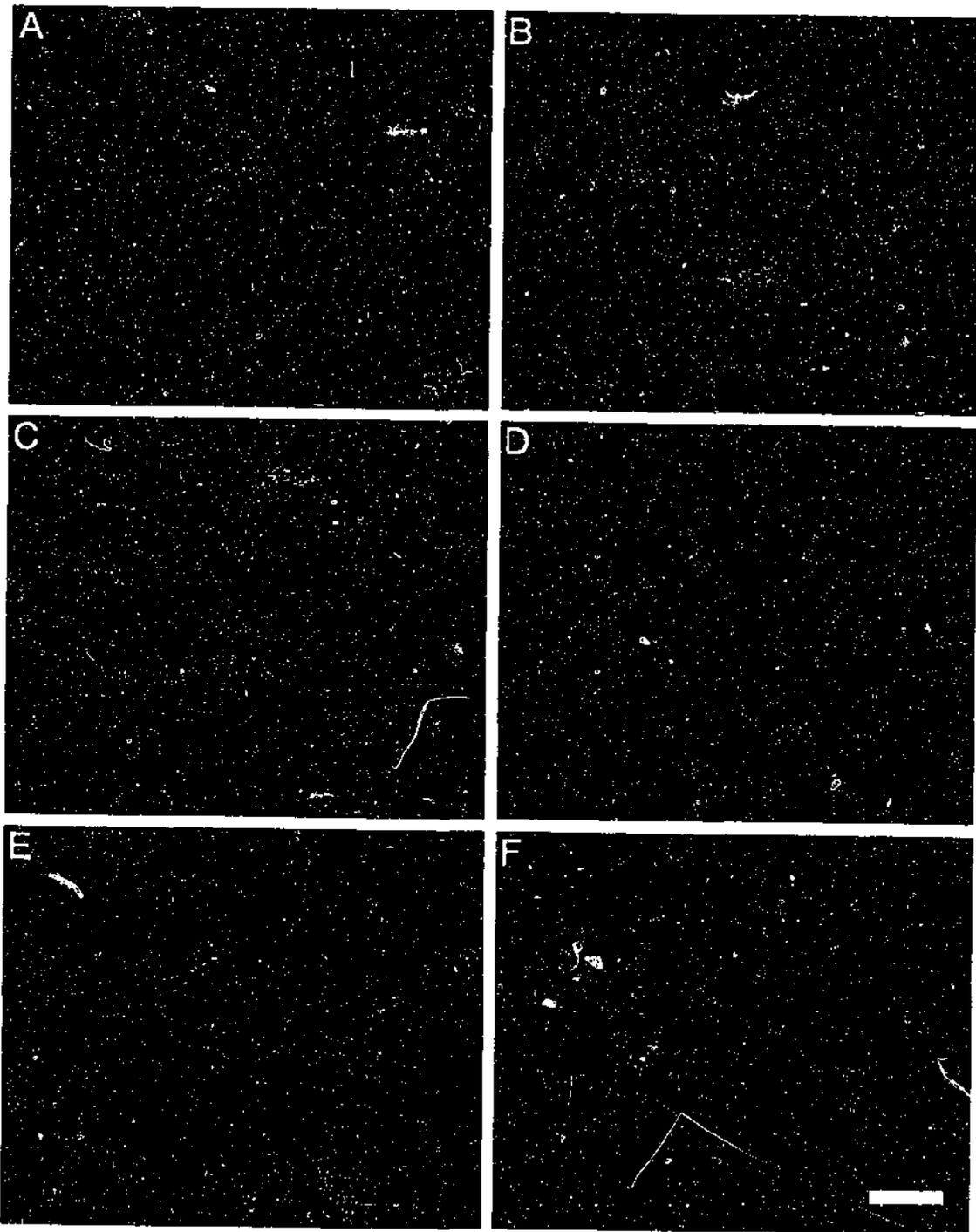
Figure 2.18 PI incorporation (necrosis) after Glu agonist exposure

Fluorescent micrographs taken on *div* 8 1-2h after exposure to 100μM Glu (B) demonstrated a marked increase in the number of cells incorporating PI compared to control (A), labeling that was attenuated by MK-801 (10μM; C). KA exposure at lower concentrations (30μM; D) resulted in negligible PI incorporation, however more cells at higher concentrations (300μM; E) were labeled, and this labeling was completely attenuated by CNQX (50μM; F). Scale bar represents 10μm.
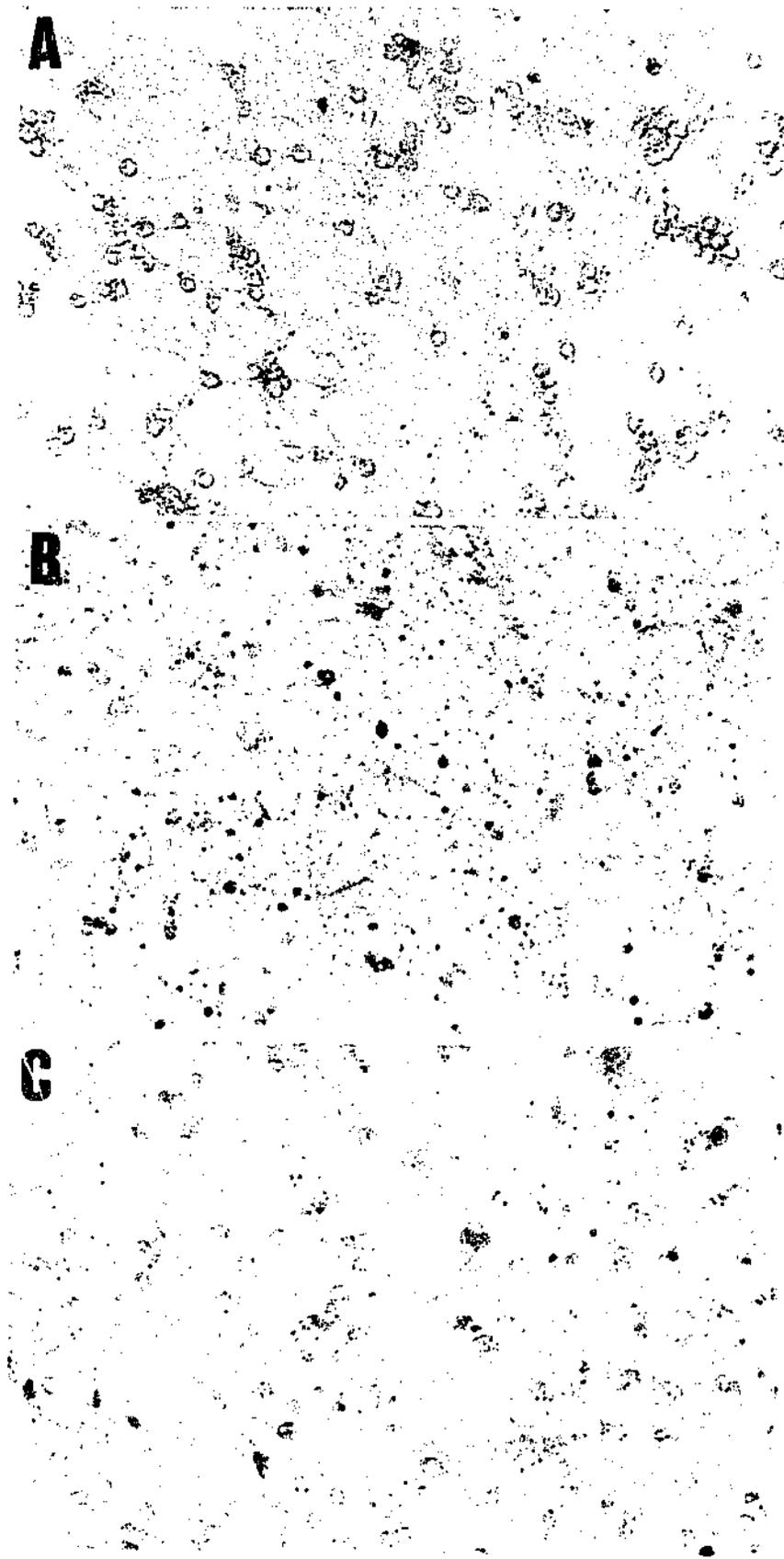
Figure 2.19 TUNEL (apoptosis) labeling of cultures exposed to KA

Bright field micrographs of cultures demonstrating expression of TUNEL-positive cells (apoptosis) following 24h exposure to (A) vehicle control, (B) 300µM KA and (C) 300µM KA in the presence of CNQX (50µM). Scale bar represents 10µm.

(data not shown), indicative of KA receptor-mediated apoptosis and corresponding with the MTT data.

2.4 DISCUSSION

Primary neuronal cultures are a convenient model system in which biochemical pathways and receptor-mediated responses of the central nervous system can be examined in a controlled milieu. Studies of cell survival and death processes *in vitro* are advantageous due to the absence of phagocytes, which rapidly remove dying cells from all major organ systems (Savill *et al.*, 1993), and due to the absence of other complex interferences from the central and peripheral nervous systems. CGCs are a particularly useful model, due to the homogeneity of the cells (Messer, 1977), making them a suitable model system for examining gene and protein expression. In the present study, cerebellar granule cell cultures were successfully maintained in the absence of serum and represented a viable monolayer of pure neurones, with minimal spontaneous cell death and contamination from glial cells. This even distribution mode of the culture is especially suitable for morphological and immunocytochemical analysis. While older cultures (> *div* 10) did demonstrate some signs of degeneration, the majority of cells were intact with no morphological signs of cell damage. Microscopic examination confirmed that the cells maintained in culture possessed identical morphologies to those previously attributed to CGCs (Schousboe *et al* 1985; Messer, 1977). Moreover, previous studies in our laboratory using these neurones cultured in NMDA with B27 supplements have confirmed their identity to be glutamatergic (Carroll *et al.*, 1998a)

CGCs are classically grown in growth media containing serum and inhibitors of cellular proliferation (Gallo *et al.*, 1987; Gunnmoore & Tavare, 1998; Messer, 1977). In

many studies neuronal apoptosis is actually induced by serum-withdrawal (Gunnmoore & Tavare, 1998; Villalba *et al.*, 1997; Miller & Johnson, 1996), whereby the cultures are established such that they are dependent upon serum. Serum strongly favours the proliferation and survival of glial and astrocytes (Torrens *et al.*, 1986), and therefore media that contain serum must also contain cytotoxic inhibitors of proliferation, such as cytosine arabinoside, to attenuate the stimulation of non-neuronal cell proliferation. Unfortunately, many mitotic inhibitors are indeed toxic to neurones (Enokido *et al.*, 1996; Dessi *et al.*, 1995), further confounding studies involving the analysis of neurotoxicity. Therefore, the growth of cultures in conditions that favour neuronal growth eliminates the requirement of potentially neurotoxic mitotic inhibitors. The current study did in fact use a mitotic inhibitor, aphidicolin, this compound, however, was not toxic to cultures at the concentrations employed (Miller & Johnson, 1996). Aphidicolin was employed as later studies examine the effects of apoptosis of various genes that control the cell cycle, and the rationale was that glial cells are capable of mitotic division and could give false positive results. B27-supplemented growth media was employed in this study as it is a specially designed media that has been optimized for the growth of neurones and not glia (Brewer *et al.*, 1993). While reports have indicated that CGCs do not require a depolarizing concentration of K^+ in B27 supplemented media (Brewer, 1995), under the present experimental conditions neurones underwent apoptotic cell death in physiological concentrations (5.4mM) of K^+.

In this study the ability of non-NMDA receptor agonists to induce toxicity was examined in primary cultures of CGCs. NMDA and KA, but not AMPA, produced a significant, concentration-dependent cellular injury. Sensitivity of the cultures to the various agonists differed dependent upon the time in culture. Cultured neurones appeared to be insensitive to exposure to iGluR agonists up until *div* 3, where KA exposure

significantly reduced cell viability. NMDA exposure reduced cell viability from *div* 6, whereas before this time a trophic effect was noted as previously described (Favaron *et al.*, 1993; Balazs *et al.*, 1990; Balazs *et al.*, 1989). This delayed appearance of toxicity is most likely due to the time taken for the receptors to be synthesized, assembled and inserted into the plasma membrane. The cultures developed sensitivity to NMDA and KA at different days *in vitro* suggesting discrete regulatory mechanisms for receptor expression between the iGlu receptor subtypes. While KA and NMDA produced a notable reduction in cell viability, there was no significant change in cells treated with the AMPA receptor agonists AMPA and FW. However, unlike the culture conditions presented here, AMPA toxicity has previously been reported in CGCs when stimulated in physiological levels of K^+ (5mM) (Cox *et al.*, 1990). However, AMPA receptor-mediated toxicity has also been reported in cultures grown in the presence of high K^+ (25mM) and serum after *div* 9, with maximal toxicity at *div* 13-16 (Hack *et al.*, 1995). Here no significant AMPA receptor-mediated toxicity was found even when the rapid receptor desensitization was attenuated by the addition of CTZ. These data suggest that there is an absence of receptor expression in the present serum-free system of CGCs, in agreement with past observations (Hack *et al.*, 1995; Resink *et al.*, 1994). Culture conditions have a profound effect on receptor expression in primary cultures of CGCs. NMDA, receptors for instance, are actually down regulated under depolarizing conditions including K^+ treatment or exposure to NMDA (Resink *et al.*, 1995; Resink *et al.*, 1994). This evidence may be one explanation for the relatively high concentrations of NMDA required to cause toxicity when compared to other culture systems (Choi *et al.*, 1988). Another explanation may be the presence of Mg^{2+} ions in the experimental media, although the depolarizing conditions (25mM K^+) should remove the voltage-dependent Mg^{2+} block present in the NMDA receptor complex, as previously reported (Resink *et al.*, 1994). Interestingly, Glu-mediated toxicity was found to

be concentration-dependent and mainly mediated by the NMDA receptor, but only slightly by the non-NMDA receptors, suggesting another component by which Glu toxicity occurs, possibly involving the disruption of ions or GluTs dysfunction.

While toxicity induced by AMPA and FW has previously been reported in neuronal cells to be mediated by the AMPA receptor (John *et al.*, 1999; Jensen *et al.*, 1998; Larm *et al.*, 1996; Yoshioka *et al.*, 1995), AMPA-mediated toxicity is also thought to be in part mediated by the NMDA receptor (Jensen *et al.*, 1998). In contrast to the current findings, AMPA-induced toxicity has been previously observed in CGCs grown under serum-containing conditions (Cebers *et al.*, 1997). AMPA receptor-mediated toxicity has been reported to be slow and apoptotic in nature (Limatola *et al.*, 2000; John *et al.*, 1999; Larm *et al.*, 1997b; Dugan *et al.*, 1995), and requires some 24-48h exposure and is often accelerated by the presence of CTZ before toxicity is widespread. Under the current conditions 24h exposure to AMPA in the presence of CTZ induced no toxicity in primary cultures of CGCs. AMPA receptor expression is highly influenced by growth factors, culture conditions and the time *in vitro* (Cebers *et al.*, 1997; Hack *et al.*, 1995; Resink *et al.*, 1994; Condorelli *et al.*, 1993), so it is quite possible that serum-free maintenance conditions were not optimal for the expression of AMPA receptors in the current model system.

KA-receptor mediated toxicity has previously been reported in many studies and model systems, and KA is considered to be a more potent than Glu as a neurotoxin (Olney *et al.*, 1974). While KA has previously been shown to cause toxicity through the AMPA receptors in cultured hippocampal neurones (Ohno *et al.*, 1997), the current model system detected no AMPA receptor expression and therefore the toxicity induced by KA was mediated by the KA receptor since the NMDA receptor antagonist, MK-801, was ineffective at attenuating injury. KA receptor-mediated cell death has previously been

shown to occur by apoptosis (Cheung *et al.*, 1998a; Gillardon *et al.*, 1995; van Lookeren Campagne *et al.*, 1995) and/or necrosis (Moldrich *et al.*, 2000a; van Lookeren Campagne *et al.*, 1995 Garthwaite & Wilkin, 1982). The present study demonstrated the mechanism of cell injury induced by KA was almost exclusively apoptotic in nature, with only a few swollen cells evident 2-3h after exposure to high concentrations of KA. Since KA mediated toxicity was only evident after *div* 6, it can be implied that receptor expression and synthesis was occurring before this time. Maximal toxicity was evident at *div 8*, while after this time the extent of cellular injury was substantially less. This differential *div*-dependent neurotoxicity may be explained by the mild and chronic acidosis, which occurs in older cultures, by a reduction of NMDA receptor channel function, and which previously has been found to be neuroprotective (Leahy *et al.*, 1994; Giffard *et al.*, 1990). While the current study demonstrated no significant neuroprotection by MK-801 against KA-induced toxicity, a similar mechanism may be occurring with a reduction of KA receptor channel function under chronically mild conditions. Another mechanism which may have reduced the sensitivity of the cultures to KA may be the accumulation in the culture media over time of various growth factors, which are capable of protecting the cultures from excitotoxic insult.

NMDA exposure resulted in neuroprotection (*div* 1-6) and neurotoxicity (*div* 6-14), dependent on the age of the cultures. Neuroprotection mediated by NMDA in primary cultures of CGCs has been previously reported and thought to occur by depolarizing the cells (Balazs *et al.*, 1990; Balazs *et al.*, 1989), activating phosphatidylinositol 3-kinase (Zhang *et al.*, 1998) and subsequently increasing the expression of various growth factors such as brain derived neurotrophic factor (Favaron *et al.*, 1993). This effect was most pronounced on *div* 3, whereas before this time expression of the receptors would have been negligible and at *div* 6 some cells may have been susceptible to toxicity compensating for

the protective effect. The transition of the NMDA response coincides with the expression of the KA receptors. While NMDA receptor function is dependent upon the removal of the voltage-dependent Mg^{2+} block, which may be achieved by the depolarizing conditions achieved by the presence of K^+. Interestingly, the high K^+-dependence of CGCs occurs from *div* 5 (Resink *et al.*, 1995), approximately coinciding with NMDA receptor-mediated toxicity. At earlier times the NMDA receptor stimulation may not exceed the threshold necessary to induce toxicity, but may have been sufficient to stimulate growth factor expression and therefore protection. Studies that have previously reported NMDA receptor-mediated toxicity in cultured CGCs have employed serum in the growth media (Eimerl & Schramm, 1991; Didier *et al.*, 1990; Frandsen & Schousboe, 1990a; Lysko *et al.*, 1989). Serum contains various growth factors and nutrients that may stimulate the expression of NMDA receptors at an earlier time *in vitro* than found under serum-free conditions.

Overall, the present investigation has demonstrated that iGluR-mediated cell death is complex, with likely time-dependent expression of the various subtypes of receptor and different mechanisms of mediating cell death. AMPA-mediated toxicity was not evident over the time period examined as previously reported (Carroll *et al.*, 1998a), making the current model system ideal to examine KA receptor-mediated responses. NMDA produced a neuroprotective effect at earlier time points *in vitro* and induced a necrotic cell death at later time points, both effects being attenuated by MK-801. Glu-mediated cell death at *div* 8 was largely mediated by the NMDA receptor and predominantly necrotic in nature. Toxicity mediated by the KA receptor was apoptotic in nature, with toxicity only evident after 6 days *in vitro*. The remainder of this thesis will now concentrate on defining and analysing cell death mediated by the KA receptor.

CHAPTER THREE

ANALYSIS OF PUTATIVE LOW-AFFINITY KAINATE

RECEPTOR AGONISTS

3.1 INTRODUCTION

L-Glutamate (Glu) receptor-mediated neurotoxicity, termed excitotoxicity, is well documented, and is likely to be involved in numerous neurological diseases (Fonnum, 1998; Leist & Nicotera, 1998; Coyle & Puttfarcken, 1993). Glu receptor-mediated excitotoxicity appears to involve two forms of neuronal injury, necrosis and apoptosis (Cheung *et al.*, 1998b; Ankarcrona *et al.*, 1995). Necrosis is a passive process characterized by cellular swelling (Clarke, 1990), whereas apoptosis involves complex mechanisms requiring energy and protein synthesis (Dessi *et al.*, 1994a; Deckwerth & Johnson, 1993). Morphological changes induced by apoptosis are characterized by cell shrinkage, nuclear condensation, oligonucleosomal fragmentation of genomic DNA and neurite degeneration (Clarke, 1990). Apoptosis is mediated by various pathways involving caspases and various families of kinases (Francois & Grimes, 1999; Thornberry & Lazebnik, 1998; Ross, 1996; Xia *et al.*, 1995).

Glu acts on two types of receptors the G-protein coupled mGluRs and the ion-channel gated iGluRs. The iGluRs, which are named after their preferring agonists, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) (Hollmann & Heinemann, 1994), in particular are widely implicated in numerous neurodegenerative and acute neurological conditions (Leist & Nicotera, 1998; Fonnum, 1998; Ikonomidou & Turski, 1995; Lipton & Rosenberg, 1994; Coyle & Puttfarcken, 1993). Results from a considerable body of neurophysiological and neurochemical studies have provided evidence for the existence of discrete subtypes of Glu receptors (see Chapter One). Cloning experiments have played an essential role in the understanding of the various Glu receptor subunits and their ion permeabilities, and led to the discovery of the various families of subunits including GluR1-4 (AMPA), NR1 and

NR2A-D (NMDA), KA1-2 (high-affinity KA receptors) and GluR5-7 (low-affinity KA

receptors) (Hollmann & Heinemann, 1994).

Historically, studies conducted in rat forebrain membranes, demonstrated high (K_D

$\approx$ 4-16nM) and low ($K_D \approx$ 27-66nM) affinity binding for [^{3}H]KA (London & Coyle, 1979).

Much later expression cloning and *in situ* hybridization were employed to identify the KA

receptor subtypes, with GluR5 the first to be discovered (Bettler *et al.*, 1990) and the later

identification of GluR6, GluR7, KA1 and KA2 soon followed. GluR6, KA1 and KA2

share 80%, 74%, 43% and 44% sequence homology with GluR5, respectively (Egebjerg *et*

al., 1991). GluR5 and 6 subunits can form heteromeric and homomeric ion channels that

are activated by KA, but not AMPA (Egebjerg *et al.*, 1991). While GluR7 subunits were

initially believed to only form heteromeric receptor assemblies, recently they too have

been shown to form homomeric assemblies (Schiffer *et al.*, 1997). Low-affinity receptors

contain sites for RNA editing in both native and recombinant receptors: GluR5 and 6 (Q/R)

and GluR6 (I/V and Y/C) (Bernard *et al.*, 1999; Seeburg *et al.*, 1998; Belcher & Howe,

1997). Both edited and unedited forms of the GluR5 and 6 receptor subtypes exist in the

mammalian brain (Belcher & Howe, 1997), and differ in expression throughout the

development of the nervous system (Schmitt *et al.*, 1996). Structure-function relationships

indicate that in the transmembrane domain II of these KA receptors, a Q or R residue

determines ion selectivity with the unedited and edited versions of GluR6 exhibiting

different Ca^{2+}-permeability, where GluR6(Q) is considerably more Ca^{2+}-permeable

(Bernard *et al.*, 1999). Moreover, the extent of editing seems to dependent of the cell type

with GluR5 editing predominant in the peripheral nervous system and GluR6 editing

highest in the forebrain and cerebellum (Bernard *et al.*, 1999). Low-affinity KA receptors

are widely distributed throughout the rodent and primate brains (Bernard *et al.*, 1999;

Carroll *et al.*, 1998b; Bettler *et al.*, 1990) and evidence indicates that low-affinity KA

receptors are likely to play significant physiological roles in synaptic transmission and long-term potentiation (Reid & Bliss, 2000; Bortolotto *et al.*, 1999; Vignes *et al.*, 1998; Clarke *et al.*, 1997). GluR5-7 have been associated with various disease states including Alzheimer's disease (Aronica *et al.*, 1998), schizophrenia (Breese *et al.*, 1995; Kerwin *et al.*, 1990), amyotropic lateral sclerosis, cerebral ischaemia and epilepsy (Bernard *et al.*, 1999; Paschen *et al.*, 1996). There is some evidence to suggest a differential regulation of the RNA editing of the low-affinity KA receptor subunits, particularly GluR5 and 6, after KA-induced seizures in the hippocampus (Bernard *et al.*, 1999). GluR5 editing is upregulated in the striatum, but not the hippocampus or the cortex, in animal models of ischaemia (Paschen *et al.*, 1996). Interestingly, GluR6 editing is significantly reduced in the cortex, hippocampus and striatum after transient cerebral ischaemia (Paschen *et al.*, 1996), suggesting regulation of RNA editing is dependent upon cell type and local factors.

Recently novel Glu analogues have been developed and studies employing recombinant receptor model systems have demonstrated selectivity for the low-affinity KA receptor subunits (Clarke *et al.*, 1997; Jane *et al.*, 1997; Zhou *et al.*, 1997). These compounds include (*RS*)-2-amino-3-(hydroxy-5-*tert*-butylisoxazol-4yl)propanoic acid (ATPA), (*S*)-iodowillardiine (IW), (2*S*,4*R*,6*E*)-2-amino-4-carboxy-7-(2-naphthyl)hept-6-enoic acid (LY339434) and (2*S*,4*R*)-4-methylglutamate (4-MG; also commonly termed SYM 2081). Limited studies have been reported on the excitotoxic profiles of 4-MG, ATPA, IW and LY339434. However, recombinant receptor studies have revealed some of the physiological and binding characteristics of these Glu analogues. ATPA, an analogue of AMPA, is an effective AMPA agonist, and has activity at GluR5 but not GluR6 (Wahl *et al.*, 1998b; Clarke *et al.*, 1997), while IW, like other willardiines (Watkins *et al.*, 1990) has some AMPA activity (Patneau *et al.*, 1992), but is a potent GluR5 receptor agonist (Jane *et al.*, 1997). Binding studies have revealed 4-MG has higher affinity for GluR6 than

NMDA or AMPA receptors and displaces [^{3}H]KA binding more effectively than KA itself (Zhou *et al.*, 1997). 4-MG may also be employed as a GluR6 receptor antagonist as it rapidly inhibits inward KA-activated currents by desensitizing the receptor (Jones *et al.*, 1997). While ligand binding studies demonstrated that LY339434 is 1000-fold more selective for GluR5 than GluR6 (Smail *et al.*, 1998), functional studies have demonstrated activity at the AMPA and NMDA receptors (Moldrich *et al.*, 2000b; Small *et al.*, 1998). IW is some 4,000-fold more selective for GluR5 over GluR4, and > 400,000-fold more selective for GluR5 over GluR6 in binding studies employing homomeric receptor assemblies (Jane *et al.*, 1997). Studies conducted in primary cultures of cortical neurones demonstrated that these novel compounds exhibited complex excitotoxic profiles, involving actions not only at KA receptors, but also at AMPA and NMDA receptors (Moldrich *et al.*, 2000a; Moldrich *et al.*, 1999; Small *et al.*, 1998). IW is some 4,000-fold more selective for GluR5 over GluR4, and > 400,000-fold more selective for GluR5 over GluR6 in binding studies employing homomeric receptor assemblies (Jane *et al.*, 1997).

The current study examines these putative compounds (Figure 3.1) in primary cultures of CGCs. CGCs are a particularly useful model system in many respects, as they are homogenous cell preparation containing > 95% neurones when maintained under serum-free conditions (Cheung *et al.*, 1998a) and at 8 days *in vitro* possess no functional AMPA receptors (Hack *et al.*, 1995; Chapter Two). In addition, the current model system is particularly sensitive to KA receptor-mediated toxicity (Cheung *et al.*, 1998a; Chapter Two). This study demonstrates for the first time the excitotoxic profiles of these novel compounds at native KA receptors in an AMPA receptor-free environment using primary cultures of murine CGCs.

**LY339434**

IW

4-MG
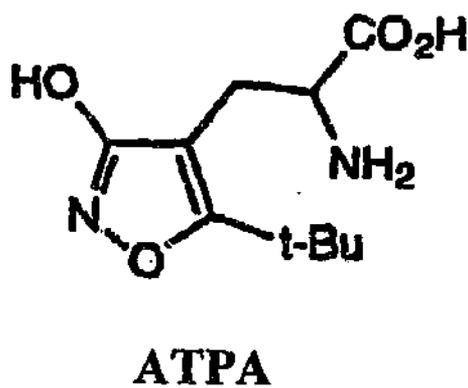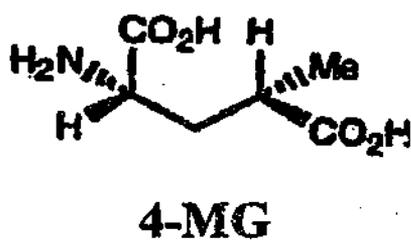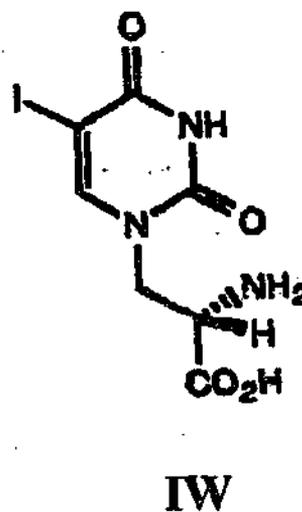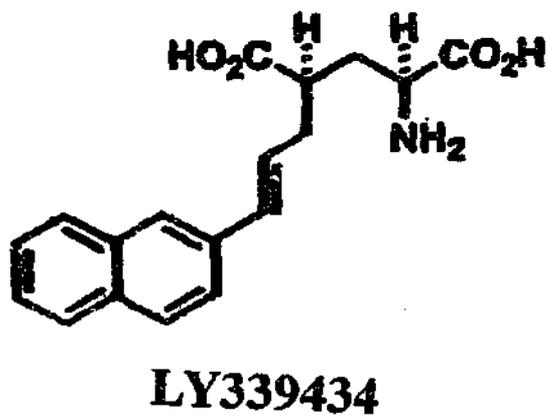
ATPA

Figure 3.1 Chemical structures of novel, putative low-affinity kainate receptor agonists used in the current study.

3.2. METHODS

3.2.1 Reagents

KA, 4-MG, ATPA, AMPA, NMDA and CNQX were purchased from Tocris Cookson (Bristol, UK). NBM, B27 nutrients, N2 supplements and Ca^{2+}-free HBSS were purchased from GibcoBRL Life Technologies (Melbourne, Australia). GYKI 52466 and MK-801 were purchased from Sigma RBI (Sydney, Australia). LY339434 was a kind gift from Dr. R. Baker (Eli Lilly; Windlesham, U.K.). KA2 and GluR6/7 antibodies were purchased from Upstate Biotechnology (Lake Placid, USA). All other reagents were purchased from Sigma or Boehringer Mannheim (Sydney, Australia) and were of cell culture or molecular biology grade.

Experiments were performed in accordance with the ethical code of the National Health and Medical Research Council (Australia) with permission from the standing Committee for Ethics in Animal Experimentation (Monash University).

3.2.2 Cell Culture

CGCs were prepared from 7d old Swiss White mice and cultured as previously described in Chapter Two. CGCs were grown in NBM containing 3% B27 components (Brewer *et al.*, 1993), 25.4mM K^+, 500µM Gln and 100U/ml penicillin-streptomycin and exposed to 10% dialysed fetal calf serum for the first 24h and left in serum-free conditions from *div* 1. Cells were seeded at a cell density of 0.3 x 10^6 cells/cm^2 in 24 well NUNC™ plates (Denmark) precoated with poly-D-lysine (50µg/ml). Aphidicolin (1µg/ml) was added to the medium 18-24h after plating to inhibit non-neuronal cell proliferation (Miller

& Johnson, 1996). Immunocytochemistry previously established that > 95% of the cells were neurones (Cheung *et al.*, 1998a; Chapter Two).

3.2.3 *Immunocytochemical Identification of KA Receptor Subunits*

CGCs were fixed in 4% paraformaldehyde in 0.1M phosphate buffered saline (pH 7.4) at room temperature for 15min. Cultures were subsequently washed in Tris buffered saline (TBS; 50mM Tris, 0.9% NaCl; pH 7.6), permeabilized in 2% Triton X-100 (TX-100) for 5min, and blocked in 10% normal goat serum (NGS), 0.1% TX-100 in TBS for 1h at 4°C. Cells were then incubated with the primary antibodies, rat polyclonal anti-KA2 (1:1000 dilution) or rat polyclonal anti-GluR6/7 (1:1000) in solution with 2% NGS and 0.1%TX-100 in TBS, overnight at 4°C. CGCs were subsequently washed in TBS and incubated with anti-rabbit-IgG horseradish-peroxidase conjugated secondary antibody (1:1000) for 3h at room temperature. After further washing, cultures were incubated with 3',3'-diaminobenzidine in stable peroxidase buffer and colour was allowed to develop for 1h at room temperature. Immunopositive labeling was visualized by bright field microscopy with an Olympus inverted microscope (Olympus, IMT-2) and photographed using Kodak Gold 100 ISO film.

3.2.4 *Agonist Exposure and Cell Viability Assays*

Cultures were exposed to the low-affinity KA agonists (10-1000µM) alone or in the presence of the non-NMDA receptor antagonists CNQX (50µM) and GYKI 52466 (20µM), or the NMDA receptor antagonist MK-801 (10µM), for 24h at 8 *div* in N2 supplemented NBM containing 100U/ml penicillin-streptomycin, 0.25% BSA, 83µM D(+) galactose, 16µM ethanolamine, 6µM L-carnitine, 0.4µM biotin and 25.4mM K^+

(Bottenstein & Sato, 1979). Conditions used for toxicity studies mediated by NMDA and AMPA (see Chapter Two) were used for the current studies, which mitigated against AMPA receptor-mediated neurotoxicity. Cellular viability was determined at 24h by the reduction of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cheung *et al.*, 1998a; Chapter Two). MTT was incubated with the cells for 30min at 37°C and the reduced formazan product was lysed from the cells in a solution containing 20% sodium dodecyl sulphate and 40% dimethylformamide, and absorbance was subsequently measured at 590nm (Ceres UV900c microplate reader; Biotek Instruments, USA). Cultures treated with 500μM Glu for 24h were taken as 100% cell death and the results were expressed as percentage of control (untreated).

After drug exposure (18-24h) cultures were examined by phase contrast microscopy for morphological changes consistent with apoptosis (cellular shrinkage, neurite blebbing), necrosis (loss of cellular density and the presence of cellular debris) and neuroprotection in relation to antagonist co-exposure (relative to control cultures, with the preservation of neurites and cellular shape) using an Olympus inverted microscope (Olympus, IMT-2).

3.2.5 Propidium Iodide Labeling of Necrotic Cells

Necrotic cell death was analysed by propidium iodide (PI) labeling of cells with damaged cell membranes (Moldrich *et al.*, 2000a). PI (1μg/ml) was incubated at *div* 8 1-2h after agonist exposure for 30min at 37°C as previously described (Moldrich *et al.*, 2000a; Chapter Two). Cultures were subsequently viewed using fluorescent microscopy (excitation: 535nm, emission: 617nm) and random yet representative fields photographed with Kodak Ektachrome P1600 film.

3.2.6 In situ Labeling of Nuclear DNA Fragments

Apoptosis was analysed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick end labeling (TUNEL) as previously described (Cheung *et al.*, 1998a; Chapter 2). CGCs were fixed overnight in 4% paraformaldehyde, permeabilized with 2% TX-100 in TBS. Cultures were subsequently washed in TBS and blocked for 1h at room temperature in solution with 10% NGS and 0.1% TX-100 in TBS and incubated with TdT reaction mixture as previously described (Cheung *et al.*, 1998a) for 3h at 37°C. DIG labeled dUTP was detected by anti-DIG alkaline phosphatase (AP; 1:1000 dilution) in solution with 2% NGS and 0.1% TX-100 and TBS. TUNEL-positive cells were detected using AP substrate solution (170mM 5-bromo-4-chloro-3-indolyl-phosphate, 60mM nitroblue tetrazolium chloride, 100mM Tris-HCl, 0.5mM $MgCl_2$; pH 9). Control cultures included the above treatment with the omission of TDT. Cells were visualized under bright field microscopy and random and representative fields photographed on Kodak Gold 100 ISO film.

3.2.7 Data Analyses

Data are given as mean ± S.E.M. from at least quadruplicate experiments across 4-6 independent cultures and concentration-response curves were generated by non-linear regression using computer-assisted curve fitting (GraphPad Prism™). Statistical significance ($p < 0.05$) of data was examined by two way ANOVA. Characteristics of cell death, determined by PI incorporation and TUNEL labeling, were conducted over 2-3 independent experiments and representative photomicrographs are presented here.

3.3 RESULTS

3.3.1 Characterization of Receptor Expression

KA2 expression levels were much less abundant than that for GluR6/7, with few immunopositive cells present at *div* 1 (Figure 3.2A) and at *div* 12 (Figure 3.2C). Immunocytochemical staining of the cultures revealed extensive expression of the GluR6/7 receptors from *div* 1 in CGCs (Figure 3.2D). Expression of GluR6/7 remained high throughout the period examined up to *div* 12, (Figure 3.2F). Immunopositive cells for KA2 and GluR6/7 were present at day 8 *in vitro*, the day the cultures were exposed to the agonists (Figure 3.2B and 3.2E respectively).

3.3.2 Low-affinity KA Receptor-mediated Toxicity

The present study found widespread, concentration-dependent cell death mediated by the putative low-affinity KA receptor agonists (Figure 3.3). Rank order potency and EC_{50} values (μM): IW (0.2 ± 0.5) > 4-MG (36 ± 0.2) > LY339434 (46 ± 0.2) > KA (74 ± 0.3) > ATPA (88 ± 0.7) [$F(4,205) = 32.24$, $p < 0.0001$]. These data coincide with the rank order for excitotoxicity found in primary cultures of cortical neurones (Moldrich *et al.*, 2000a).

ATPA-mediated toxicity was somewhat less potent than that induced by the other compounds, with a maximal cell death of 25% (1000μM) compared to that of 100% cell death induced by 4-MG (1000μM), 50% by IW (1000μM) and 90% by LY339434 (1000μM; Figure 3.3). Like KA (Chapter Two), the neurotoxicity produced by ATPA was completely attenuated by the non-NMDA receptor antagonists GYKI 52466 (20μM) and
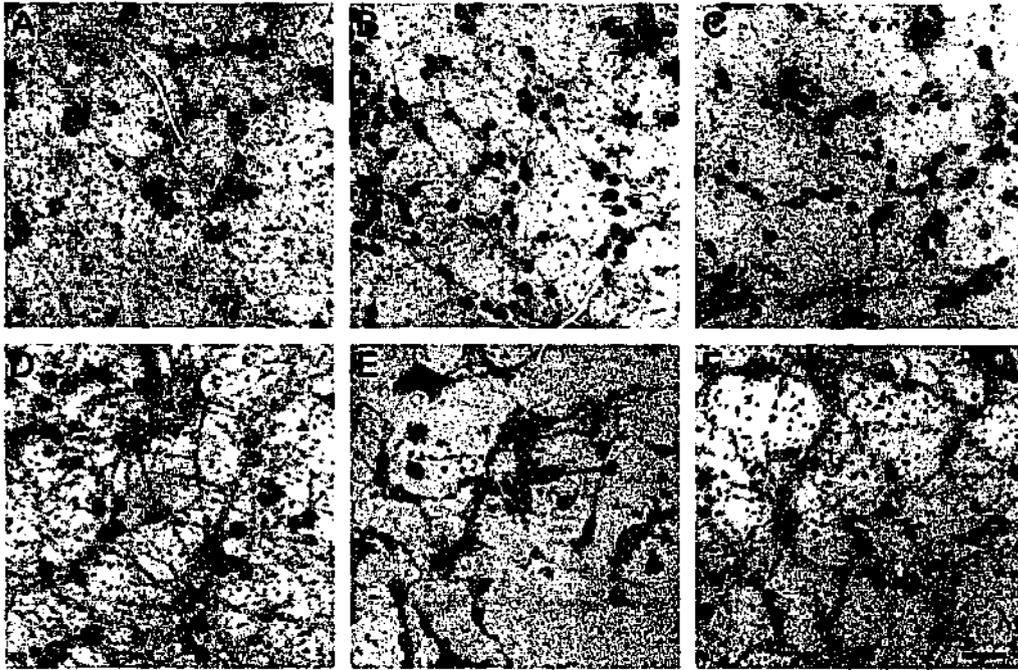
Figure 3.2 Immunostaining for KA receptor subunits

Immunopostive labeling of KA2 receptors was less extensive than that of GluR6/7 over the examined time period, with few immunopositive labeling on day 1 *in vitro* (A), with increasing levels on day 8 *in vitro* (B) and levels remained constant until day 12 *in vitro* (C). Immunocytochemical staining of cultures at various days *in vitro* demonstrated extensive expression of GluR6/7 receptor subtypes from day 1 *in vitro* (D), extending to day 8 *in vitro* (E), and remaining high at day 12 *in vitro* (F). Scale bar represents 10μm.
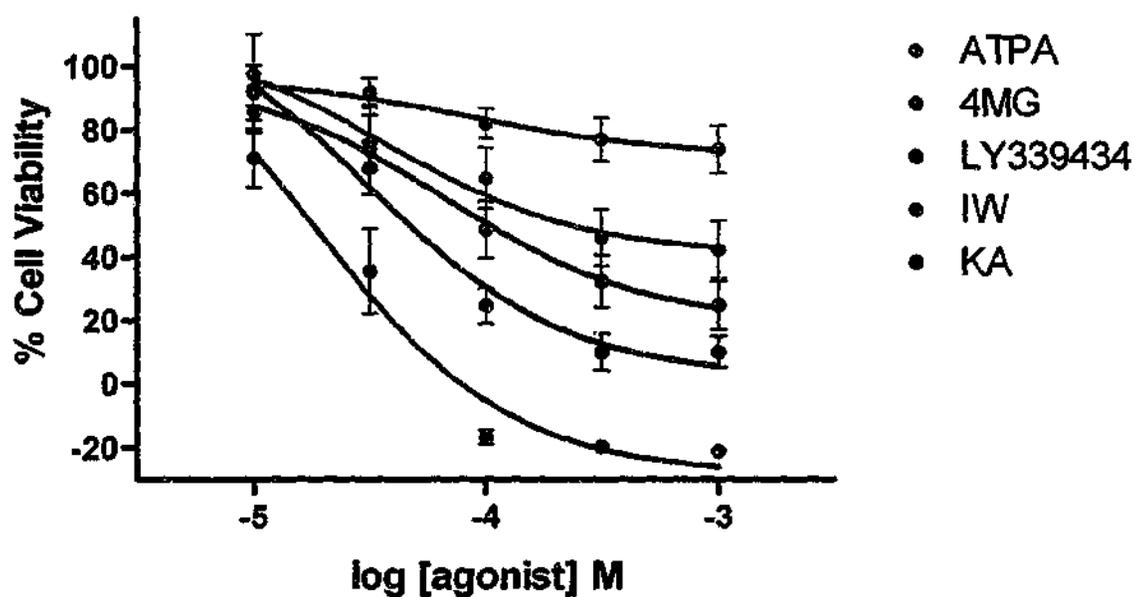
Figure 3.3 Concentration-response curves of the low-affinity KA receptor agonists

All agonists produced a concentration-dependent cell death $[F_{(5,225)} = 30.57, p < 0.0001]$, except ATPA, with rank order potencies and EC_{50} values (μM): IW (0.2) > 4-MG (36) > LY339434 (46) > KA (74) > ATPA (88).

CNQX (50μM), but not by MK-801 (10μM) (Figure 3.4). The receptors involved in mediating IW toxicity were of the non-NMDA subtype, where toxicity was attenuated completely by CNQX (50μM) and partially by GYKI 52466 (20μM) (Figure 3.5). In contrast to previous findings (Moldrich *et al.*, 2000a), MK-801 (10μM) did not attenuate IW-mediated toxicity $[F(1,70) = 0.02, p = 0.88]$, implying that IW acts through the non-NMDA receptors in the current model system. Neurotoxicity mediated by 4-MG was not attenuated by MK-801 (10μM), GYKI 52466 (20μM), or CNQX (50μM), to any great extent, implicating a non Glu receptor-mediated mechanisms as the route by which the majority of toxicity occurs (Figure 3.6). However, injury induced by lower concentrations of 4-MG (< 30μM) was significantly attenuated by MK801 $[F(1,70) = 5.01, p < 0.0001]$, CNQX $[F(1,70) = 1.34, p < 0.05]$, but not 20μM GYKI 52466 $[F(1,70) = 0.45, p = 0.77]$. Combinations of the iGluR antagonists were employed to overcome the ineffectiveness of the antagonists alone, and to evaluate whether 4-MG-induced neurotoxicity was mediated by both NMDA and KA receptors. While the concentration response curve to 4-MG is significantly shifted to the right in the combined presence of CNQX, MK-801, and GYKI 52466 $[F(1,70) = 9.77, p < 0.0001]$, attenuation of neurotoxicity was greatest at lower concentrations of 4-MG (< 100μM) and is only partial at higher concentrations. Neurotoxicity mediated by LY339434 was completely attenuated by MK801 (10μM; $[F(1,80) = 46.10, p < 0.0001]$), and while the concentration-response curve was slightly shifted to the right by CNQX (50μM) (Figure 3.7), this effect was not statistically significant (p > 0.05).
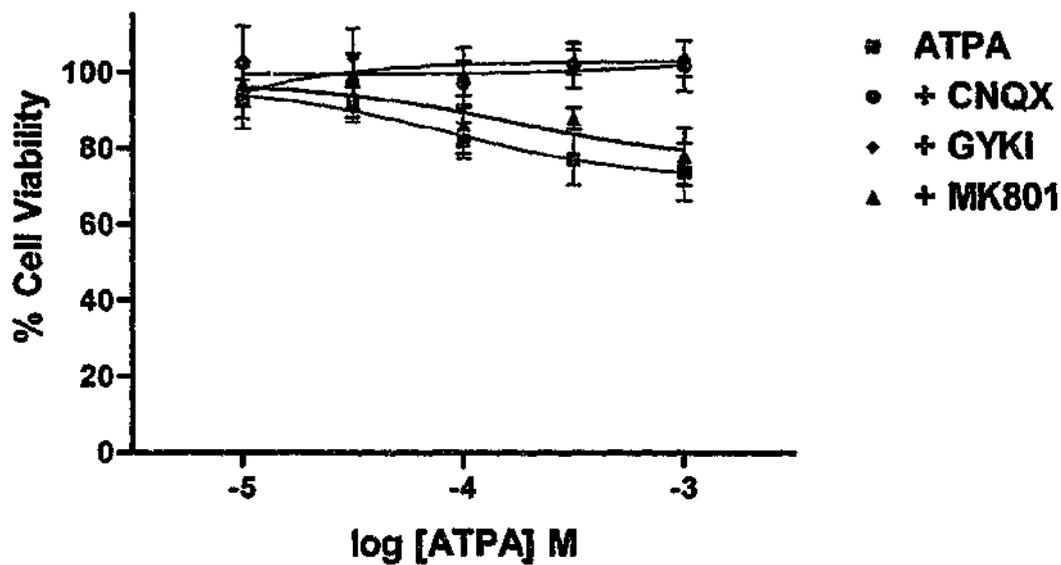
Figure 3.4 Neurotoxicity mediated by ATPA

Neurotoxicity mediated by ATPA was not concentration-dependent [$F_{(4,115)}$ = 2.29, p = 0.47], however some reduction in cellular viability was evident. Neurotoxicity was attenuated by the non-NMDA receptor antagonists CNQX ($50\mu M$) and GYKI 52466 ($20\mu M$) [$F_{(2,95)}$ = 18.17, $p < 0.001$], but not by the NMDA receptor antagonist MK-801 ($10\mu M$) [$F_{(1,65)}$ = 1.77, p = 0.24].
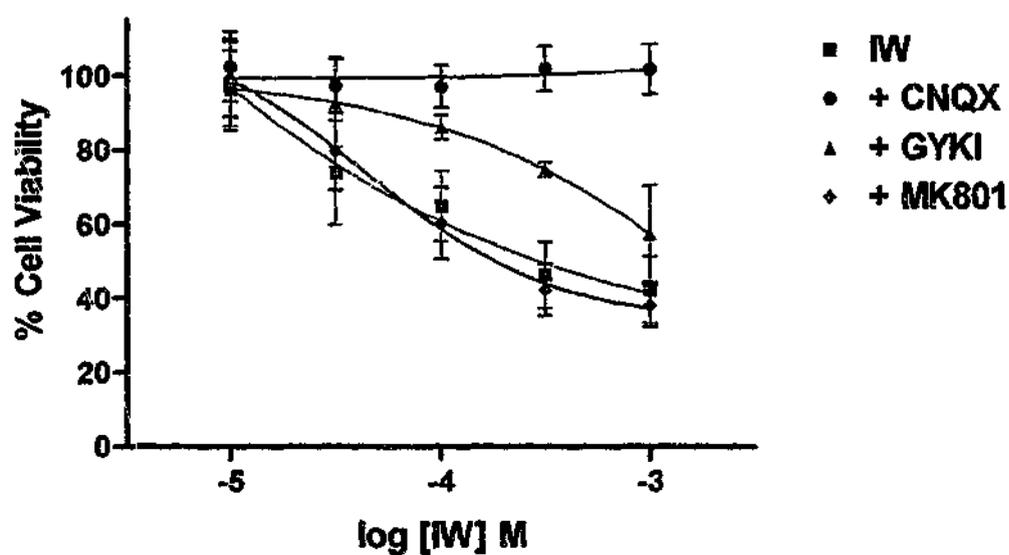
Figure 3.5 Neurotoxicity mediated by IW

Neurotoxicity mediated by IW was concentration-dependent [$F_{(4,115)}$ = 15.29, $p < 0.0001$], and was significantly attenuated by the non-NMDA receptor antagonists CNQX (50μM) and GYKI 52466 (20μM) [$F_{(2,90)}$ = 21.22, $p < 0.0001$], but not MK-801 (10μM) [$F_{(1,70)}$ = 0.02, $p = 0.875$].
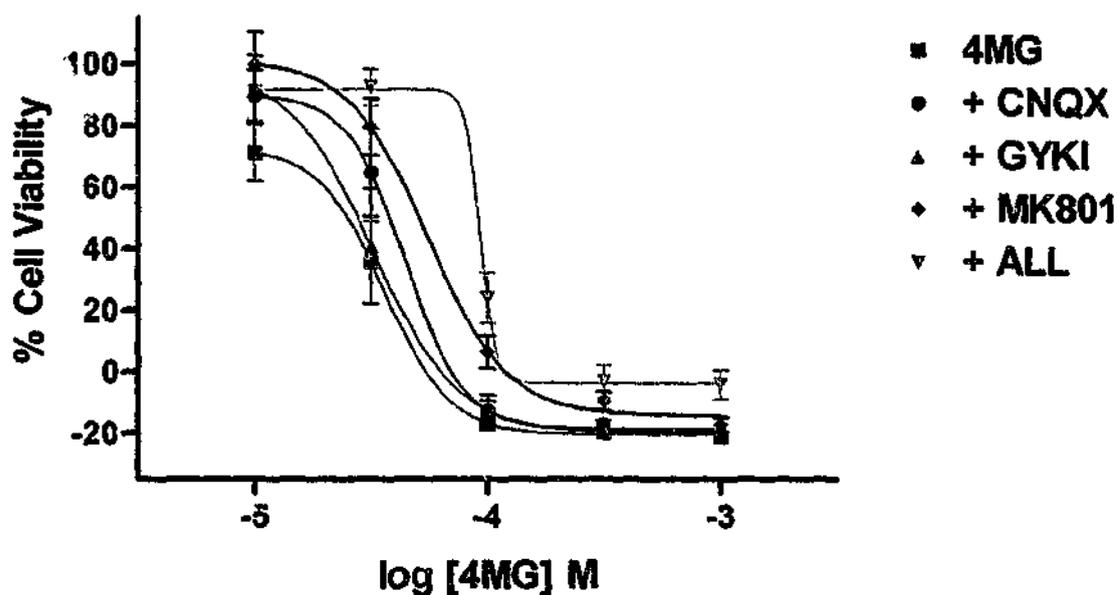
Figure 3.6 Neurotoxicity mediated by 4-MG

Neurotoxicity mediated by 4-MG was concentration-dependent $[F_{(4,145)} = 76.28, p < 0.0001]$ and was not attenuated by the non-NMDA receptor antagonists CNQX (50μM) and GYKI52466 (20μM) $[F_{(2,95)} = 0.98, p = 0.1014]$, but slightly by the NMDA receptor antagonist MK-801 (10μM) $[F_{(1,70)} = 5.01, p < 0.0001]$. Toxicity was not completely attenuated by any of the antagonists, even a combination of all three (referred to as "+ all" in the legend) $[F_{(1,70)} = 9.77, p < 0.0001]$.
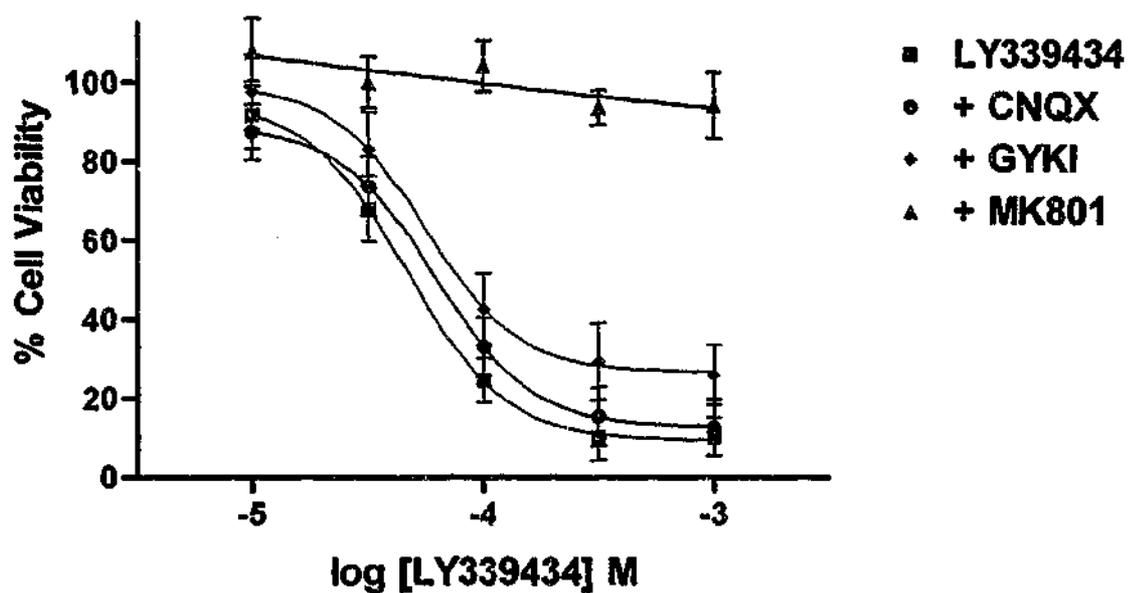
**Figure 3.7 Neurotoxicity mediated by LY339434**

Neurotoxicity mediated by LY339434 was concentration-dependent [$F_{(4,145)}$ = 33.89, $p <$ 0.0001], and was significantly attenuated by the NMDA receptor antagonist MK-801 (10μM) [$F_{(2,90)}$ = 21.22, $p <$ 0.0001], and to a lesser extent GYKI 52466 (20μM) [$F_{(1,75)}$ = 3.54, $p <$ 0.05], but not CNQX (50μM) [$F_{(1,80)}$ = 0.22, $p <$ 0.4339].

3.3.3 *Morphology of Neurotoxicity Induced by Putative Low-affinity KA Receptor Agonists*

Morphological examination of the cultures by light microscopy was undertaken to correlate cellular and pathological changes with the MTT injury data. The putative low-affinity KA receptor agonists produced concentration-dependent neuronal damage, demonstrating morphological changes consistent with apoptosis and necrosis, dependent upon the intensity of the insult. Vehicle controls demonstrated no morphological changes (see controls of Figures 3.8, 3.9, 3.10 and 3.11). Cellular swelling, indicative of necrosis, was evident in cultures 1-2h after exposure to 4-MG (10-1000µM), LY339434 (100-1000µM) and IW (30-1000µM). Cellular swelling was not evident in cells treated with lower concentrations of IW, LY339434, or any concentrations examined for ATPA (10-1000µM). After exposure (24h) of the cultures to lower concentrations of IW (10-30µM; Figure 3.8), LY339434 (10-100µM Figure 3.9), and all concentrations of ATPA (10-1000µM; Figure 3.10), cultures demonstrated morphological characteristics of apoptosis, consisting of shrunken cell bodies and neurite blebbing. IW-mediated toxicity at lower concentrations (30µM; Figure 3.8B) was completely attenuated by GYKI 52466 (Figure 3.8C), but not MK-801 (Figure 3.8D), consistent with KA receptor mediated toxicity. CGCs treated with high concentrations of LY339434, IW, and all concentrations of 4-MG examined produced high levels of debris and a diminished cell density 24h after stimulation, representative of necrosis. Treatment of CGCs with high concentrations of LY339434 (300µM; Figure 3.9B) resulted cellular swelling and some presence of shrunken cell bodies, indicative of both necrosis and apoptosis, that was completely attenuated in the presence of MK-801 (Figure 3.9C), but not by CNQX (Figure 3.9D), consistent with NMDA receptor-mediated toxicity.
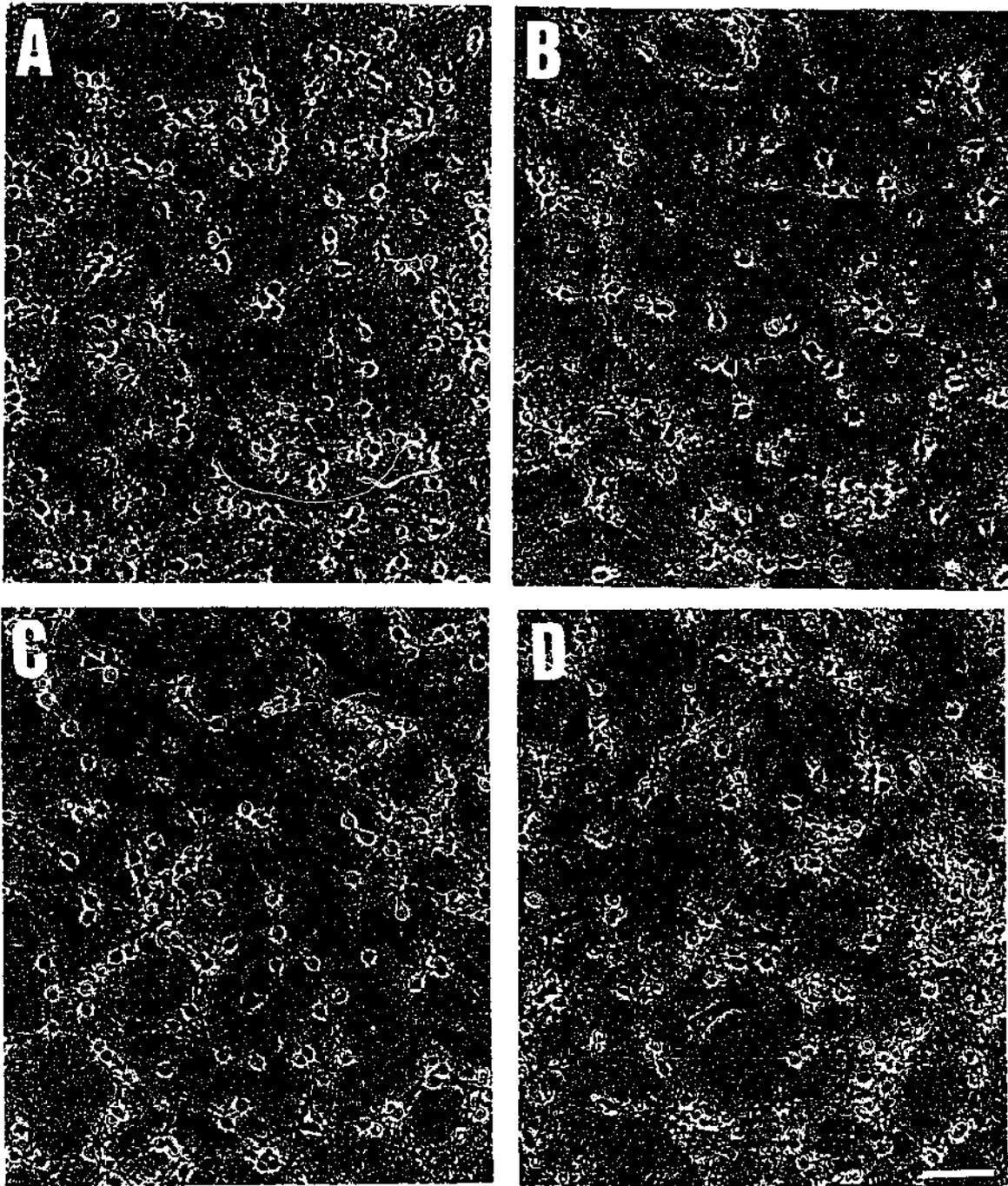
Figure 3.8 Morphological changes induced by IW

IW-mediated toxicity at lower concentrations (30µM; B) induced morphological changes consistent with apoptosis, with neurones demonstrating cellular shrinkage and neurite blebbing when compared to vehicle treated sister cultures (A). IW-mediated toxicity was completely attenuated by GYKI 52466 (20µM; C), but not MK-801 (10µM; D), consistent with KA receptor mediated toxicity. Scale bar represents 10µm.
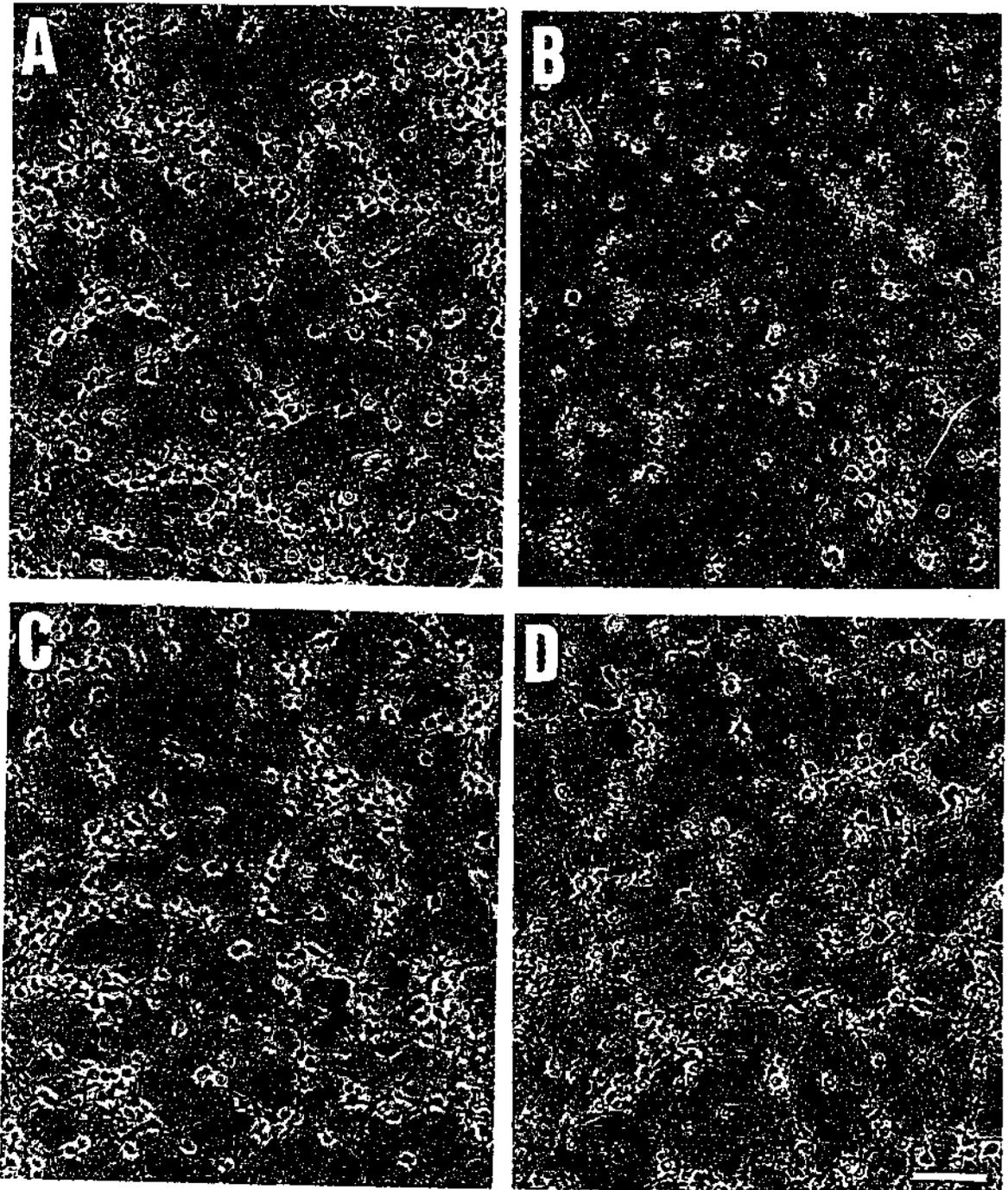
Figure 3.9 Morphological changes induced by LY339434

LY339434-mediated toxicity at higher concentrations (300µM; B) induced morphological changes consistent with necrosis, with neurones demonstrating cellular swelling and loss of cellular density, when compared to vehicle treated sister cultures (A), however some apoptotic cells are evident (arrow). LY339434-mediated toxicity was completely attenuated by MK-801 (10µM; C), but not CNQX (50µM; D), consistent with NMDA receptor mediated toxicity. Scale bar represents 10µm.

ATPA exposure resulted in the least neuronal damage out of all the agonists examined in the present study (Figure 3.10). Higher concentrations of ATPA (300µM; Figure 3.10B) resulted in some cellular shrinkage, consistent with apoptosis, but the majority of cells remained unchanged when compared to vehicle control (Figure 3.10A). Morphological changes as a result of ATPA exposure were attenuated by CNQX (50µM; Figure 3.10C), but not by MK-801 (10µM; Figure 3.10D), consistent with KA receptor-mediated neuronal injury. 4-MG-mediated neuronal injury was complex, with the majority of cells demonstrating morphological changes consistent with necrosis, i.e. cellular swelling and loss of cellular density (Figure 3.11B). 4-MG-induced toxicity was not attenuated by CNQX (50µM; Figure 3.11C), MK801 (10µM; Figure 3.11D), nor even a combination of CNQX, GYKI 52466 and MK-801 (data not shown) findings suggesting an alternative mechanism for the mediation of toxicity mechanism to iGluR overstimulation. These findings are summarized in Table 3.1.

3.3.4 Low-affinity KA Receptor Agonists: Mode of Cell Death

The DNA binding dye, PI, only penetrates cells with damaged cellular membranes and when used after short incubation times is a reliable index of rapid necrosis, especially when compared with findings from analyses using the TUNEL technique for apoptosis. The modes of cell death for the putative low-affinity KA agonists were somewhat different to that found for KA itself (see Chapter Two). Neurotoxicity mediated by ATPA was apoptotic rather than necrotic in nature as determined by the presence of TUNEL-positive cells (300µM; Figure 3.12D), compared to the vehicle control (Figure 3.12C), and with few PI-positive cells observed 1-2h after stimulation (Figure 3.12B). Consistent with morphological observations and MTT injury data, TUNEL labeling was attenuated by
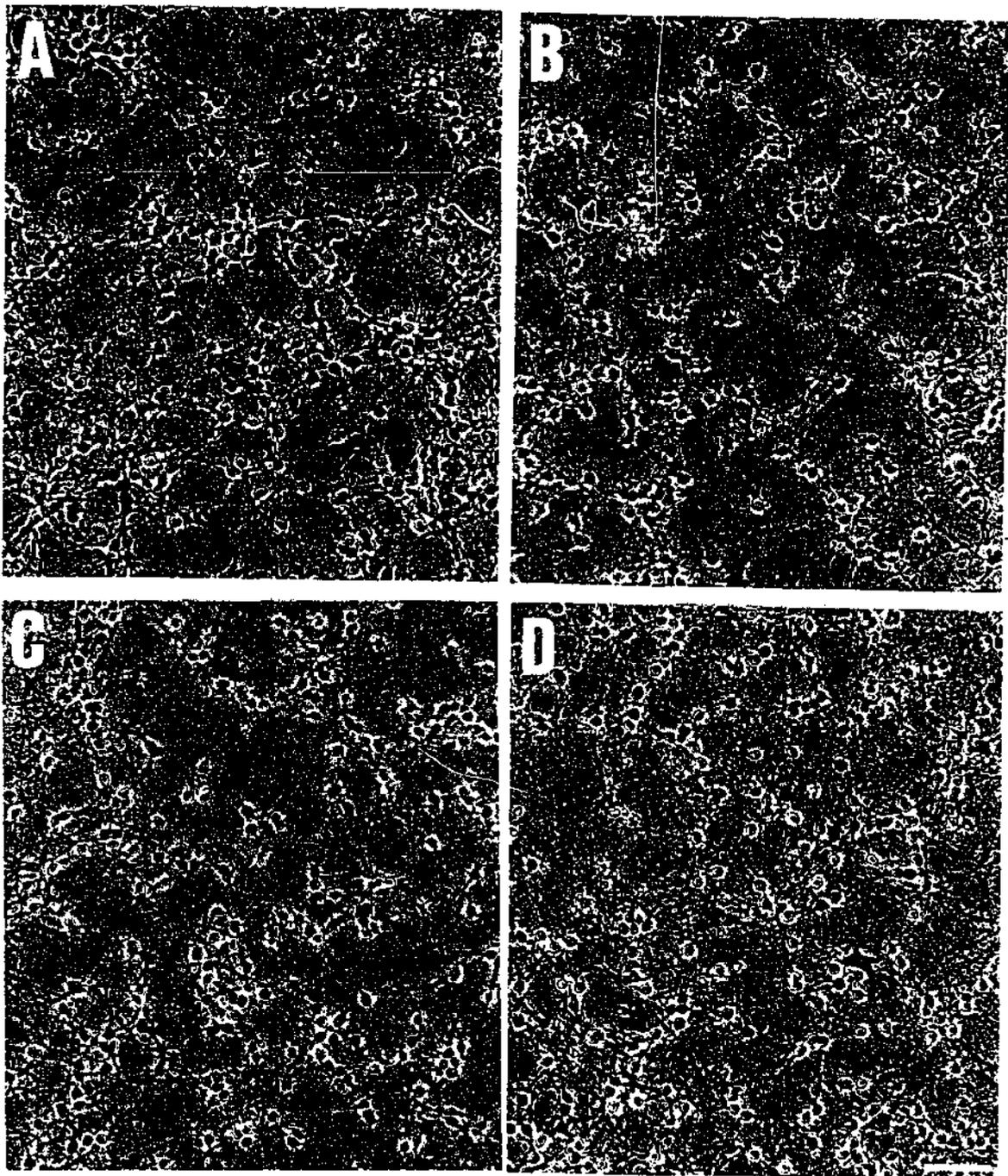
Figure 3.10 Morphological changes induced by ATPA

ATPA exposure resulted in the least neuronal damage when compared to the other putative low-affinity agonists. Higher concentrations of ATPA (300µM; B) resulted in some cellular shrinkage, consistent with apoptosis, but the majority of cells remained unchanged when compared to vehicle control (A). Morphological changes as a result of ATPA exposure were attenuated by CNQX (50µM; C), but not MK-801 (10µM; D), consistent with KA receptor-mediated neuronal injury. Scale bar represents 10µm.
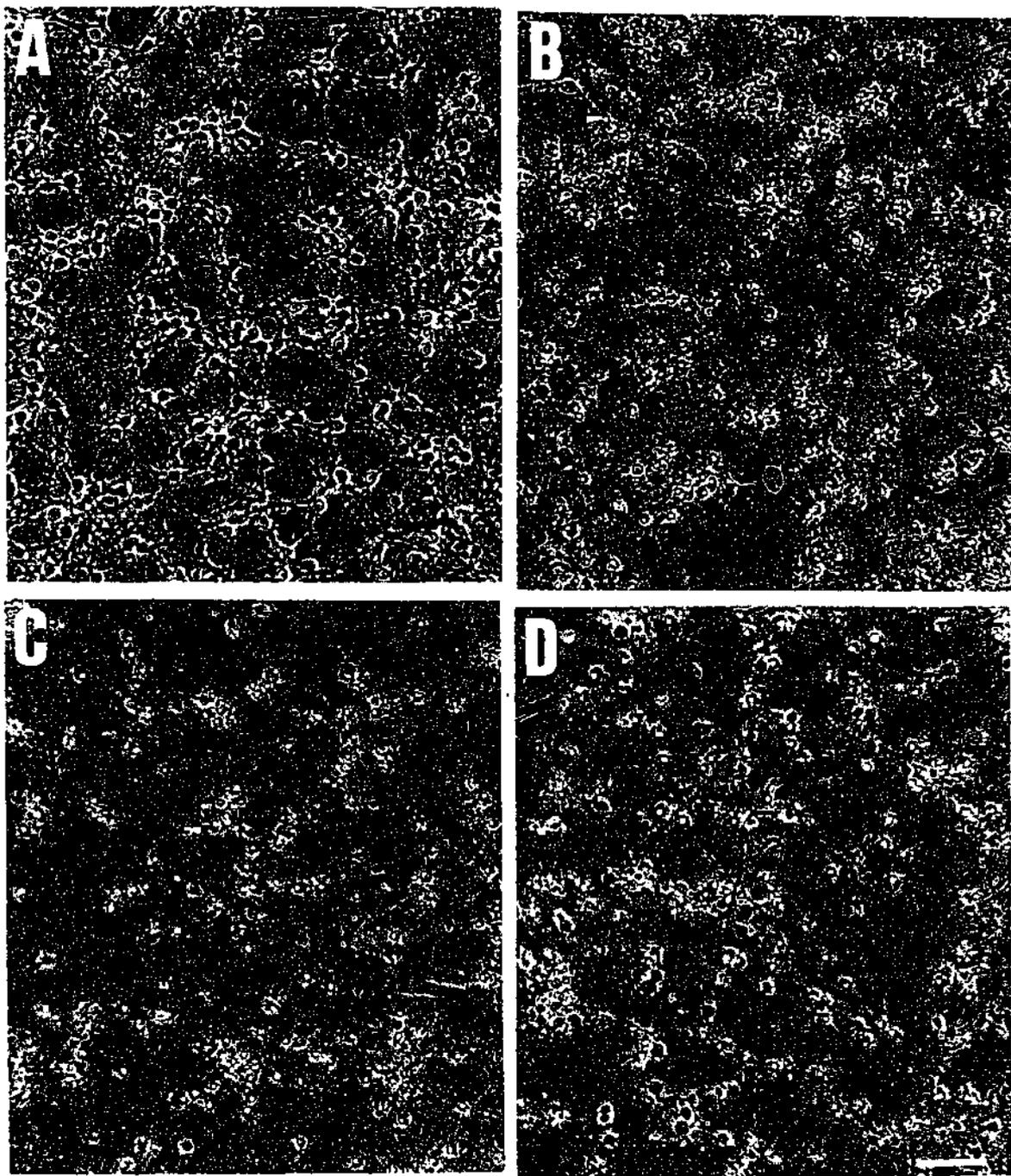
Figure 3.11 Morphological changes induced by 4-MG

4-MG-mediated neuronal injury was complex, with the majority of cells demonstrating morphological changes consistent with necrosis, i.e. cellular swelling and loss of cellular density (100μM; B) when compared to the vehicle control (A). 4-MG-induced toxicity was not attenuated by CNQX (50μM; C), or MK801 (10μM; D). Scale bar represents 10μm.

Agonist concentration (μM)	Morphological characteristics	TUNEL labeling	Propidium iodide labeling
KA(10-300μM)	Shrinkage, neurite blebbing	+++	-
KA (1000μM)	Shrinkage, neurite blebbing, some swelling and reduction in density	+++	+
ATPA (10-1000μM)	Shrinkage, neurite blebbing	++	-
IW (10-30μM)	Shrinkage, neurite blebbing	+++	-
IW (30-1000μM)	Swelling, reduction in density	+	++
4-MG (10-30μM)	Shrinkage, neurite blebbing	++	+
4-MG (30-1000μM)	Swelling, reduction in density	+	+++
LY339434 (10-100μM)	Shrinkage, neurite blebbing	+++	+
LY339434 (100-1000μM)	Swelling, reduction in density	+	++

Table 3.1 Morphological characteristic of putative low-affinity KA receptor agonists

Neuronal injury patterns induced by the putative low-affinity kainate agonists are as indicated. Characteristics of apoptosis (neurite blebbing and cellular shrinkage) and necrosis (cellular swelling and loss of cellular density) were analysed by phase contrast microscopy, TUNEL (apoptosis) and propidium iodide incorporation (necrosis). The intensity of staining was graded as follows: +++ many of the cells are staining positive; ++ significant portion of cells are staining positive; + staining slightly more intense than that of the vehicle control; - represents no significant change when compared to vehicle control.
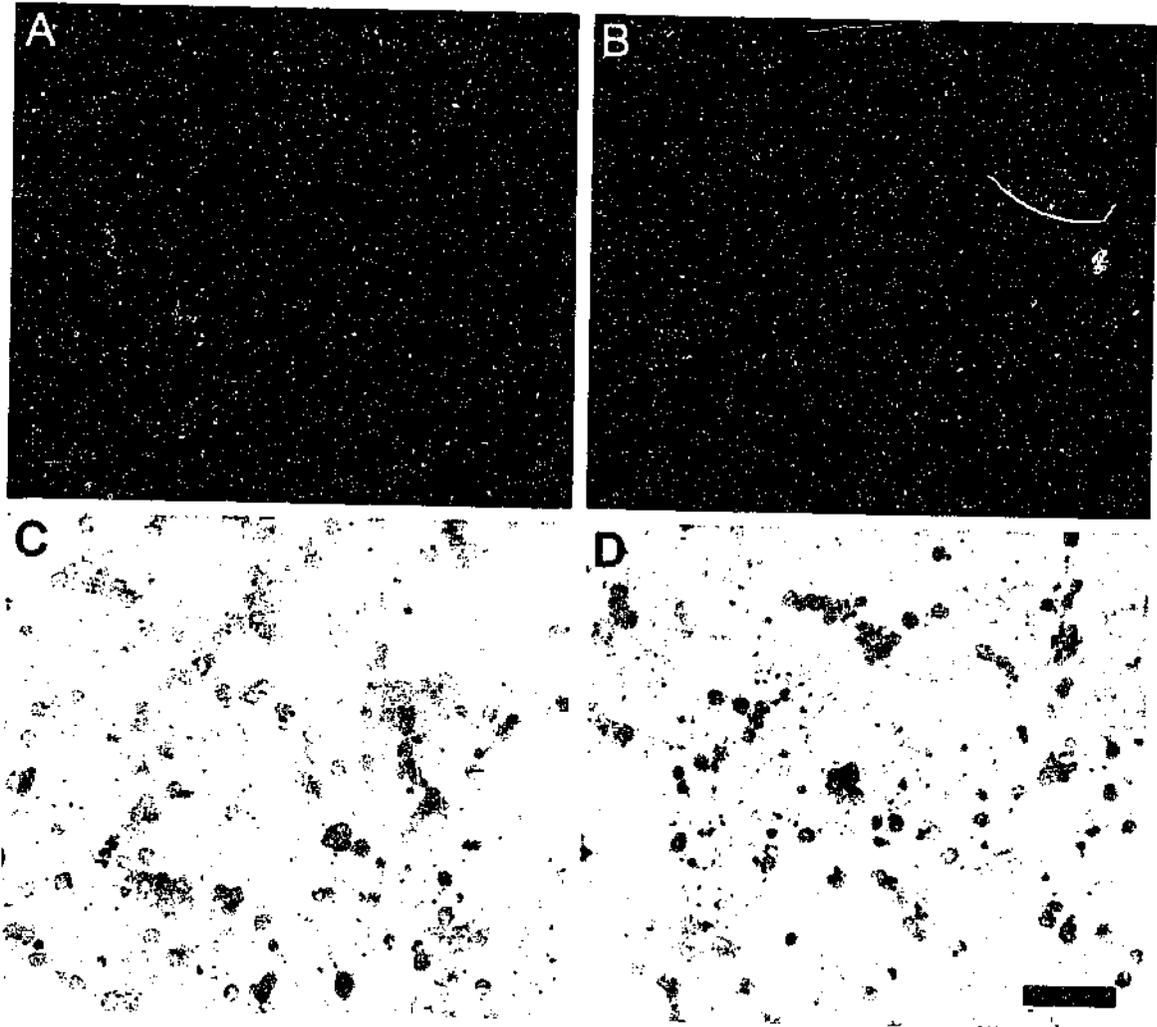
Figure 3.12 Determination of the mode of cell death induced by ATPA using PI and TUNEL labeling

The extent of necrotic damage induced by ATPA was determined by PI incorporation 1-2h after exposure. TUNEL labeling was employed 24h after insult to determine apoptotic cell bodies. ATPA exposure did not increase PI incorporation (300μM; B), compared to the vehicle control (A), however an increase in TUNEL labeling is evident (300μM; C) compared to control (D). Scale bar represents 10μm.

CNQX (50μM; data not shown) but not MK-801 (10μM; data not shown). Neuronal injury patterns found with LY339434 and IW were more complex, demonstrating apoptotic (TUNEL-positive) nuclei in cells treated with lower concentrations (Figure 3.14B and Figure 3.16B, respectively), and labeling with PI indicative of necrosis at higher concentrations (Figure 3.13B and Figure 3.15B, respectively). IW-mediated TUNEL labeling was completely attenuated by GYKI 52466 (Figure 3.16C), while the presence of MK-801 did not alter the labeling pattern (Figure 3.16D). IW exposure, at high concentrations (< 100μM), led to necrosis with the majority of cells incorporating PI (300μM; Figure 3.15B) 1-2h after exposure. This pattern of labeling was attenuated by the presence of CNQX (Figure 3.15C), but not MK-801 (Figure 3.15D), suggesting a KA-receptor mediated mechanism. Toxicity induced by 4-MG was largely necrotic in nature as shown by wide-spread labeling of cells with PI (30μM; Figure 3.17B) and few cells staining TUNEL-positive for apoptosis 24h after stimulation (100μM; Figure 3.18B). The observations that 4-MG-induced PI and TUNEL labeling were not completely attenuated by any of the iGluR antagonists (Figures 3.17 and 3.18), and were consistent with findings from the MTT data and morphological examination. These findings are summarized in Table 3.1.

3.4 DISCUSSION

The current study is the first to analyse the excitotoxic profile of the putative low-affinity KA receptor agonists ATPA, IW, 4-MG and LY339434 in an essentially AMPA-receptor free, physiologically relevant milieu (see Chapter Two). CGCs are a particularly useful model system for pharmacological evaluation of compounds as they are an essentially homogenous cell preparation under serum-free conditions, with a negligible
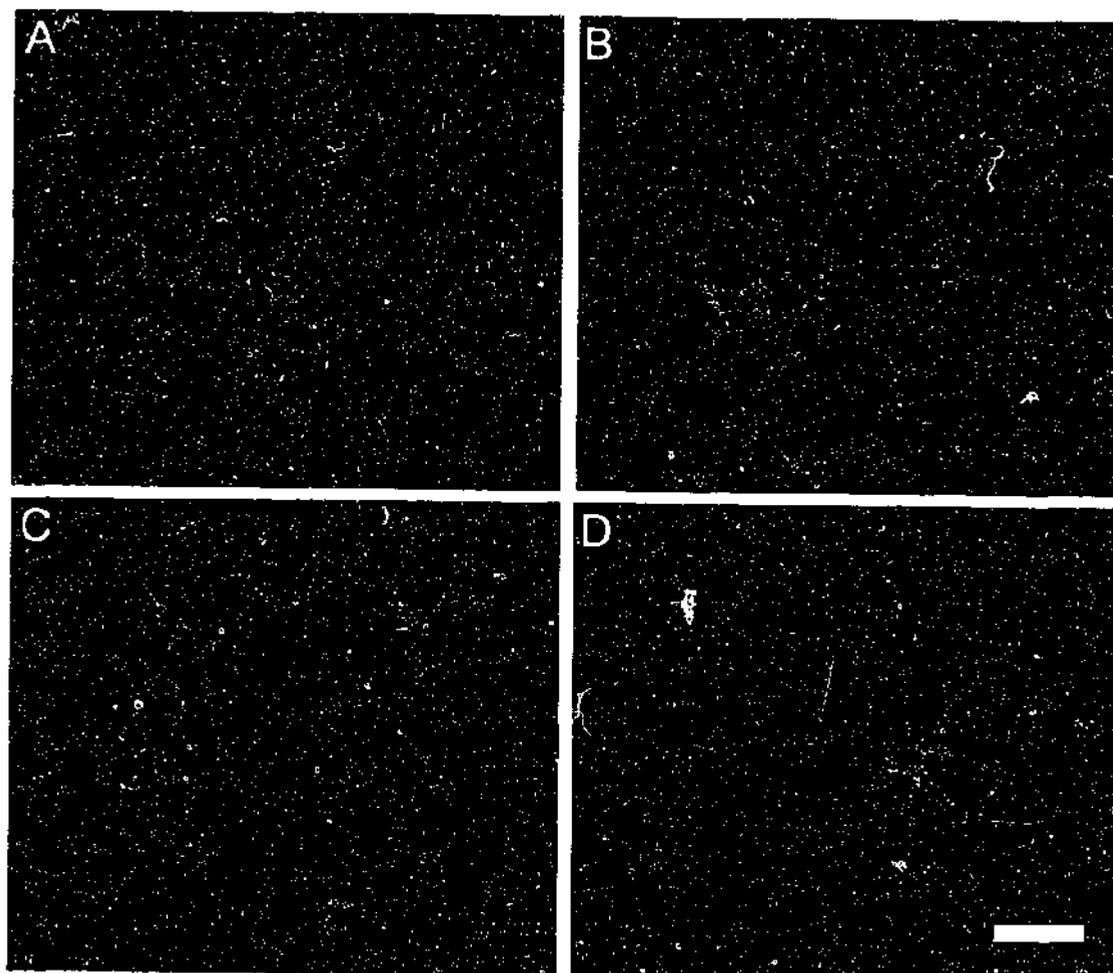
Figure 3.13 Determination of necrosis induced by LY339434 using PI labeling

The extent of necrotic damage induced by LY339434 was determined by PI incorporation 1-2h after exposure. LY339434 exposure increased PI incorporation (300µM; B) compared to the vehicle control (A). PI incorporation induced by LY339434 was completely attenuated by MK-801 (10µM; C) but not CNQX (50µM; D). Scale bar represents 10µm.
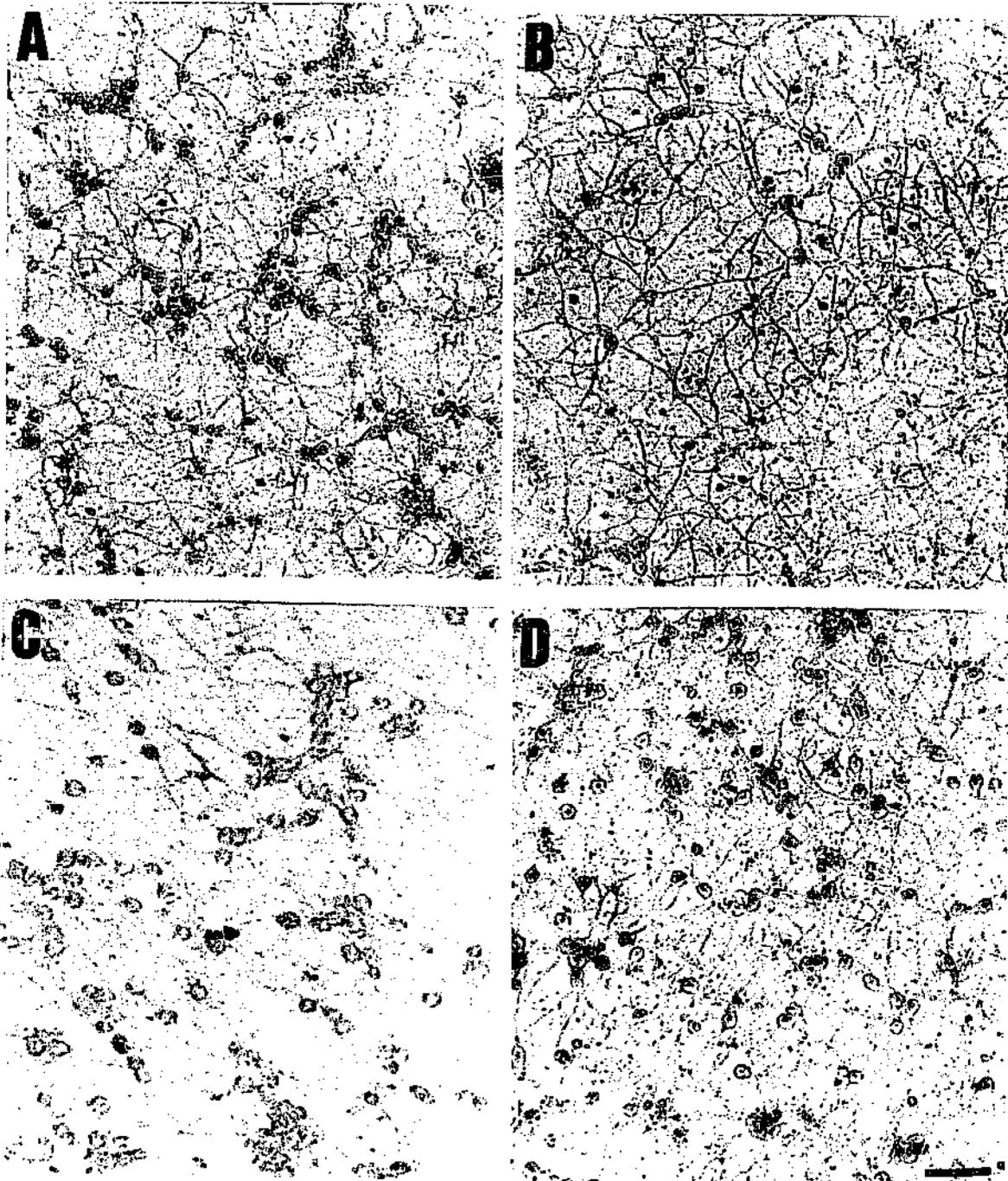
Figure 3.14 Determination of apoptosis induced by LY339434 using TUNEL labeling
The extent of apoptotic damage induced by LY339434 was determined by TUNEL labeling 18-24h after exposure. LY339434 exposure increased TUNEL labeling (100µM; B) compared to the vehicle control (A). Apoptotic (TUNEL) labeling induced by LY339434 was completely attenuated by MK-801 (10µM; C) but not CNQX (50µM; D). Scale bar represents 10µm.
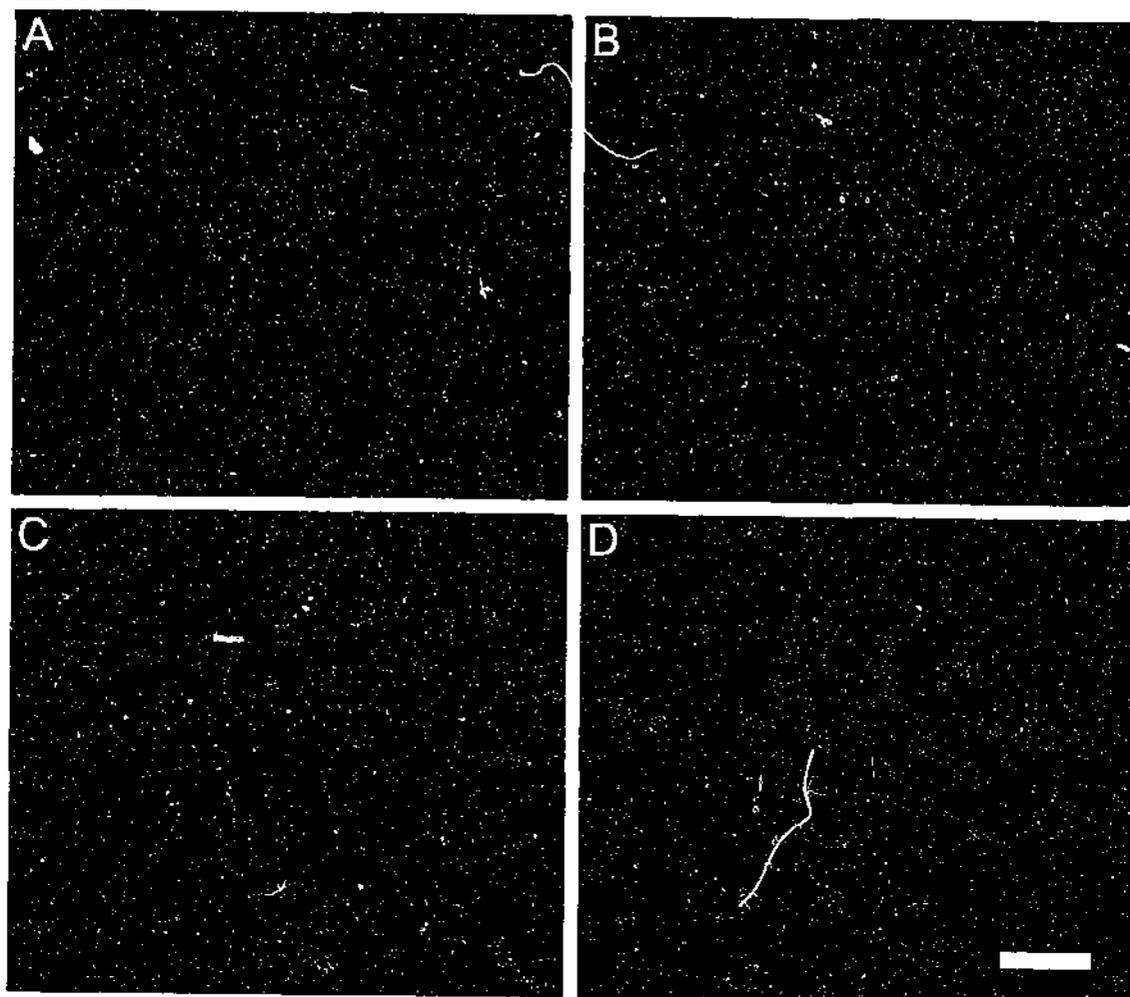
Figure 3.15 Determination of necrosis induced by IW using PI incorporation

The extent of necrotic damage induced by IW was determined by the incorporation of the DNA binding dye PI 1-2h after exposure to IW. IW exposure increased PI incorporation (300µM; B) compared to the vehicle control (A). Necrotic (PI) labeling induced by IW was attenuated by CNQX (50µM; C) but not MK-801 (10µM; D). Scale bar represents 10µm.
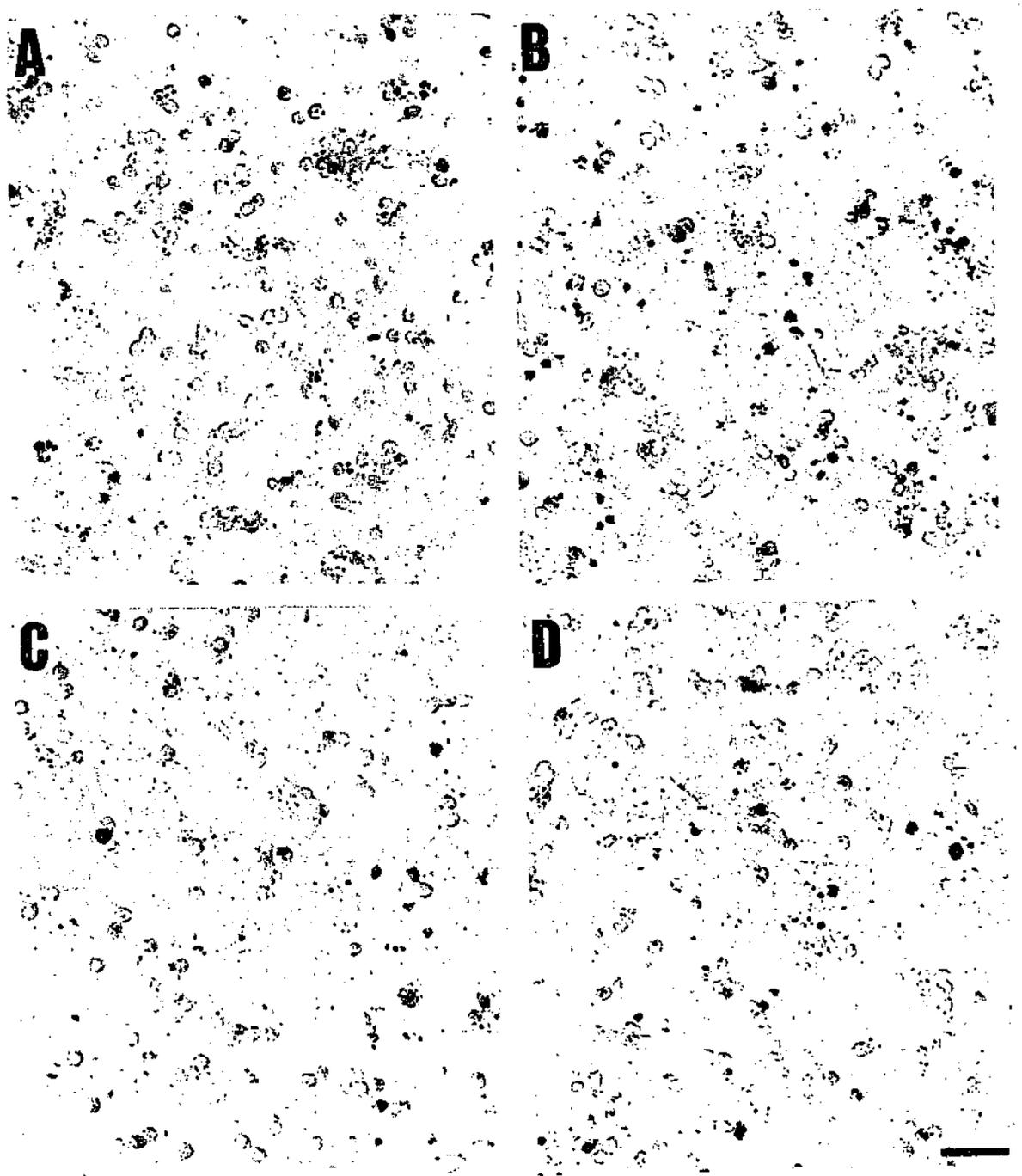
Figure 3.16 Determination of apoptosis induced by IW using TUNEL labeling

The extent of apoptotic damage induced by IW was determined by TUNEL labeling 18-24h after exposure. IW exposure increased apoptotic (TUNEL) labeling (100µM; B) compared to the vehicle control (A). TUNEL labeling induced by IW was completely attenuated by GYKI 52466 (20µM; C) but not MK-801 (10µM; D). Scale bar represents 10µm.
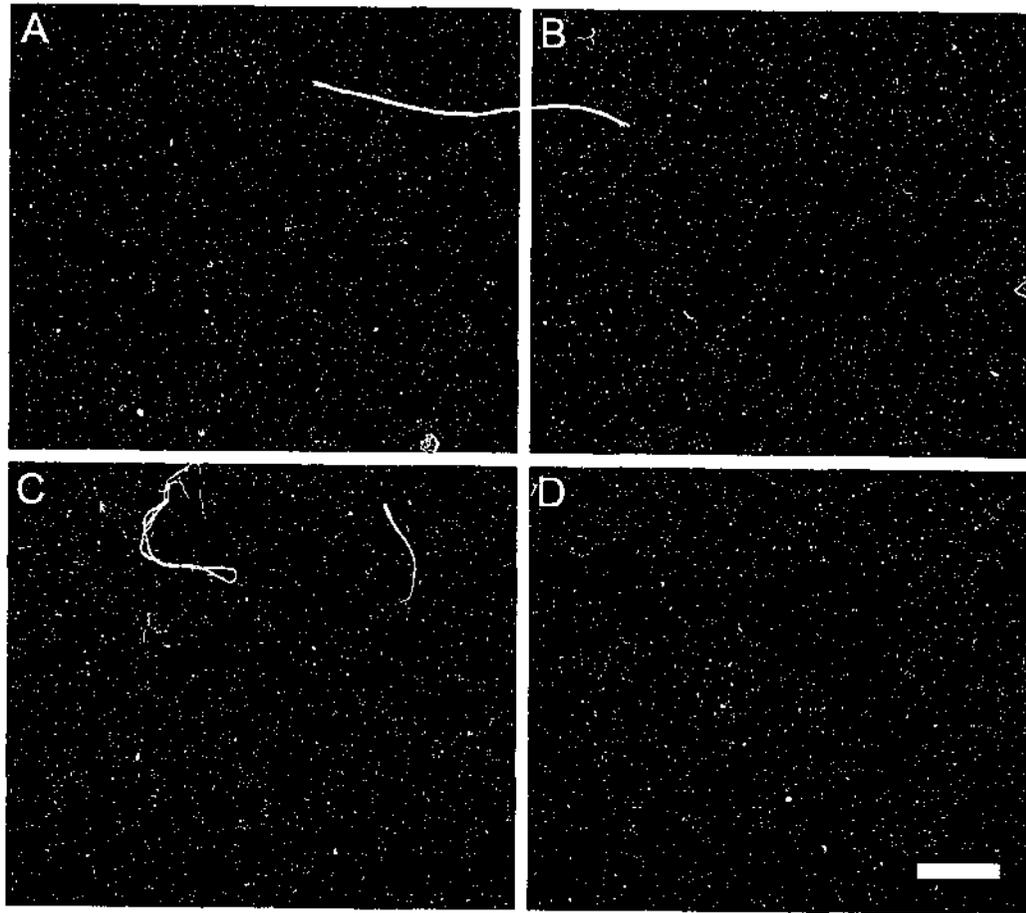
Figure 3.17 Determination of necrosis induced by 4-MG using PI incorporation

The extent of necrosis induced by 4-MG was determined by PI incorporation 1-2h after exposure. 4-MG exposure greatly incre.sed necrotic (PI) labeling (100µM; B) compared to the vehicle control (A). PI labeling induced by 4-MG was only slightly reduced by MK-801 (10µM; C) but not CNQX (50µM; D). Scale bar represents 10µm.
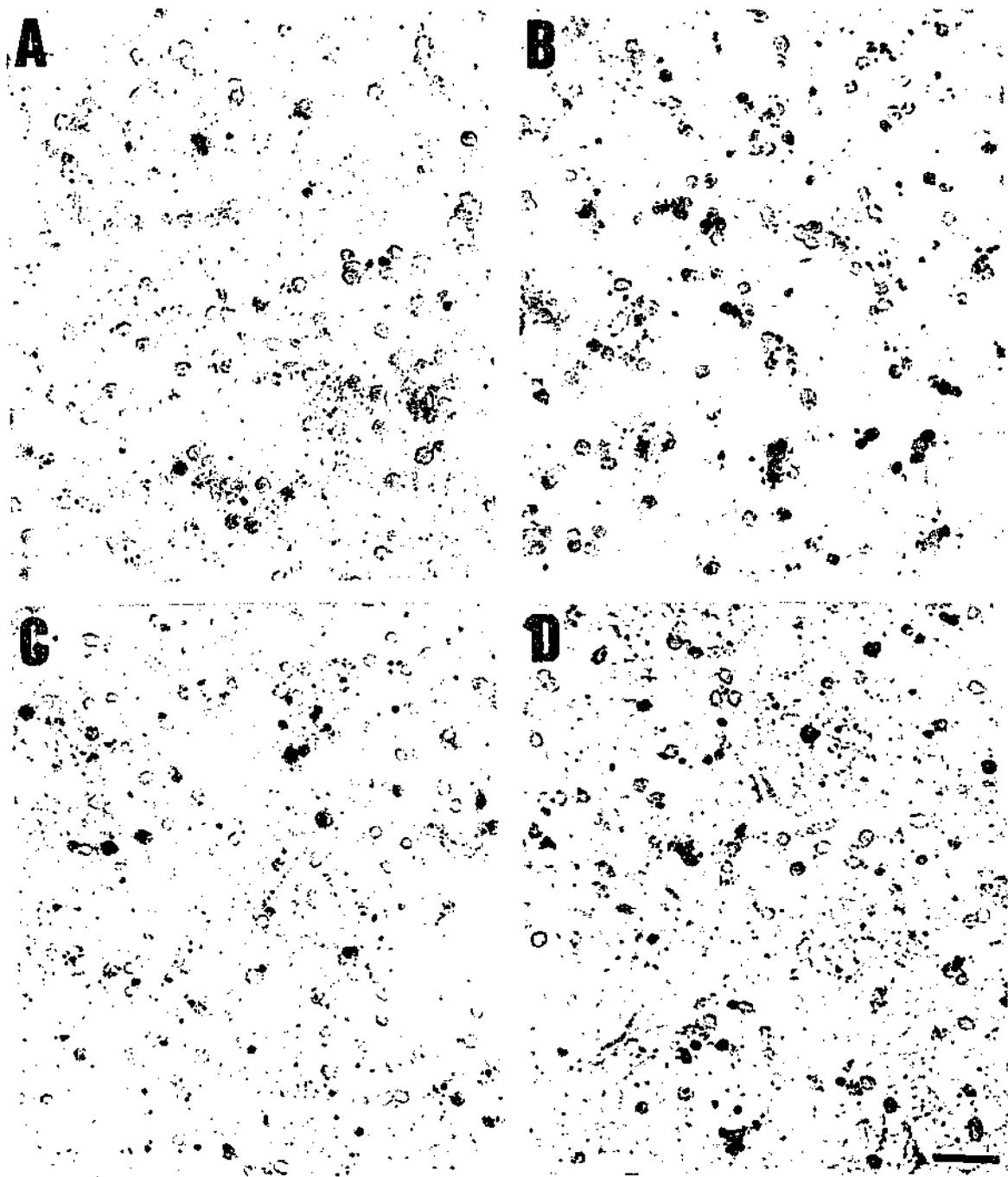
Figure 3.18 Determination of apoptosis induced by 4-MG using TUNEL labeling

The extent of apoptosis induced by 4-MG was determined by TUNEL labeling 18-24h after exposure. Exposure of cell cultures to 4-MG increased apoptotic (TUNEL) labeling (30µM; B) compared to the vehicle control (A). 4-MG-mediated TUNEL labeling was only slightly reduced by MK-801 (10µM; C) but not CNQX (50µM; D). Scale bar represents 10µm.

glial cell population, and naturally express KA receptor subunits GluR5 and 6 (Belcher & Howe, 1997) and high affinity subunits (Pemberton *et al.*, 1998), but not GluR7 (Hollmann & Heinemann, 1994). While KA2 and GluR6 expression occurs in equal abundance in the intact cerebellum (Porter *et al.*, 1997), the present study demonstrated differing expression patterns dependent upon the time in culture. This difference is most likely to be due to culture conditions not entirely mimicking the *in vivo* system, directly affecting receptor expression as previously described (Resink *et al.*, 1994). The much higher abundance of GluR 6 and 7 expression suggests that low-affinity KA receptors are likely to be the site of action of the various KA agonists. These cultures also do not express functional AMPA receptors at the time in culture employed, as AMPA fails to exhibit any loss of cell viability, even in the presence of CTZ (Chapter Two), in agreement with previous findings (Hack *et al.*, 1995), and making them a particularly favourable system to examine KA receptor-mediated responses. Under these experimental conditions all the putative low-affinity KA agonists produced neuronal cell death that was concentration-dependent with the rank order of potency being IW > 4-MG > LY339434 > KA > ATPA. The patterns of injury produced by the various agonists showed considerable variation involving apoptosis and/or necrosis apparently dependent on their different pharmacological activities at KA and/or NMDA receptors.

Under the present experimental conditions IW and ATPA seemed likely to exert a considerable portion of their excitotoxicity via KA receptors in CGCs, given the neuroprotective actions of the receptor antagonists and the abundant expression of these receptors found by immunocytochemistry. ATPA was somewhat less potent than IW and has previously been shown to be a quite potent AMPA receptor agonist (Stensbol *et al.*, 1999), whilst IW has recently been suggested to possess activity at NMDA receptors in primary cortical cultures (Moldrich *et al.*, 2000a). Since MK-801 was ineffective at

preventing IW-mediated neurotoxicity, such a NMDA receptor-related effect was not operative in CGCs. This discrepancy may not be due to the immediate action of IW, but rather a response secondary to necrotic cell death leading to the release of Glu which subsequently acts on neighboring NMDA receptors to elicit further toxicity in cortical neurones. While IW and ATPA were more and less potent than KA itself, respectively, neurotoxicity mediated by these compounds was not attenuated by MK-801. Indeed, NMDA under the present Mg^{2+}-containing conditions was a weak excitotoxin only causing appreciable cell death at concentrations >100μM (see Chapter Two). Thus the experimental milieu would appear to mitigate against secondary mechanism subsequently dependent upon NMDA receptor activation. While KA produces apoptosis at lower concentrations and necrosis is a rare event, only evident at quite high concentrations, IW-mediated cell death was largely necrotic in nature except at low concentrations. ATPA did not produce any necrotic death at the concentrations examined and the cell death that was evident was apoptotic as determined by TUNEL and morphological analyses, however even at concentrations as high as 1mM ATPA only reduced cellular viability to 75%, implying it is indeed a weak excitotoxin. The differences in the toxicity profiles of ATPA and IW when compared to KA are likely to be explained by their affinities these agents have for the KA receptor, rather than different sites of action.

4-MG has previously been shown to be a selective and potent GluR5 and 6 KA receptor agonist in studies that expressed recombinant receptors in HEK293 cells (Zhou *et al.*, 1997). The present study demonstrated the toxicity mediated by 4-MG was not mediated to any great extent by the iGluRs (≤ 30%), as non-NMDA and NMDA receptor antagonists failed to attenuated toxicity except at concentrations < 30μM. One explanation for this discrepancy may be the blocking activity of 4-MG at Glu transporters (Vandenberg *et al.*, 1997). Blocking Glu transporter hinders uptake of Glu, resulting in an accumulation

of extracellular Glu that in turn is free to act on various Glu receptors, causing excitotoxic damage. While damage caused by this mechanism should be attenuated by the various Glu receptor antagonists, this was not evident in the present study, again indicating a non-iGluR mediated mechanism for 4-MG. 4-MG produced a cellular loss that was predominantly necrotic in nature and perhaps the mechanism of neurotoxicity involves cellular swelling and ultimately necrosis, that arise from the disruption of associated ion transport (Ankarcrona *et al.*, 1995; Kroemer *et al.*, 1998).

The pattern of neurotoxicity induced by LY339434, although consistent with studies conducted at native Glu receptors present in other neuronal cultures (Moldrich *et al.*, 1999; Small *et al.*, 1998), contrasted with studies conducted in recombinant receptor model systems (Small *et al.*, 1998). Excitotoxicity mediated by LY339434 was only attenuated by MK-801, with no significant protection from the non-NMDA receptor antagonists, and thus was likely to be mediated by NMDA receptors. While NMDA-induced toxicity was significantly less than observed with LY339434, it was also mediated purely by the NMDA receptor (Chapter Two). Exposure of cultures to LY339434 resulted in a pattern of neuronal loss that was typical of NMDA receptor stimulation (Bonfoco *et al.*, 1995; Portera-Cailliau *et al.*, 1997a), with lower concentrations producing some apoptosis, and higher concentrations resulting in a marked cellular loss, attributed to necrosis. Recent evidence from our laboratory suggests that LY339434 may also have actions on Glu transporters (Aprico *et al.*, 2000). Thus the mechanism discussed above for 4-MG may contribute to the increased neurotoxic potency of LY339434 relative to NMDA. While LY339434 was initially thought to be a selective low-affinity KA receptor agonist, recent evidence suggests that it is indeed a NMDA receptor agonist (Moldrich *et al.*, 2000b; Small *et al.*, 1998). How LY339434 overcame the Mg^{2+} block, present due to the experimental medium containing Mg^{2+}, to allow NMDA receptor stimulation is

unknown, but may have occurred by an initial stimulation of the non-NMDA receptors, resulting in membrane depolarization, releasing the Mg^{2+} block and subsequently allowing the activation of the receptor.

While the pharmacological profiles of these agonists were quite different, all produced an apoptotic-necrotic continuum of cell death, except for ATPA, where the pattern of neuronal injury only exhibited apoptotic characteristics. Morphologically apoptotic cell bodies were evident at low concentrations for the agonists, with evidence of shrunken cell bodies and neurite blebbing indicative of apoptosis (Clarke, 1990). Apoptosis is a complex mechanism involving cascades of events largely initiated by an increase in intracellular Ca^{2+} (Lipton & Nicotera, 1998; Orrenius *et al.*, 1992; Toescu, 1998) leading to the activation of various caspases and kinases (Szalai *et al.*, 1999). Hence apoptosis is slower mechanism than necrotic cell death and cellular damage is not evident within the first hour of stimuli, unlike necrosis, allowing the manipulation of labeling with various dyes that only penetrate cells with damaged membranes at earlier time points for identification of necrotic cells. Necrotic cell death, as demonstrated at higher concentrations of IW, 4-MG and LY339434, was evident 1-2h after stimulation where the cells appeared large and swollen and stained positive with PI. After 24h of agonist exposure only debris and a fraction of the cells remained, characteristic of necrosis (Clarke, 1990), mediated, in large, by an influx of Na^+ and Cl^- ions followed by a secondary passive influx of water (Choi *et al.*, 1987). While Glu receptor-mediated toxicity has be shown to largely occur by necrosis (Ankarcrona *et al.*, 1995; Cheung *et al.*, 1998b; Dessi *et al.*, 1994b; Nicotera *et al.*, 1997), cellular damage that occurs from the overstimulation of the non-NMDA receptors generally occurs by apoptosis (John *et al.*, 1999; Larm *et al.*, 1997b; Portera-Cailliau *et al.*, 1997b), except when extremely severe insults are used (van Lookeren Campagne *et al.*, 1995). This pattern of injury is consistent

with the current findings for IW and KA, which produced extensive apoptotic cell death at lower concentrations, but at higher concentrations these agonists resulted in necrosis.

The current study highlights the importance of examining pharmacological profiles of various novel compounds in native receptors, and the vast difference that can exist between artificially combined receptors and those that are formed in the presence of other receptor subunits, allowing the construction of heteromeric receptors for example, and other protein conformational "tools" such as enzymes responsible for RNA editing (Belcher & Howe, 1997; Puchalski *et al.*, 1994). Therefore, while recombinant techniques are invaluable for the initial characterization of compounds it is essential to examine the pharmacological properties in native receptors, where primary cell·culture is useful due to the lack of influences from other neuronal inputs and cell types such as glia. Thus whilst 4-MG and LY339434 are clearly unsuitable for the study of low-affinity KA receptors in a physiological milieu, IW and ATPA appear to be useful for this purpose, especially in the CGC preparation used herein. Clearly very long exposure times (24-48h) will be needed for ATPA, although the excitotoxic injury should be purely apoptotic and relevant to the slow neuronal loss seen in neurodegenerative diseases. Using this strategy new roles have identified for low-affinity KA receptors, which appear additional to their involvement in synaptic transmission (Clarke *et al.*, 1997; Reid & Bliss, 2000), and whereby GluR5-7 via apoptosis and/or necrosis are likely to contribute to neurodegeneration.

CHAPTER FOUR

THE INVOLVEMENT OF THE CELL

CYCLE GENES AND BAX IN KAINATE-MEDIATED

APOPTOSIS

4.1 INTRODUCTION

Glu receptor-mediated neurotoxicity is well documented and is considered to occur through both necrosis and apoptosis (Portera-Cailliau *et al.*, 1997a), dependent upon the intensity of the insult (Cheung *et al.*, 1998b). KA-mediated cell death has been reported previously to be predominantly apoptotic in nature (Simonian *et al.*, 1996; van Lookeren Campagne *et al.*, 1995), however in higher concentrations may cause necrosis (Portera-Cailliau *et al.*, 1997a; van Lookeren Campagne *et al.*, 1995). Morphological changes induced by apoptosis are characterized by cell shrinkage, nuclear condensation, oligonucleosomal fragmentation of the DNA and neurite degeneration, whereas necrosis is a passive process involving cellular swelling and lysis of the intracellular contents (Clarke, 1990). Apoptosis involves complex mechanisms requiring energy and protein synthesis (Dessi *et al.*, 1994a), leading scientists to examine the genetic control of apoptosis. Apoptosis is now affiliated with various genes being either up or down regulated including the Bcl-2 and the cell cycle families, which will be discussed in this chapter.

The Bcl-2 family of genes have been well documented in various cells types undergoing apoptosis (Desagher & Martinou, 2000; Nicholls & Budd, 2000; Chao & Korsmeyer, 1998). BAX is a well studied, pro-apoptotic member of the Bcl-2 family, that plays a central role in apoptosis, linking caspase activation to mitochondrial dysfunction (Chao & Korsmeyer, 1998). The ratio of BAX to Bcl-2 in a cell is thought to determine whether a cell will survive or die (Oltvai *et al.*, 1993). However, recent evidence suggests that indeed it is not the ratio between Bcl-2 and BAX that produces the anti-apoptotic function, but rather Bcl-2 acts as an enzyme in either homo- or heteromeric configurations, and acts through interactions with lipids and ions (Otter *et al.*, 1998). Recently it has been

shown that BAX initiates cyto c release from the mitochondria (Skulachev, 1998), an action that is inhibited by Bcl-2.

BAX has been found to be expressed after numerous apoptotic stimuli, including, in neurones after kainate induced apoptosis (Gillardon *et al.*, 1995), after global ischaemia (Honkaniemi *et al.*, 1996), and in normal developmental neuronal cell loss (Vekrellis *et al.*, 1997). Although studies employing various apoptotic models have found an elevated level of BAX expression many have not, including, c-myc-induced apoptosis in Rat 1A MycER cells (Kangas *et al.*, 1998), and cortical neurones undergoing hypoxia-induced apoptosis (Tamatani *et al.*, 1998). Interestingly, p53 overexpression or camptothecin treatment does not elevate BAX expression, however Glu exposure leads to marked increase in BAX protein in both cortical and hippocampal cultures (Xiang *et al.*, 1998). This same study demonstrated a dependence of the p53 gene for BAX expression, implicating the cell cycle in the regulation of BAX.

More recently, some of the genes that control proliferation have been implicated in neuronal apoptosis (Chapter One). Moreover, some neuronal cell populations, which were once thought to be incapable of proliferation, have since been found to divide after various toxic stimuli (Luo & Miller, 1998; Bengzon *et al.*, 1997; Gould & Tanapat, 1997). Apoptotic stimuli have been speculated to force post-mitotic cells into the first phase of the cell cycle, namely the G1 phase. Evidence to suggest that the early stage of the cell cycle is involved in apoptosis has come from studies whereby various G1/S phase inhibitors have attenuated various apoptotic insults in neurones (Farinelli & Greene, 1996; Park *et al.*, 1996a) (see Chapter Five).

Recently, genes that control the cell cycle have been found to cause apoptosis and/or to be activated in apoptotic cells in various cell lines (King & Cidlowski, 1998). Initial evidence suggesting the involvement of cell cycle regulating proteins in apoptosis

came from investigations performed in cytotoxic granules derived from T lymphocytes, where there was a notable activation of CDK2 (Shi *et al.*, 1994). Furthermore, overexpression of Bcl-2, an anti-apoptotic protein, suppresses levels of CDK2 and hinders apoptosis in HeLa cell lines (Meikrantz *et al.*, 1994). Remarkably little is known about the relationship between the cell cycle and neuronal apoptosis, although cyclin B seems to be involved in dopamine-induced apoptosis in sympathetic neurons (Shirvan *et al.*, 1997a), and cyclin D1 has been implicated in KA-mediated neuronal cell death *in vivo* (Liu *et al.*, 1996) and serum withdrawal-induced apoptosis *in vitro* (Miller & Johnson, 1996).

Recent studies *in vivo* have demonstrated NMDA receptor-mediated apoptosis is accompanied by increased neuronal proliferation in the hippocampal dentate gyrus (Gould & Tanapat, 1997), implicating excitotoxicity in insult-dependent neuronal proliferation. Emerging studies suggest that little differs between the biochemistry of apoptosis and mitosis, and that apoptotic stimuli may result in mitosis in neurons (Ross, 1996). Thus the study of cell cycle regulating genes represents a logical progression in the attempts to understand the cellular mechanisms involved in neuronal apoptosis. The expression of BAX was studied in parallel as this pro-apoptotic member of the Bcl-2 family should represent a marker of cellular death cascades.

4.2 METHODS

4.2.1 Materials

CNQX was purchased from Tocris Cookson (Bristol, UK) and KA from Sigma (Sydney, Australia). NBM, Gln, penicillin-streptomycin, dNTPs, superscript reverse transcriptase II and HBSS were purchased from GibcoBRL Life Technologies (Melbourne,

Australia). Red Hot Taq polymerase was purchased from advance biotechnologies (Surrey, UK). Cyclin D1 Antibodies were from Santa Cruz Biotechnology (CA, USA), and cyclin A and B1 antibodies were purchased from Calbiochem (Melbourne, Australia). RNAeasy™ mini kit was purchased from Qiagen (Melbourne, Australia). Proteinase K was purchased from Boehringer Mannheim (Sydney, Australia). All other reagents were purchased from Sigma or Boehringer Mannheim (Sydney, Australia) and were of cell culture or molecular biology grade.

Experiments were performed in accordance with the ethical code of the National Health and Medical Research Council (Australia) with permission from the standing Committee for Ethics in Animal Experimentation.

4.2.2 Cell Culture

CGCs were prepared from 7d old Swiss White mice and cultured as described in Chapter Two. CGCs were grown in NBM containing B27 components (Brewer *et al.*, 1993), 25.4mM K^+, 500µM Gln and 100U/ml penicillin-streptomycin and exposed to 10% dialysed fetal calf serum for 24h on *div* 0. Cells were seeded at a cell density of 0.3 x 10^6 cells/cm^2 in 24 well NUNC™ plates (Denmark) precoated with poly-D-lysine (50µg/ml/well). Aphidicolin (2µg/ml) was added to the medium 18-24h after plating to inhibit non-neuronal cell proliferation (Miller & Johnson, 1996).

4.2.3 Agonist Exposure and Cell Viability Assays

CGCs were exposed to KA (10-1000µM) alone or in the presence of the non-NMDA receptor antagonist CNQX (50µM), for various times (1-24h) at 8 *div* in N2 supplemented NBM containing 100U/ml penicillin-streptomycin, 0.25% BSA, 83µM D(+)

galactose, 16µM ethanolamine, 6µM L-carnitine, 0.4µM biotin and 25.4mM K$^+$ (Bottenstein & Sato, 1979).

4.2.3.1 Determination of Cellular Viability

Cellular viability was determined at 24h by the reduction of MTT as described previously (Chapter Two). Injury in control cultures left in low K$^+$ for 24h were taken as 100% cell death and the results were expressed as percentage of control (untreated). Morphological changes were examined by phase contrast microscopy.

4.2.4 Detection of Apoptosis

4.2.4.1 Determination of DNA Fragmentation in situ

Apoptosis was analysed by TUNEL as previously described (Chapter Two). CGCs, after treatment with KA(10-1000µM), were fixed overnight in 4% paraformaldehyde, permeablized with 2% TX-100 in TBS, and incubated with TdT reaction mixture for 3h at 37°C. DIG labelled dUTP was detected using anti-DIG alkaline phosphatase (AP) and detected using AP substrate solution as described in Chapter Two. Cells were visualized under bright field microscopy.

4.2.4.2 Determination of DNA Fragmentation by Gel Electrophoresis

Genomic DNA was extracted from cells grown in 6-well plates (NUNC™, Denmark) that were treated with various concentrations of KA (10-1000µM) in the presence or absence of CNQX (50µM) for 24h. Cells were lysed in 700µl extraction buffer (10mM Tris, 20mM EDTA and 0.5% SDS; pH 8) for 1h at 37°C. The extraction buffer was subsequently removed from the wells and added to microtubes containing proteinase

K (160μg). Digestion was carried out for 1h at 55°C and subsequently stored at -20°C overnight. Phenol:chloroform:isoamyl alcohol (25:24:1 ratio; 800μl) was added to the cell lysate, vortexed, and centrifuged for 15min at 13,000 x g. The aqueous phase was collected and 220μl ammonium acetate (7.5M) was added followed by vortex mixing. DNA was precipitated with absolute, ice-cold ethanol overnight at -20°C. The suspension was subsequently spun, 13,000 x g at 4°C and the supernatant removed and the pellet allowed to air dry. The pellet was dissolved in 20μl 8mM NaOH, and DNA concentration was determined at 260nm using an UV spectrophotometre (GeneQuant).

Agarose gels were prepared with TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.3). DNA samples (10μg) were loaded onto 1.5-2% (w/v) agarose gels, containing 0.5μg/ml ethidium bromide, with 1/5th volume of loading dye (Sigma) and run at 100V for 1-2h. Gels were visualized under UV light and images captured using the Eagle Eye II still video system (Stratagene, CA, USA).

4.2.6 Protein Analyses of Cyclins A, BI and D1 and BAX Expression

4.2.6.1 Changes in Protein Expression as Determined by Immunocytochemistry

After exposure of the cultures to KA (1-1000μM), culture medium was aspirated after various times (1-24h) and immediately fixed in 4% paraformaldehyde for 20min at room temperature, and subsequently washed in TBS. Cultures were then permeabilized in 0.1% TX-100, and blocked for 1h with 10% NGS in solution with 0.1% TX-100 and TBS. Cultures were then exposed to primary antibodies: cyclin D1 (1:1000), cyclin A (1:250), cyclin B1 (1:250), or BAX (1:1000) overnight at 4°C in solution with 0.1% TX-100 and 2% NGS in TBS. Cultures were subsequently washed in TBS and incubated with secondary antibodies, anti-mouse HRP for cyclin D1 and anti-rabbit HRP for cyclins B1

and A, for 3h at room temperature. Immunopositive cells were detected using DAB, as previously described (Chapter Two) and colour was allowed to develop for up to 30min at room temperature. Immunopositive cells were visualized under bright field microscopy and photographed on 100 ISO Kodak™ film. Random, yet representative fields were photographed at low magnification (x 100) and cells counts conducted for cyclin D1 and BAX protein expression.

4.2.6.2 Changes in Protein Expression as Determined by Western Blot Analysis

After KA stimulation (1-24h), CGCs were lysed in 0.4% SDS solution containing protease inhibitors (Boehringer Mannheim) for 1h at 4°C. Prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein content was determined by a commercially available protein assay (Biorad). Protein separation was performed on a 10% SDS-polyacrylamide gel, whereby equal amounts of protein (10μg) were separated at 100V for 1-2h. Protein was then transferred to nitrocellulose membrane (Biorad) with a semi-dry transblotter (Biorad), blocked in 5% BSA in TBS and incubated with the antibodies for cyclin D1 (1:1000 dilution) or BAX (1:1000 dilution) in 1% BSA/TBS solution, overnight with constant agitation at 4°C. The membranes were subsequently washed in TBS and incubated with the secondary antibodies anti-mouse (1:1000) for cyclin D1 and anti-rabbit (1:1000) for BAX, for 3h with constant agitation at room temperature. The signal was detected using DAB and the resultant protein size compared to rainbow standard ladder (GibcoBRL Life Technologies).

4.2.7 RT-PCR Analysis of Cyclins A, B1 and D1.

PCR primers were specific for the cell cycle genes and other mRNAs, using the respective published murine sequences (Table 4.1). RNA was isolated from cultures using

Gene	Genbank Reference	5' primer 3'primer	Product size
Cyclin A	X75483	5'-CTCCTCCATGTCTGTGTTAA-3' (829-848) 5'-CAACCTTACAGTTTGCAGGC-3' (1069-1088)	259bp
Cyclin B1	L11995	5'-AGAAGGTGCCTGTGTGTGAA-3' (373-392) 5'-TTGGTCTAACTGACTGCTCTTC-3' (681-660)	306bp
Cyclin D1	X75207	5'-GATGAAGGAGACCATTCCCT-3' (473-491) 5'-TCTGCTTGTTCTCATCCGC-3' (648-666)	193bp
GAPDH	M32599	5'-TGAAGGTCGGTGTGAACGGATTTGGC-3' (51-76) 5'-CATGTAGGCCATGAGGTCCACCAC-3' (1033-1010)	983bp
β-Actin	X03765	5'-GTGGGCCGCTCTAGGCACCAA-3' (25-45) 5'-CTCTTTGATGTCACGCACGATTTC-3' (541-564)	540bp

Table 4.1
Table showing primer sequences used for RT-PCR studies.

the RNAeasy™ mini kit (Qiagen) performed as per manufacturer's instruction. RNA concentration was determined using an UV spectrophotometer (GeneQuant). Total RNA was subsequently converted to cDNA by reverse transcriptase as per manufacturer's instructions (GibcoBRL Life Technologies). In brief, 2µg of RNA was incubated with oligo-dt (40ng/ml) for 10min (70°C), followed by incubation with first strand buffer (50mM Tris-HCl, 75mM KCl, 5mM MgCl₂), 0.2mM DTT, and 2mM dNTP mix for 2min (42°C). Superscript II™ (200 units) was then added to the solution and incubated at 42°C for 50min. The reaction was inactivated by heating (70°C, 10min) and the resultant cDNA was stored at -20°C until required. Master mixes consisted of 0.2mM each dNTP, 1 x reaction buffer (50mM KCl, 10mM Tris-HCl, pH 8.3), 2mM MgCl₂, 120ng of each primer, 2µg cDNA and 1 unit Red Hot taq DNA polymerase. PCR was performed on a Touchdown™ thermal cycler (Hybaid, Middlesex, UK) with the following temperature profile: denaturation at 94°C for 1min, primer annealing for 57-62°C for 1min, and primer extension at 72°C for 2min, for 25 cycles as previously described (Oka *et al.*, 1996). Samples (10µl) were separated on 1.5-2% agarose gel electrophoresis, prepared as described previously for DNA gel electrophoresis, and products compared against a 100bp standard (GibcoBRL Life Technologies).

4.2.8 Data Analyses

Concentration-response curves were generated as previously described (Chapter Two). Cell counts were taken from random and representative fields and ≥ 800 cells were counted. Values are the mean $\pm$ S.E.M. from 4-6 independent cultures and experiments. Statistical significance ($p < 0.05$) of data was examined by two way ANOVA with a post-hoc Newman-Keuls test.

4.3 RESULTS

4.3.1 KA Receptor-mediated Neurotoxicity

KA exposure resulted in cell death as determined by morphological analysis (Figure 4.1), with CGCs exhibiting characteristic apoptotic morphology including shrunken cell bodies and neurite blebbing (Figure 4.1B) that was attenuated by CNQX (Figure 4.1C). Rapid cellular swelling was not evident up to 2h after KA exposure indicating the absence of necrotic cell death. KA caused a significant concentration-dependent cell death as determined by the MTT assay ([$F_{(5,36)} = 36.07$, $p < 0.0001$]; $EC_{50} = 200 \pm 50\mu M$). CNQX significantly shifted the concentration-response curve for KA to the right ($EC_{50} = 3400 \pm 20\mu M$), consistent with the reduction in cell death ($F_{[1,36]} = 106.3$, $p < 0.0001$; Figure 4.2). Cell death was confirmed to be apoptotic by the extensive positive labeling using the TUNEL technique at 24h (Figure 4.3). Counts of TUNEL positive cells indicated a concentration-dependent increase in KA-mediated cell death [$F_{(4,36)} = 29.47$, $p < 0.0001$], which was attenuated by CNQX ([$F_{(1,36)} = 32.41$, $p < 0.0001$]; Figure 4.4). TUNEL-labeling was confirmed by DNA gel electrophoresis where KA-induced fragmentation, consistent with apoptosis, that was attenuated by CNQX. KA exposure resulted in a clear DNA "laddering", characteristic of apoptosis, whereas no smaller fragments of DNA were evident in control treated cultures (Figure 4.5).

4.3.2 Analysis of Protein Expression of the Cell Cycle Genes and BAX After KA Receptor-mediated Apoptosis

After documenting that the pattern of neuronal injury induced by KA was apoptotic, investigations into whether the activation of cyclins D1, B1 and A protein
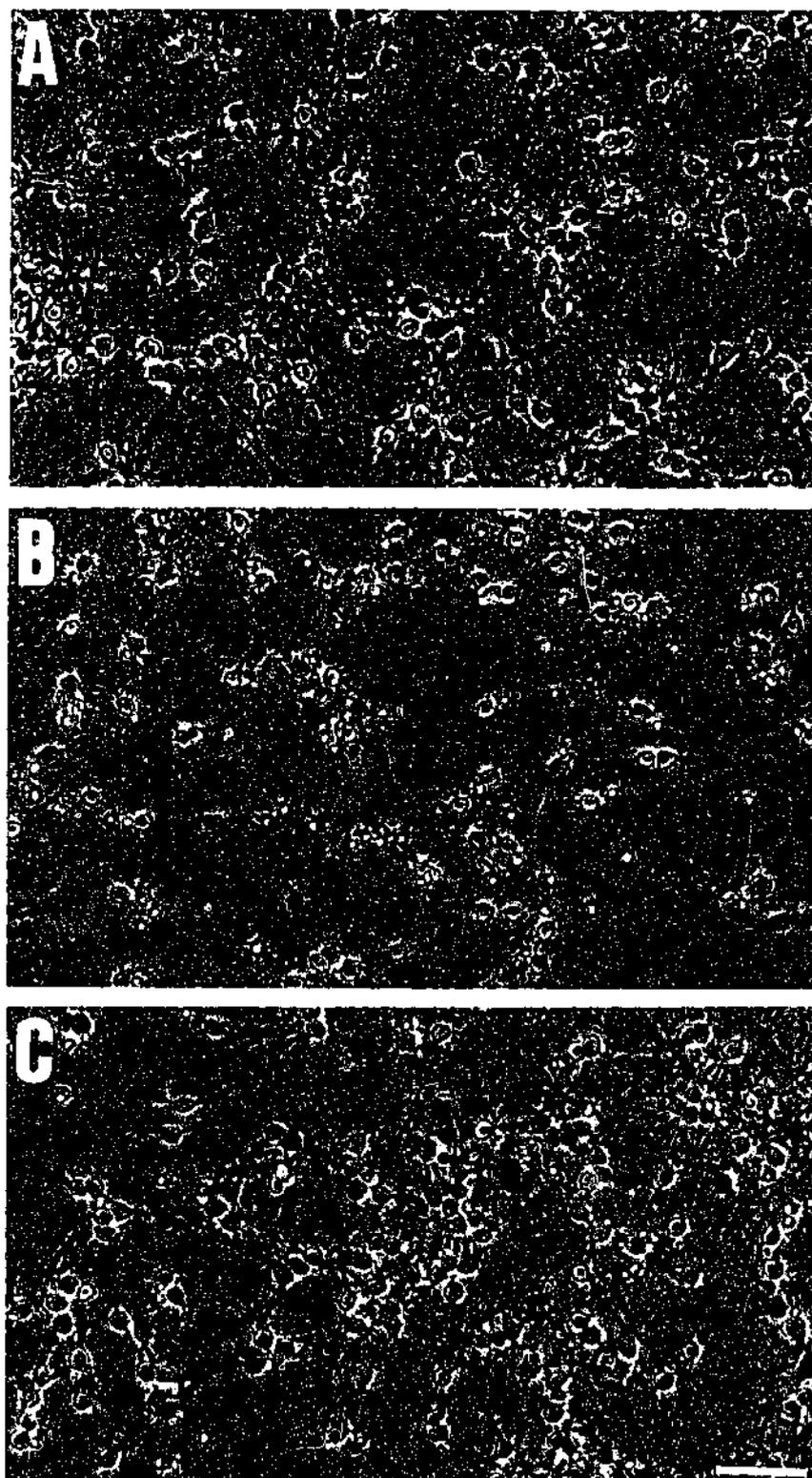
Figure 4.1 Morphological analyses of KA exposure to cerebellar granule cells

Cultures exposed to KA (100µM; B) demonstrate shrunken cellular bodies and a breakdown in neurites (neurite blebbing), compared to control cultures (A) where extensive neurite networks are evident and the cellular bodies are rounded. KA-mediated changes in morphology were completely attenuated by CNQX (50µM; C). Scale bar represents 10µm.
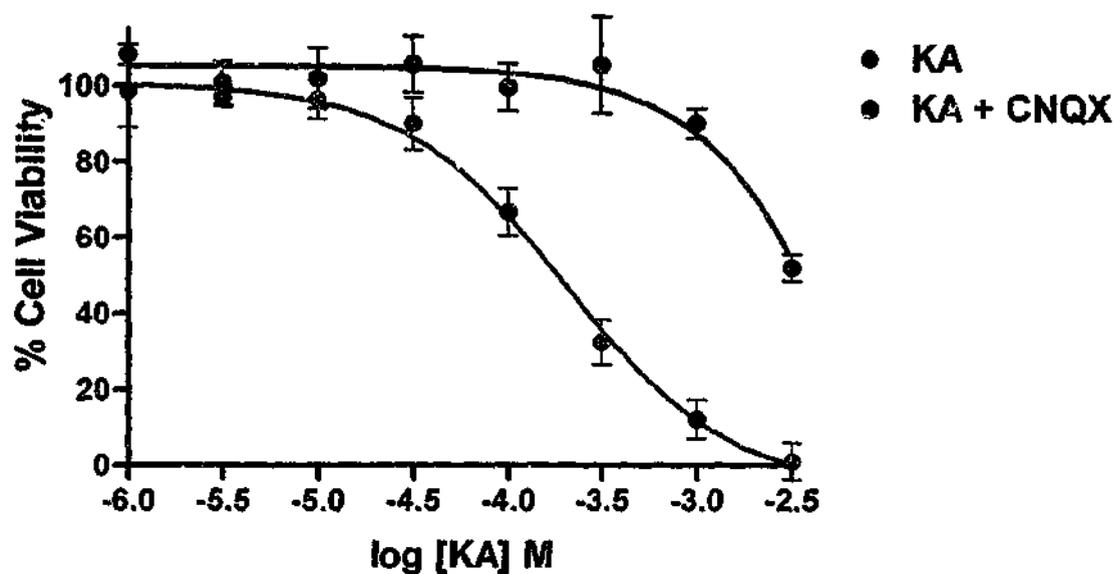
Figure 4.2 Neurotoxic profile of KA in cerebellar granule cells

Neuronal viability was assessed by MTT assay. Each point represents the means of 4-6 experiments across 4-6 independent cultures (mean ± SEM). MTT data indicated KA caused a concentration-dependent cell death ($F[5,36] = 36.07$, $p < 0.0001$), that was attenuated by CNQX ($50\mu M$; $F[1,36] = 106.3$, $p < 0.0001$).
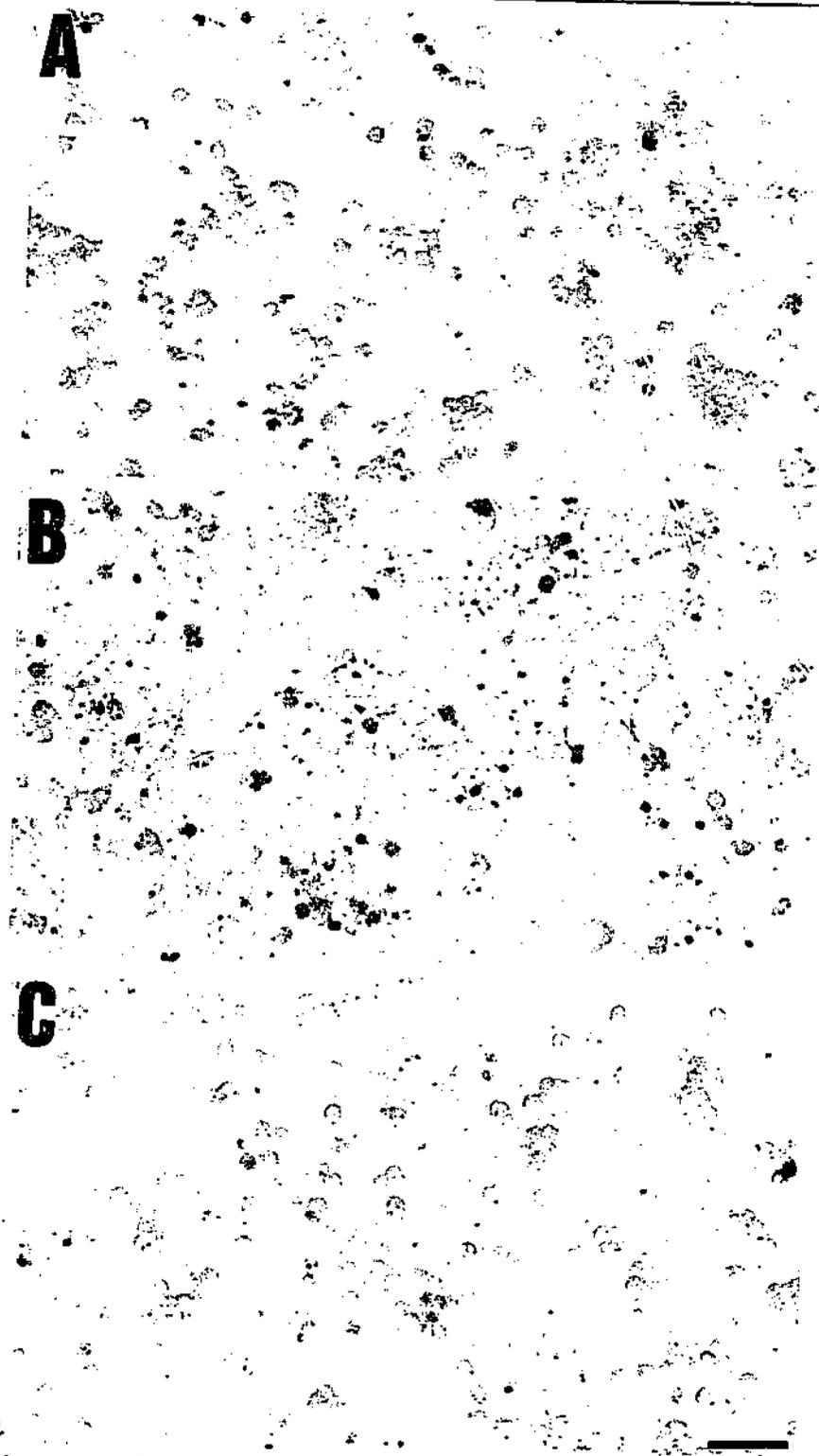
Figure 4.3 Apoptotic labeling (TUNEL) induced by KA in cerebellar granule cells

Apoptotic labeling was assessed by the TUNEL technique. Bright field photomicrographs demonstrate a marked increase in labeling induced by KA (300μM; B), compared to control cultures (A). KA-induced TUNEL labeling was attenuated by CNQX (50μM; C). Scale bar represents 10μm.
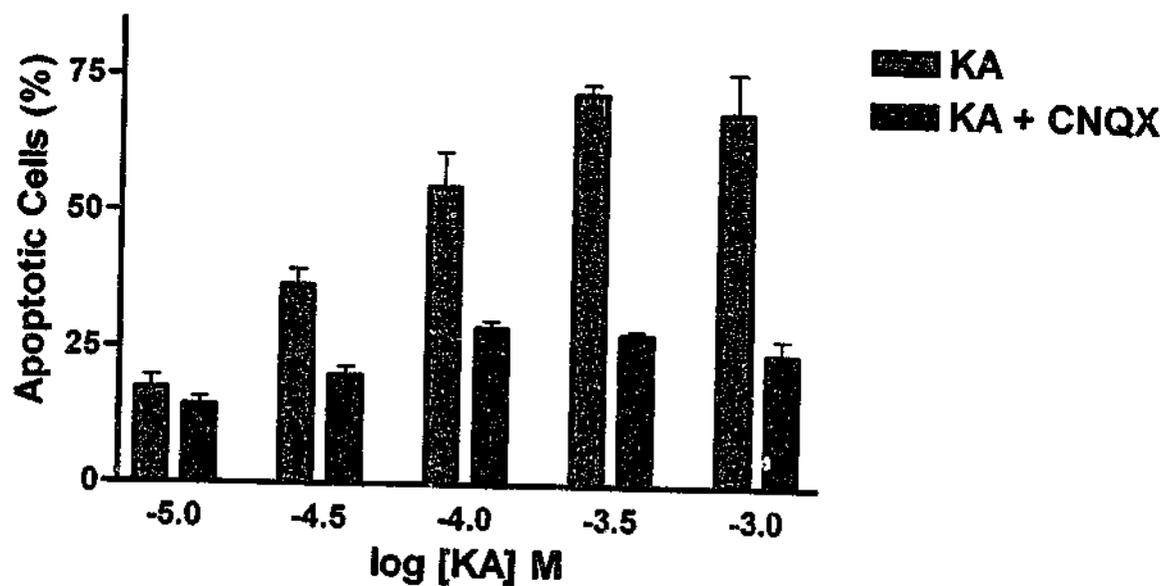
Figure 4.4 Apoptotic labeling (TUNEL) induced by KA in cerebellar granule cells
Cell counts for apoptotic labeling, determined by the TUNEL technique, revealed a
concentration-dependent ($F[4,36] = 29.47$, $p < 0.0001$), labeling that was CNQX-sensitive
($F[1,36] = 32.41$, $p < 0.0001$).

1 2 3

21.2kbp →

5.2kbp →

0.9kbp →
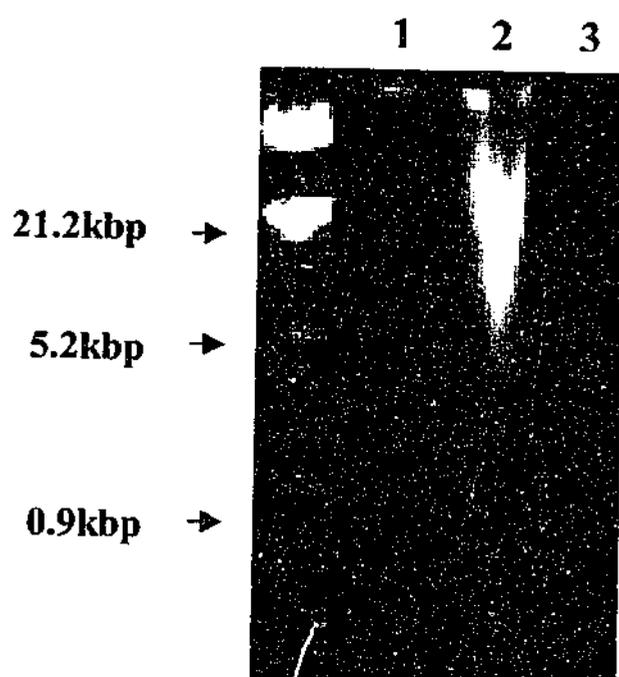
Figure 4.5 Detection of apoptosis by DNA gel electrophoresis

DNA was extracted from cultures 18-24h after KA exposure for gel electrophoresis. Exposure of KA (300μM; lane 2) to cultures resulted in gels with DNA laddering, indicative of apoptosis, which was attenuated by CNQX (50μM; lane 3). Negligible laddering is evident in control cultures (lane 1). 123bp DNA marker.

expression accompanied KA-mediated apoptosis were conducted. Many immunopositive profiles for cyclin D1 were noted 2h after KA exposure (Figure 4.6), and counting of CGCs expressing cyclin D1 and immunoreactivity indicated a time-dependent effect of exposure to KA [$F_{(5,30)}$ = 9.60, $p < 0.0001$], with peak expression of 70 ± 0.6% of cells above control after 2h (Figure 4.7A) for cyclin D1. In subsequent studies, a KA exposure time of 2h resulted in a concentration-dependent increase in cyclin D1 protein expression ([$F_{(4,18)}$ = 33.93, $p < 0.0001$]; Figure 4.7B). Interestingly, the KA-mediated increases in cyclin D1 protein and apoptotic labeling followed a very similar pattern [$F_{(5,36)}$ = 47.736, $p < 0.0001$], indicating that both responses were dependent on the severity of insult. Negligible changes in expression of cyclin B1 and A were noted in the examined time period (1-24h) after KA-mediated apoptosis (Figure 4.8).

Western blot analysis confirmed immunocytochemical observations, indicating a maximal expression of cyclin D1 protein at 2h, with protein levels returning to control levels 24h after KA exposure (Figure 4.9A). A concentration-dependent effect for cyclin D1 was also evident upon Western blot analysis (Figure 4.9B).

4.3.3 Analysis of BAX Protein Expression After KA Receptor-mediated Apoptosis

. Extensive immunolabeling of BAX was evident 12h after KA receptor-mediated apoptosis, that was CNQX-sensitive (Figure 4.10). Cell counting for BAX expression after KA receptor-mediated apoptosis indicated a time-dependent effect, with maximal expression 12h after KA (100μM) exposure ([$F_{(5,12)}$ = 28.69, $p < 0.01$]; Figure 4.11A). After 12h KA exposure, cell counts for BAX expression revealed a concentration-dependent pattern [$F_{(4,19)}$ = 13.79, $p < 0.05$] that was CNQX-sensitive ([$F_{(1,19)}$ = 56.77, $p < 0.0001$]; 50μM) (Figure 4.11B), and consistent with the immunocytochemical data.
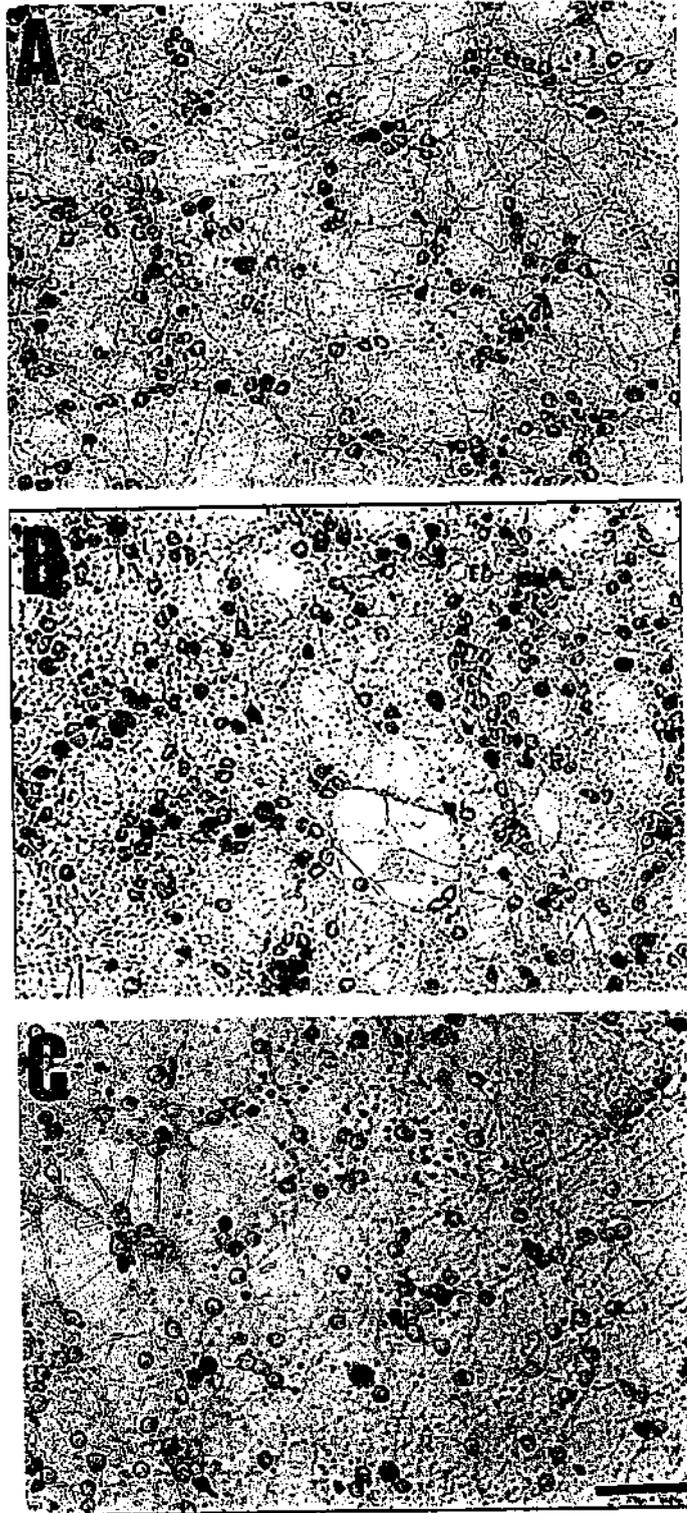
Figure 4.6 Immunocytochemical staining of cyclin D1 after KA exposure

Cerebellar granule cells exposed to KA (300μM; B) demonstrated a marked increase in cyclin D1 protein expression compared to untreated controls (A), 2h after KA exposure. Co-exposure of cultures to KA and CNQX (2h; 50μM; C) resulted in a marked attenuation of cyclin D1 protein expression. Scale bar represents 10μm.
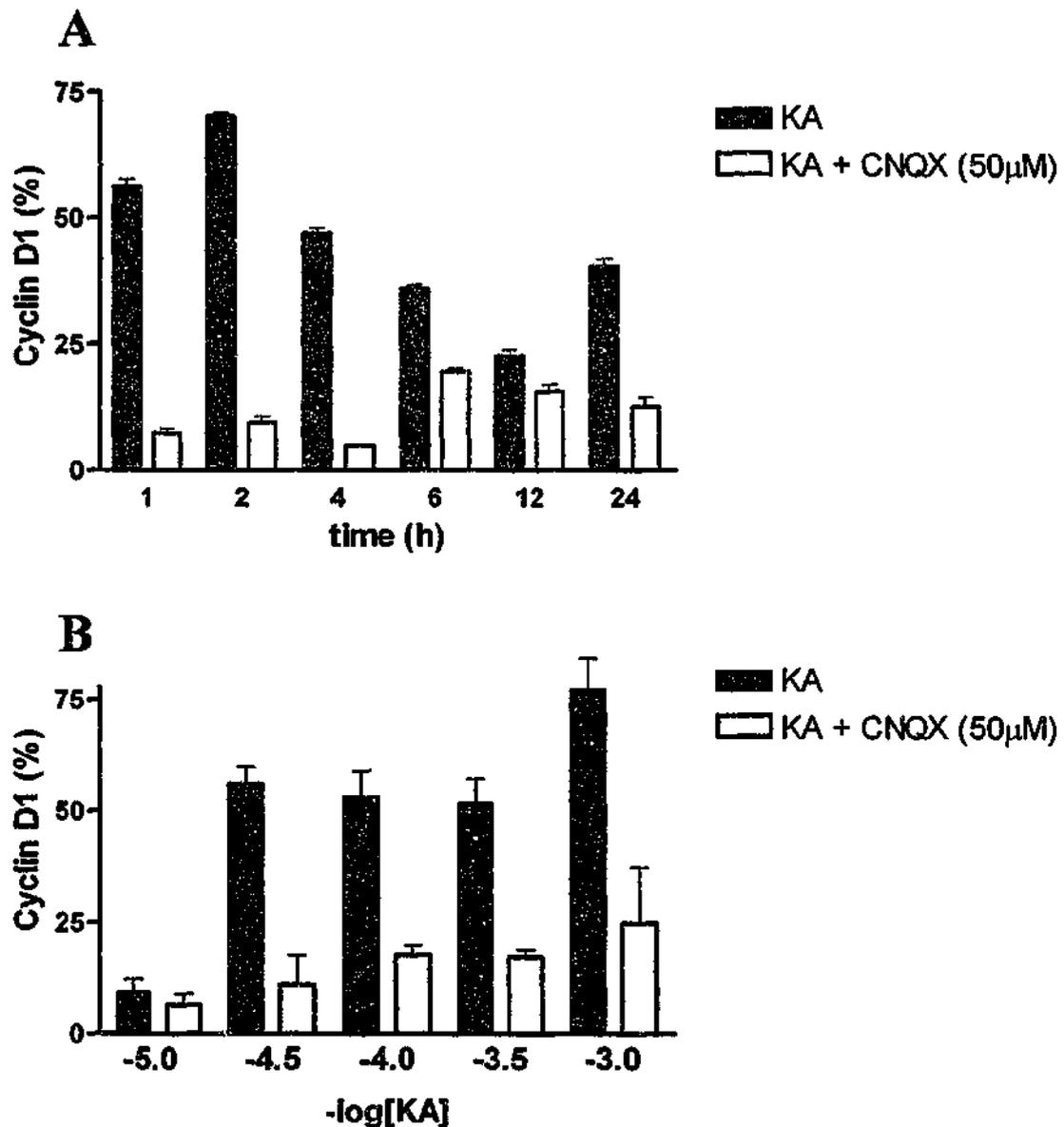
Figure 4.7 Immunocytochemical staining of cyclin D1 after KA exposure

Cerebellar granule cells exposed to KA demonstrated a marked increase in cyclin D1 protein expression as determined by cell counts for immunopositive cells. KA produced a time-dependent (A; $[F(5,30) = 9.60, p < 0.0001]$) change in cyclin D1 expression, with maximal expression after 2h. Cyclin D1 expression was attenuated by CNQX ($50\mu M$; $[F(1,30) = 69.24, p < 0.001]$, indicating a KA receptor-mediated mechanism. KA exposure (2h; B) resulted in a marked, concentration-dependent, increase in cyclin D1 $[F(4,18) = 33.93, p < 0.0001]$, that was significantly attenuated by CNQX ($50\mu M$; $[F(1,18) = 50.27, p <0.0001$).
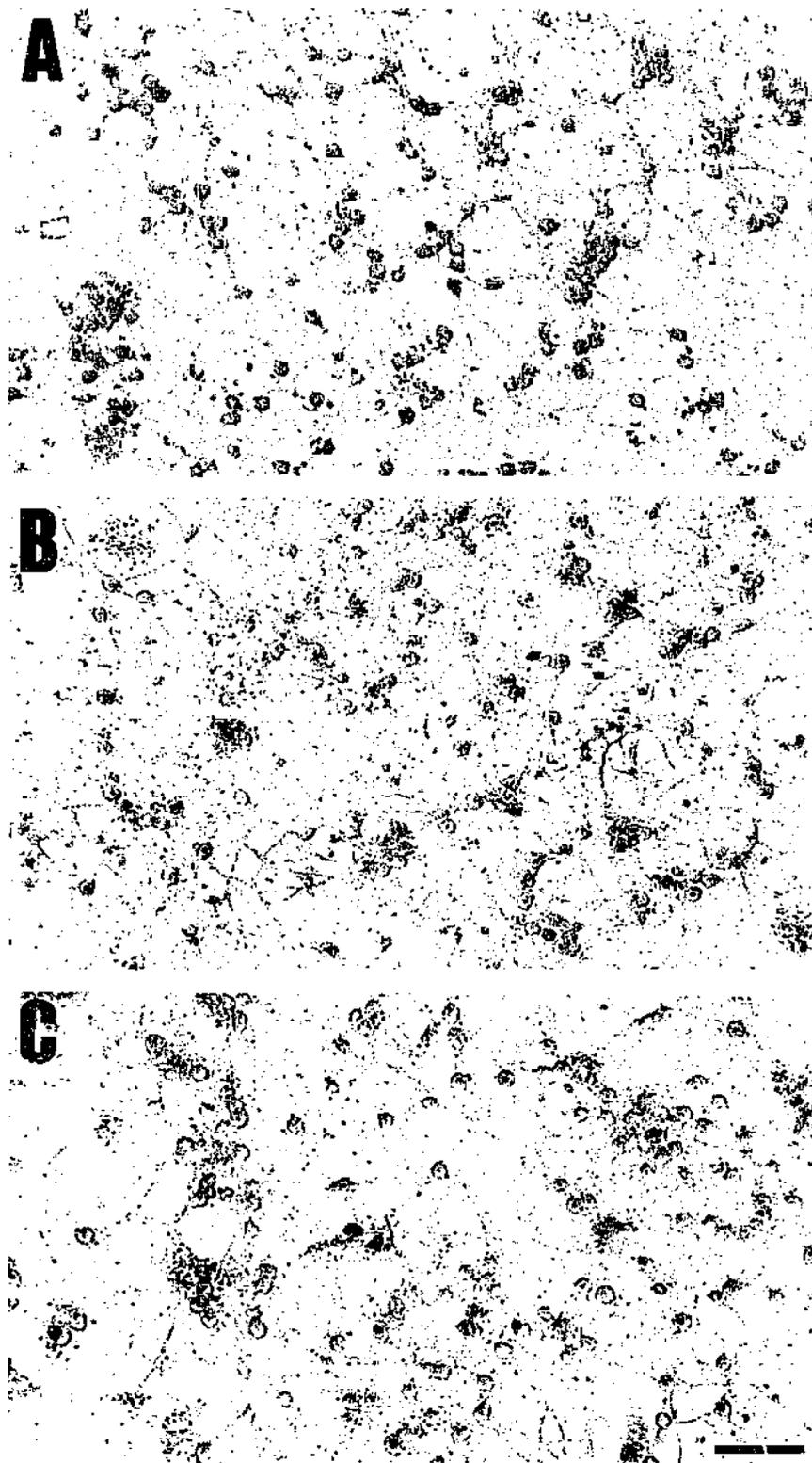
Figure 4.8 Immunocytochemical staining of cyclins A and B1 after KA exposure

Cerebellar granule cells exposed to KA (300µM) demonstrated no change in expression of cyclins A (B) and B1 (C), 12h after KA exposure, compared to vehicle alone treated cultures (A). Scale bar represents 10µm.
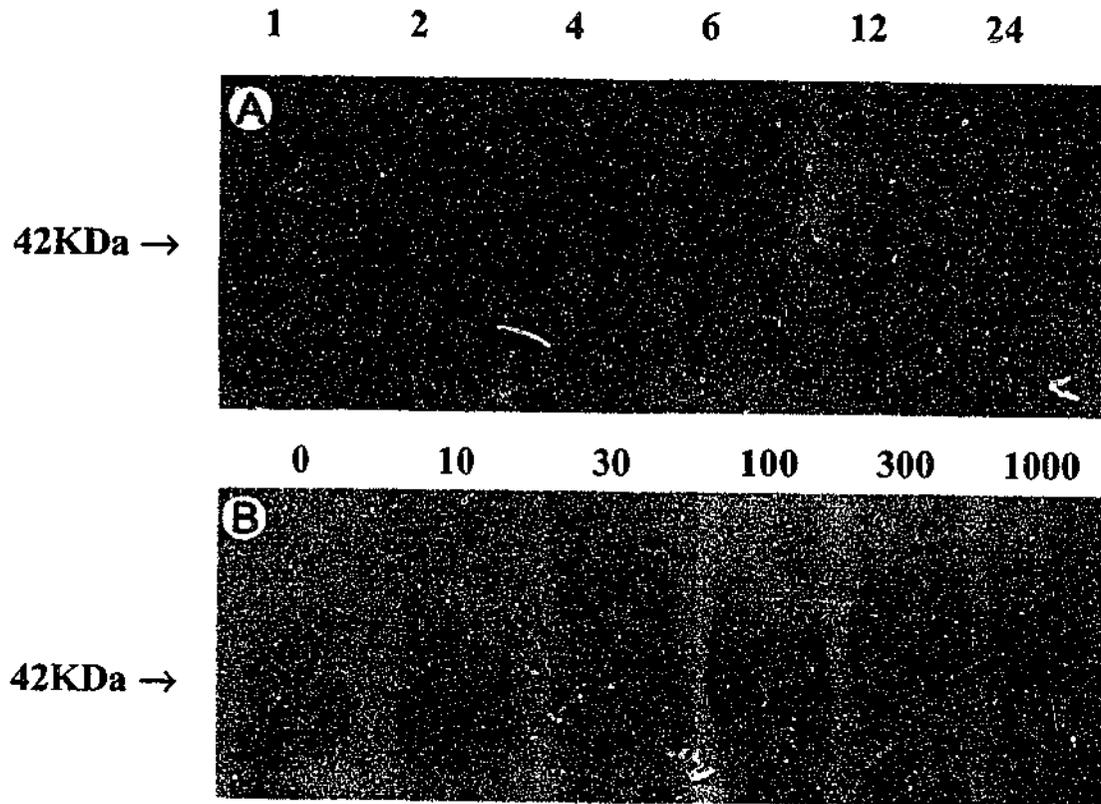
Figure 4.9 Western Blot analysis cyclin D1 expression after KA exposure

Cerebellar granule cells exposed to KA (100µM) for varying times demonstrated an increase, and time-dependent expression of cyclin D1 protein expression as determined by Western Blot analysis (A). Maximal cyclin D1 expression was found at 2h after KA exposure and at this time KA-induced a concentration-dependent expression of cyclin D1 (B).
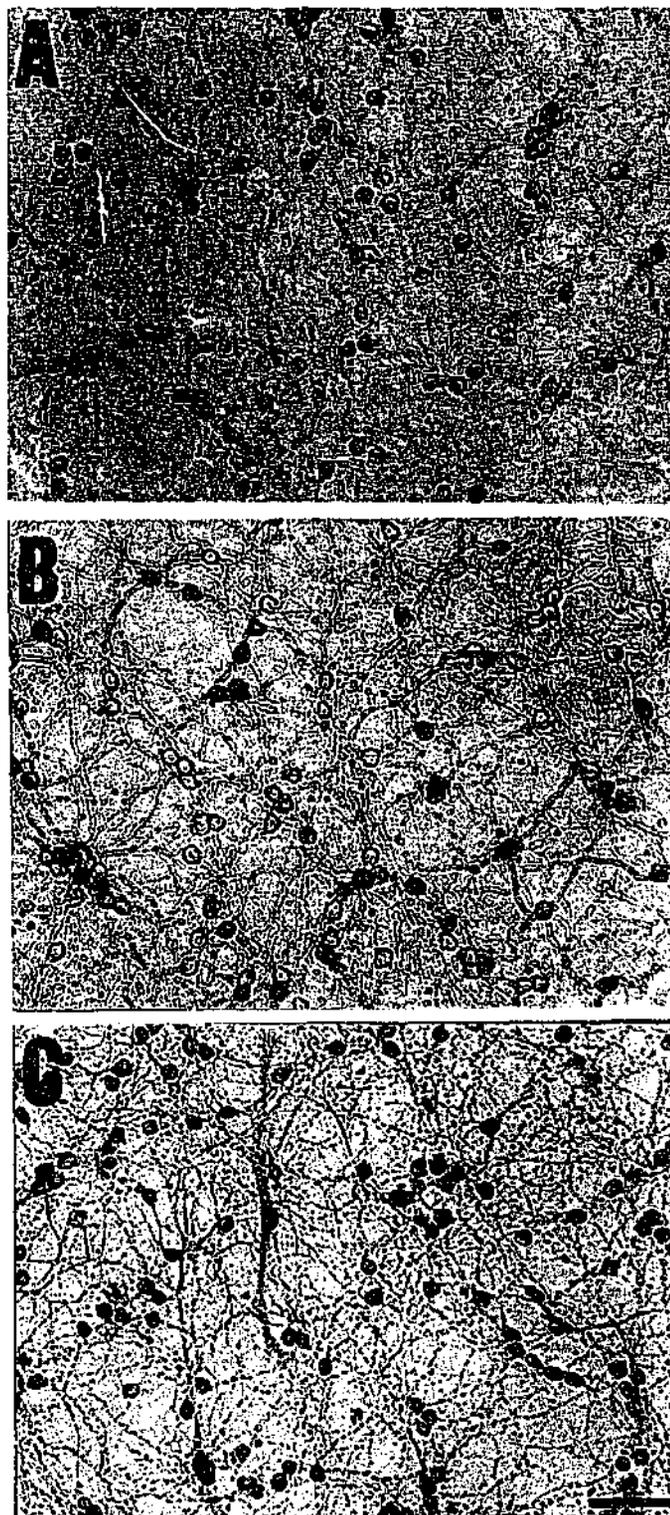
Figure 4.10 Immunocytochemical staining of BAX after KA exposure

Cerebellar granule cells exposed to KA (300µM; B) demonstrated a marked increase in BAX protein expression compared to untreated controls (A), 12h after KA exposure. Co-exposure of cultures to KA and CNQX (50µM; C) resulted in a marked attenuation of BAX protein expression. Scale bar represents 10µm.
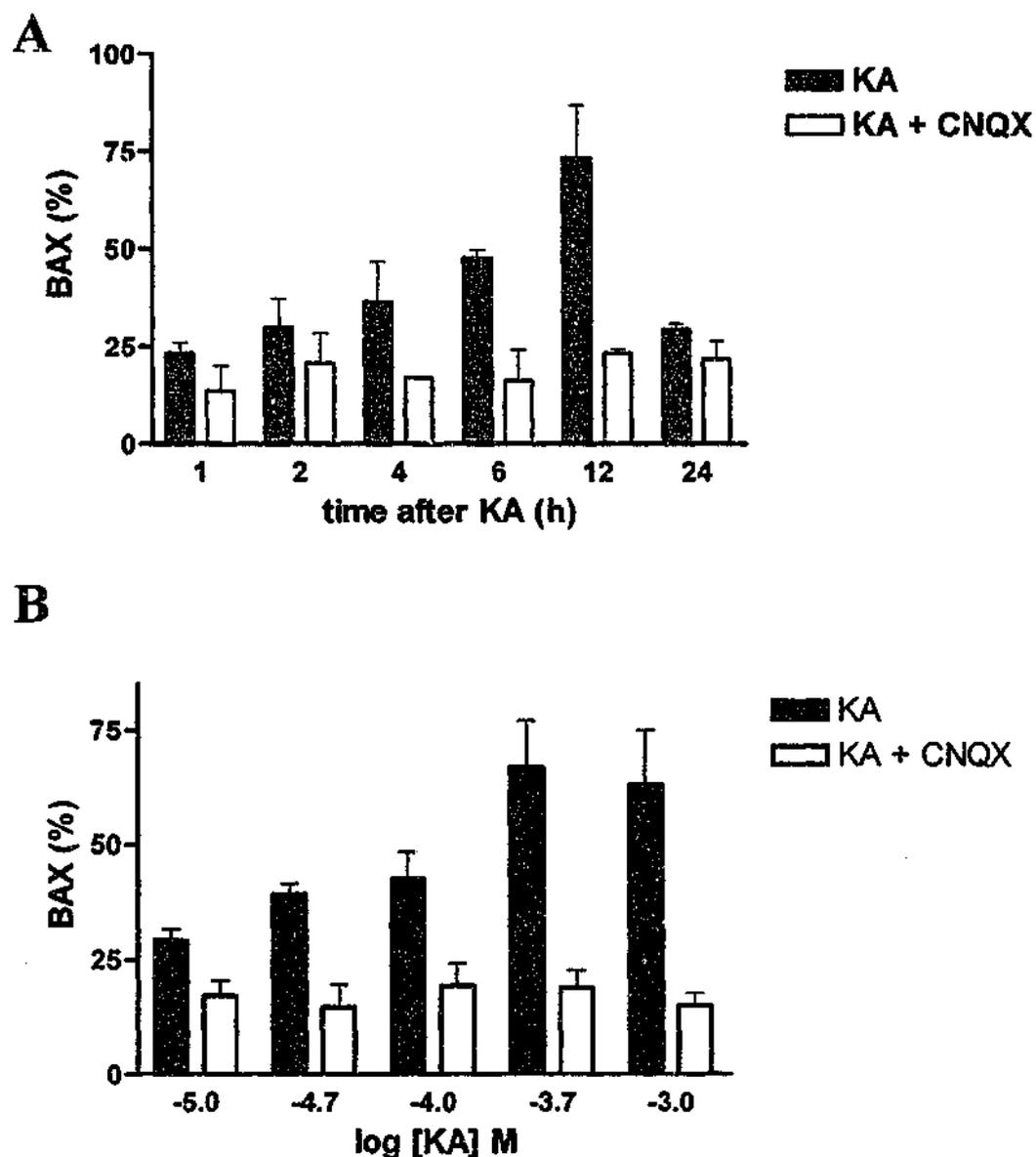
Figure 4.11 Immunocytochemical staining of BAX after KA exposure

Cerebellar granule cells exposed to KA demonstrated a marked increase in BAX protein expression as determined by cell counts for immunopositive cells. KA produced a time-dependent (A; $[F(5,12) = 28.69, p < 0.0001]$ change in BAX expression, with maximal expression after 12h. BAX expression was attenuated by CNQX (50μM; $[F(1,12) = 37.08, p < 0.0001])$, indicating a KA receptor-mediated mechanism. KA exposure (12h; B) resulted in a marked, concentration-dependent, increase in BAX $[F(4,19) = 13.79, p < 0.001]$, that was significantly attenuated by CNQX (50μM; $[F(1,19) = 56.77, p < 0.001])$.

Western blot analyses of BAX expression after KA exposure corresponded with findings from immunocytochemical labeling, with time- and concentration-dependent increases in expression, with maximal expression 12h after KA exposure (Figure 4.12A). In subsequent studies, a KA exposure time of 12h resulted in a concentration-dependent increase in BAX expression as determined by Western blot analysis (Figure 4.12B).

4.3.3 Analysis of the Cell Cycle mRNA After KA Receptor-mediated Apoptosis

In many cases neuronal apoptosis is attenuated by inhibitors of RNA synthesis (Dessi *et al.*, 1994a; Martin *et al.*, 1988), and previous studies have shown for these inhibitors to be effective they must be used no later than 7h after the noxious stimuli, implying the genetic "switch" to apoptosis must occur before this time. In view of the findings with cyclin D1, further studies were undertaken (despite the immunocytochemistry above) to determine if cell cycle activation proceeds beyond the G1/S transition. Examination of cyclins A, B1 and D1 expression by RT-PCR revealed a marked, early increase in the expression of cyclin D1 mRNA, with elevated levels remaining high 1-6h after KA exposure. Negligible change was evident in cyclins A and B1 (Figure 4.13), consistent with findings from immunocytochemical studies with protein expression. No change was evident in the "house keeping" genes GAPDH or β-Actin, suggesting the increase in cyclin D1 expression is selective and may be a good indicator of apoptosis.

0	1	2	4	6	8	12	24

Time after KA (h)

0	1	5	10	50	100	500	1000

KA [µM]
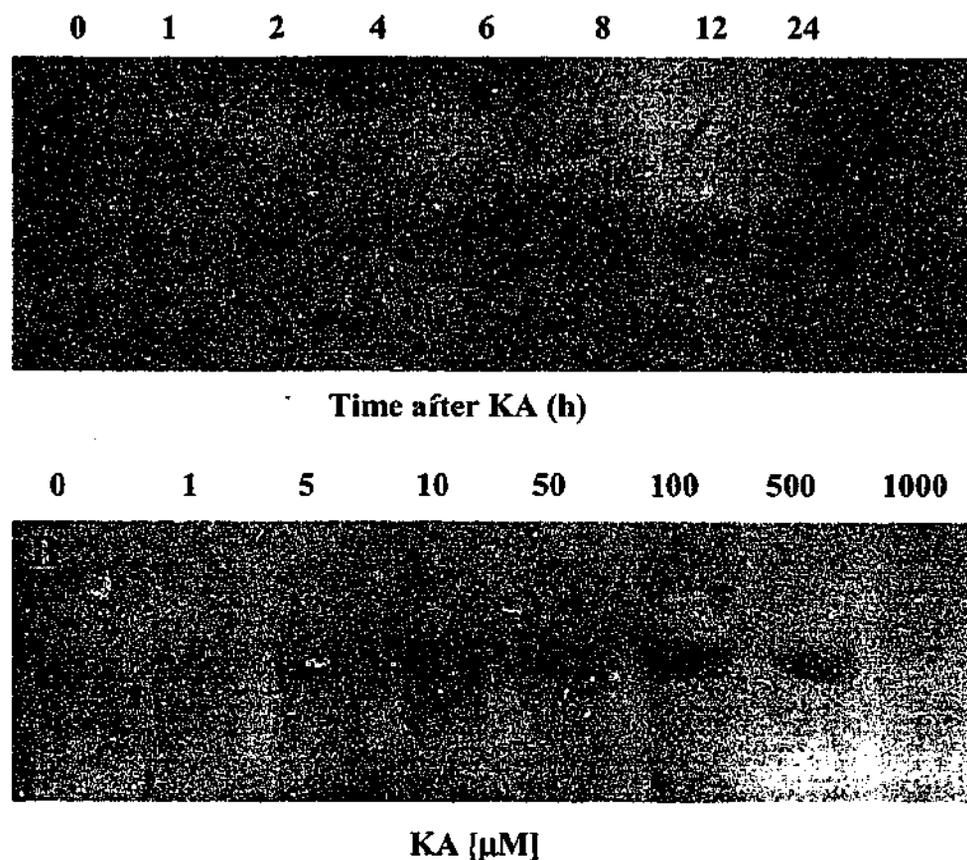
**Figure 4.12 Western Blot analysis BAX expression after KA exposure**

Cerebellar granule cells exposed to KA (100µM) for varying times demonstrated an increase, and time-dependent expression of BAX protein expression as determined by Western Blot analysis (A). Maximal BAX expression was found at 12h after KA exposure and at this time KA-induced a concentration-dependent expression of BAX (B).

Cyclin A

Cyclin B1

Cyclin D1

GAPDH

β-ACTIN

0 1 2 4 6 8 12 24
Time After KA (h)
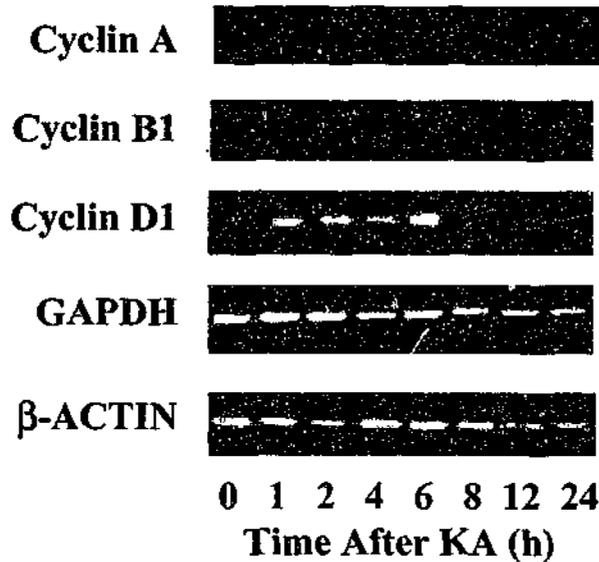
Figure 4.13 RT-PCR analysis of the cell cycle genes after KA exposure

Cerebellar granule cells exposed to KA (100µM) for varying times demonstrated negligible changes in the expression of cyclin A and cyclin B1 over the examined time period (1-24h). Cyclin D1 demonstrated a marked increase in expression 1-6h after KA exposure, with levels returning to essentially baseline values after this time. No change in expression of β-actin or GAPDH was evident over the examined time frame. Results of a single study are shown with very similar findings determined in at least triplicate, independent experiments.

4.4 DISCUSSION

The current study examined the expression patterns of members of the cell cycle genes after exposure to KA. Cultured CGCs are a particularly suitable model system for gene studies as they are a homogenous preparation (Schousboe *et al.*, 1989), and when mitotic inhibitors are employed, such as aphidicolin, the non-neuronal cell population is negligible (Chapter Two). The importance of an insignificant non-neuronal cell population in studies like presented herein, and especially those involved in proliferation, is imperative to avoid false positive results, particularly when highly sensitive procedures like RT-PCR are employed. The present study demonstrated a marked increase in cyclin D1 protein and mRNA expression, with no change in cyclins A and B1, indicating an involvement of the G1/S phase of the cell cycle. Moreover, the effect was CNQX-sensitive indicating a KA receptor-mediated effect. The results from the current study imply while neurones are capable of expressing some cell cycle genes, the neurones are incapable of progressing further through the cell cycle, and is perhaps the reason these cells die.

Neuronal cell death and the subsequent re-entry into the cell cycle has been reported in several recent studies (Timsit *et al.*, 1999; Luo & Miller, 1998; Maas *et al.*, 1998; Nuydens *et al.*, 1998; Park *et al.*, 1997; Shirvan *et al.*, 1997b; Park *et al.*, 1996; Ross, 1996; Herrup & Busser, 1995; Freeman *et al.*, 1994). Cyclin D1 is a member of the D-type of cyclins, which are responsible for progression through the G1 phase of the cell cycle (Ross, 1996). Interestingly, cyclin D1 induction in cell lines is mediated by p53, a proto-oncogene which has previously been associated with KA-mediated neuronal apoptosis (Sakhi *et al.*, 1996; Morrison *et al.*, 1996; Sakhi *et al.*, 1994). Recently, cyclin D expression has been implicated in apoptotic neuroblastoma cell lines, whereby cell death was induced by overexpression of cyclin D1 and attenuated by inhibitors of cyclin D

CDKs (Kranenburg *et al.*, 1996). Since KA induces apoptosis in cultured CGCs (Cheung *et al.*, 1998a), this model is convenient for investigating the relationship between Glu receptor-mediated apoptosis and cell cycle genes. As the pattern of cyclin D1 expression followed that of TUNEL staining, cyclin D1 may be a suitable marker for the early detection of apoptosis, since maximal expression of cyclin D1 occurred after 2h of KA exposure, but apoptosis was not morphologically evident until 12-24h after stimulation. The present results indicates that cyclin D1 expression was markedly elevated during KA receptor-mediated apoptosis in CGCs, consistent with previous findings in apoptotic sympathetic neurons (Freeman *et al.*, 1994). However, studies in post-mortem hippocampus from Alzheimer's patients have failed to demonstrate any significant increases in cyclin D1 levels, but rather increases in cyclin B protein (Nagy *et al.*, 1997), and the brain-specific CDK, CDK5, has been shown to be selectively upregulated in neurones of Alzheimer's diseased brains (Pei *et al.*, 1998). While the upregulation of cyclin D1 has been previously reported (Timsit *et al.*, 1999; Guegan *et al.*, 1997; Kranenburg *et al.*, 1996; Liu *et al.*, 1996; Freeman *et al.*, 1994), loss of cyclin D1 has also been reported in apoptotic and necrotic neurones (Small *et al.*, 1999), a discrepancy most likely due to differences in the neurodegenerative models employed. Other cell cycle genes have been implicated in neuronal apoptosis and differ depending on the type of neurone and the insult. The most convincing evidence to suggest this class of protein is implicated in neuronal apoptosis has come from post-mortem brain tissue from patients suffering various neurodegenerative conditions (Smith *et al.*, 1999; Pei *et al.*, 1998; Nagy *et al.*, 1997; Vincent *et al.*, 1997; Arendt *et al.*, 1996). Since no change was evident in cyclins A and B1, it could be suggested that neurones faced with an apoptotic stimuli attempt to re-enter the cell cycle and hence activate cyclin D1, which mediates the G1/S transition, but die or

become incapable of further progression due to cellular damage or the lack of appropriate proteins to progress further through the cell cycle.

The current study demonstrated a marked time- and concentration-dependent increase in BAX expression, with maximal expression 12h after KA exposure, implying BAX acts downstream of cyclin D1. BAX involvement has been previously been reported in various apoptotic paradigms including low K^+-induced apoptosis in CGCs (Gleichmann *et al.*, 1998; Miller *et al.*, 1997), during normal development and shaping of the nervous system (White *et al.*, 1998; Vekrellis *et al.*, 1997), and in excitotoxic neuronal death (Gillardon *et al.*, 1995). While the relationship between BAX and mitochondria has become somewhat clearer (Desagher & Martinou, 2000; Nicholls & Budd, 2000; Green & Reed, 1998), the actual mechanisms underlying the pro-apoptotic effects of BAX still remain largely unknown. Results from the current study suggest that BAX activation is downstream from cyclin D1, as expression levels become maximal 12h after KA exposure, compared to 2h for cyclin D1. While BAX has been shown to be an essential mediator of apoptosis (Johnson *et al.*, 1998), there is some evidence to suggest the apoptotic pathway is independent of BAX (Gleichmann *et al.*, 1998). While the current study demonstrated a marked concentration- and time-dependent expression of BAX that was CNQX-sensitive, suggesting a KA-receptor mediated response, whether Bcl-2 expression was coincidently reduced is not known. The ratio of Bcl-2 to BAX is thought to be the essential internal signal which determines whether a cell shall survive or die (Chao & Korsmeyer, 1998; Boise *et al.*, 1995; Oltvai *et al.*, 1993), it has been increasingly clear that the Bcl-2-related proteins play an essential role in the maintenance of the permeability transition of the mitochondria (Desagher & Martinou, 2000; Nicholls & Budd, 2000; Chao & Korsmeyer, 1998; Kroemer *et al.*, 1998; Shimizu *et al.*, 1999). Pro-apoptotic members of the Bcl-2 family, including BAX, release cyto c from the mitochondria, which subsequently activates

caspase-9 (Shimizu *et al.*, 1999; Green & Reed, 1998; Rosse *et al.*, 1998). Therefore, it is not surprising that BAX is activated in CGCs undergoing apoptosis, as BAX is central to the apoptotic pathway. There are some studies, however, that have indeed found that apoptosis is independent of BAX expression including DNA damage in CGCs (Araki *et al.*, 1998). However, like the present study, KA causes a marked increase in BAX expression, that is accompanied with a down-regulation of Bcl-2 (Gillardon *et al.*, 1995). In the interpretation of these findings, recent evidence needs to be borne in mind that the "death" cascades involved, and their pattern of recruitment seems to be dependent upon the specific insult (Budd, 2000).

Whether cyclin D1 plays an active role in the intracellular mechanisms that leads to cell death still remains to be elucidated, there is a growing body of evidence to suggest a relationship between apoptosis and mitosis (Ross, 1996). The role BAX plays in neuronal apoptosis, while more established than the role of the cell cycle, still requires further analyses as the activation of downstream events are still to be elucidated. Collectively, the current study demonstrates the KA receptor-mediated neuronal damage is apoptotic in nature and accompanied by an increase in BAX expression and an attempt to re-enter the cell cycle with the expression of cyclin D1. This study demonstrated elevated cyclin D1 expression in post-mitotic neurones in culture during excitotoxic injury suggesting that cyclin D1 may be an appropriate marker for the early detection of neuronal apoptosis. However, from patterns of expression of cyclins A and B1, it appears unlikely, at least in excitotoxic injury *in vitro*, that the cell cycle activation does not proceed further than the G1/S phase.

CHAPTER FIVE

KAINATE INDUCED APOPTOSIS: THE

INVOLVEMENT OF THE

MITOGEN-ACTIVATED PROTEIN AND CYCLIN-

DEPENDENT KINASES

5.1 INTRODUCTION

Neuronal injury mediated by overstimulation of receptors for the major excitatory transmitter, Glu, termed "excitotoxicity", is well documented and has been implicated in a variety of neurodegenerative conditions (Leist & Nicotera, 1998; Lipton & Rosenberg, 1994). Excitotoxic neuronal injury can occur quickly, resulting in lesions in the intact nervous system, or neurones can degenerate more slowly by an apoptotic mechanism, dependent upon the intensity of the insult (Cheung *et al.*, 1998b; Ankarcrona *et al.*, 1995). Exposure to neurotoxic concentrations of Glu generally leads to necrosis via the NMDA receptors (Choi *et al.*, 1988), while overstimulation of the non-NMDA receptors, KA and AMPA, commonly produces a pattern of cell death characteristic of apoptosis (Cheung *et al.*, 1998a; Larm *et al.*, 1997b; Portera-Cailliau *et al.*, 1997b; Chapter Two). Apoptosis, or programmed cell death, is morphologically distinct from necrosis, with neurones losing cellular shape and appearing shrunken, while the neurites break down (known as neurite blebbing). Examination of the DNA from apoptotic cells reveals oligosomal fragmentation, or DNA laddering, where the DNA is digested into fragments of approximately 180bp (Walker & Sikorska, 1994). Biochemically, apoptosis is dependent upon macromolecular and RNA synthesis, suggesting apoptosis requires the activation of various "death genes" to bring about the demise of the cell (Leist & Nicotera, 1998; Oppenheim *et al.*, 1990)

Recently, neuronal apoptosis has been suggested to result from a failed attempt to re-enter the cell cycle (Ross, 1996; Chapter Four). Various genes and/or proteins associated with cell cycle can promote apoptosis or demonstrate altered expression during neuronal apoptosis, the most well studied being p53 (Hughes *et al.*, 1999). Other cell cycle genes have more recently been implicated in neuronal death, namely the cyclins and their catalytic subunits the cyclin-dependent kinases (CDK). Freeman *et al.* (1994) demonstrated

a selective activation of cyclin D1 in sympathetic neurones deprived of nerve growth factor. Studies from our laboratory have also found a marked increase in the expression of cyclin D1 after KA-receptor mediated apoptosis in cultured CGCs (Chapter Four), although, loss of cyclin D1 expression has also been reported in neurones undergoing apoptosis mediated by staurosporine (Small *et al.*, 1999). CDK5 is upregulated in post-mortem brains from Alzheimer's disease patients (Pei *et al.*, 1998), and apoptosis can be attenuated in primary neuronal cultures by overexpressing endogenous inhibitors of CDKs (Park *et al.*, 1998a). Pharmacological evidence to suggest neuronal apoptosis is an abortive attempt to re-enter the cell cycle has come from the use of agents that inhibit various stages of the cell cycle. Inhibition of the G1/S transition can attenuate apoptosis in PC12 cells and sympathetic neurones, whereas agents that block the later stages of the cell cycle are ineffective (Park *et al.*, 1997; Kranenburg *et al.*, 1996; Park *et al.*, 1996a).

Recently MAP kinases have been implicated in neuronal apoptosis (Maas *et al.*, 1998; Walton *et al.*, 1998), in addition to their already established role in differentiation and proliferation (Fukunaga & Miyamoto, 1998; Chapter One). While evidence is confounding, the ERKs are generally thought to mediate anti-apoptotic signaling, while SAPKs; including the JNKs and the p38 MAP kinases, are believed to mediate pro-apoptotic signaling (Xia *et al.*, 1995). While survival of sympathetic neurones is not dependent upon ERK1 or ERK2 activation (Virdee & Tolkovsky, 1995), ERK inhibition may be essential for the execution of apoptosis (Xia *et al.*, 1995). While *c-jun* has been implicated in various apoptotic paradigms (Behrens *et al.*, 1999a; Araki *et al.*, 1998; Beer *et al.*, 1998; Cheung *et al.*, 1998a; Guegan *et al.*, 1997), and *c-jun* dominant-negative mutants attenuate apoptosis in sympathetic neurones (Ham *et al.*, 1995), the involvement of JNK in neuronal apoptosis is less clear. An increase in JNK activity has been reported in growth-factor mediated apoptosis in sympathetic neurones, but suppression of JNK activity

is not sufficient to rescue apoptotic neurones (Virdee *et al.*, 1997). Apoptosis in cultured CGCs has previously been reported to be independent of p38 and JNK (Gunnmoore & Tavare, 1998), although other studies have reported confounding results (Ikeuchi *et al.*, 1998). The most convincing evidence implicating JNK in excitotoxic neuronal death has come from studies utilizing JNK3 knockout mice, where KA-mediated seizures *in vivo* failed to cause apoptosis in hippocampal neurones, coincident with the reduction of *c-jun* phosphorylation (Yang *et al.*, 1997).

Glu activates both the SAPK and p38 MAP kinases in cultured CGCs (Kawasaki *et al.*, 1997), an effect that requires a rise in intracellular Ca^{2+} (Fukunaga & Miyamoto, 1998). Pharmacological inhibition of p38 kinase can reduce the number of dying cells in axotomized retinal ganglion cells (Kikuchi *et al.*, 2000; Castagne & Clarke, 1999) and neuronal apoptosis occurring in *in vitro* models of seizure activity is prevented by ERK kinase inhibitors (Murray *et al.*, 1998). The neuroprotective effects of protein kinase C, have been attributed, in part, to the inhibition of the p38 kinases, but not inhibition of JNK! (Behrens *et al.*, 1999a). KA and AMPA receptor stimulation results in the marked activation of the ERK kinases in oligodendrocytes (Liu *et al.*, 1999) and in striatal slices (Cruise *et al.*, 2000) by a Ca^{2+}-dependent mechanism, providing direct evidence for an association of Glu receptor-mediated activation with the MAP kinases.

Here, the effects of CDK, p38 and MAP kinase (MEK 1/2) kinase inhibitors were examined in primary neuronal cultures exposed to concentrations of KA that result in apoptosis. CGCs provide a unique model system as they are a homogenous preparation with a negligible glial population when cultured under defined conditions in a chemically defined medium (Cheung *et al.*, 1998a), and allowed the examination of the involvement of the cell cycle and the stress kinase cascades in the cellular events contributing to KA receptor-mediated excitotoxic injury.

5.2 METHODS

5.2.1 Materials

KA was purchased from Tocris Cookson (Bristol, UK). Neurobasal™ medium (NBM), B27 nutrients, N2 supplements and Ca^{2+}-free-Hank's balanced salt solution (HBSS) were purchased from GibcoBRL Life Technologies (Melbourne, Australia). All other reagents were purchased from Sigma or Boehringer Mannheim (Sydney, Australia) and were of cell culture or molecular biology grade. Olomoucine, iso-olomoucine, SB203580, PD98059, and roscovitine were purchased from Alexis Biochemicals (CA, USA) or Calbiochem (Sydney, Australia), and U0126 was purchased from Calbiochem (Sydney, Australia). Ciclopirox, silymarin, mimosine, rapamycin and desferrioxamine (DFO) were purchased from Sigma (Sydney, Australia). Flavopiridol was a kind gift from Hoechst Marrion Roussell (UK).

5.2.2 Cell Culture

Primary cultures of CGCs were established as described in Chapter Two. In brief, cells were obtained from day 6-8 Swiss-white mice and exposed to dialysed fetal calf serum for 24h in Neurobasal medium. Cells were seeded at a final density of 0.25 x 10^6 cells/cm^2, and maintained in a humidified incubator at 37°C. From *div* 1 cells were maintained in a serum-free environment in the presence of 2µg/ml aphidicolin, to inhibit non-neuronal cell proliferation (Freeman *et al.*, 1994).

5.2.3 Exposure to KA and Kinase Inhibitors

Initial investigations were carried out to examine the effects of the kinase inhibitors themselves on the viability of the cultures. A range of concentrations were employed according to those used in previous studies (Maas *et al.*, 1998; Kawasaki *et al.*, 1997; Park *et al.*, 1996a) to ensure that no changes in cell viability were evident. Optimal survival of the cultures, with no evidence of loss of cell viability in the presence of the kinase inhibitors, was determined to be a 4h exposure time and therefore KA and the inhibitors were incubated for 4h before being left in drug-free medium overnight. Studies in our laboratory have demonstrated previously that KA (10-1000µM) induces apoptosis (see Chapter Two), coincident with the activation of (Cheung *et al.*, 1998a) and cyclin D1 (see Chapter Four).

Cultures were exposed to KA (10-1000µM) alone or in the presence of flavopiridol (10-1000nM), mimosine (50-600µM), DFO (50-1000µM), ciclopirox (10-1000µM), silymarin (5-500µM), rapamycin (5-500µM), olomoucine (50-600µM), roscovitine (1-100µM), SB203580 (1-100µM), PD98059 (1-100µM) or U0126 (1-100µM) for 4h at 8 *div* in N2 supplemented NBM containing 100U/ml penicillin-streptomycin, 0.25% BSA, 83µM D(+) galactose, 16µM ethanolamine, 6µM L-carnitine, 0.4µM biotin and 25.4mM K^+ (Bottenstein & Sato, 1979). Stock solutions for olomoucine (100mM), U0126 (50mM), PD98059 (50mM), roscovitine (20mM), flavopiridol (20mM) and SB203580 (100mM) were made in 100% DMSO and further diluted as required in N2 medium immediately before use. Silymarin (10mg/ml) and rapamycin (11mM) were diluted in 100% ethanol, whereas mimosine (20mM) was dissolved in 0.1M NaOH. Stock solutions of DFO (50mM) and ciclopirox (20mM) were made in distilled water. The actions of these cell cycle and/or MAP kinase inhibitors are tabulated in Table 5.1. On the basis of preliminary

Table 5.1 Activity of cell cycle and MAP kinase inhibitors

INHIBITOR	ACTION	REFERENCE
desferroxamine	antioxidant, iron chelator	Kulp and Vulliet, 1996
flavopiridol	inhibits CDK5	Losiewicz *et al.*, 1994
iso-olomoucine	inactive form of olomoucine	Kitagawa *et al.*, 1993
olomoucine	inhibits CDC2, CDK2, CDK5, CDK6, ERK, JNK, p38	Vesely *et al.*, 1994
mimosine	iron chelator, antioxidant	Kulp and Vulliet, 1996
rapamycin	reduces cyclin D1 mRNA stability	Hashemolhosseini *et al.*, 1998
roscovitine	CDC2, CDK2, CDK5	Meijer *et al.*, 1997
SB203580	p38 MAPK inhibitor	Cuenda *et al.*, 1995
silymarin	antioxidant, inhibits CDK4, CDK6	Zi *et al.*, 1998
PD98059	MEK 1 and 2 inhibitor	Favata *et al.*, 1998
U0126	MEK 1 and 2 inhibitor	Favata *et al.*, 1998

experiments this injury time would produce a maximal injury representing approximately a 50% reduction in cellular viability, without the inhibitors causing significant toxicity. After 4h the drug containing medium was aspirated and cultures were left in fresh, drug-free N2 media overnight (for approximately 20h).

5.2.4 Morphological changes induced by KA and the Effects of the Kinase Inhibitors

After drug exposure (18-24h) cultures were examined by phase contrast microscopy for morphological changes consistent with apoptosis (cellular shrinkage, neurite blebbing), necrosis (loss of cellular density and the presence of cellular debris) or neuroprotection (relative to control cultures, with the preservation of neurites and cellular shape). All morphological changes induced by KA and the pharmacological inhibitors were visualized by phase contrast microscopy with an Olympus inverted microscope (Olympus, IMT-2).

5.2.5 Determination of Cellular Viability

Cellular viability was determined at 24h by the reduction of MTT (Chapter Two). MTT was incubated with the cells for 30min at 37°C and the reduced formazan product was lysed from the cells in 20% sodium dodecyl sulphate and 40% dimethylformamide, and absorbance was subsequently measured at 590nm (Ceres UV900c microplate reader; Biotek Instruments, USA). Cultures grown in 5.4mM K^+ from *div* 1 were taken as 100% apoptotic cell death and the results were expressed as percentage of control (untreated and

vehicle treated). Vehicle controls were conducted for all agents and included the media they were dissolved in, plus direct exposure to the inhibitors themselves to identify any inherent neuroprotective or neurotoxic actions.

5.2.6 In situ Labeling of DNA Fragmentation

Apoptosis was analysed by TUNEL labeling as previously described (Chapter Two). TUNEL labeling was investigated under the treatment conditions that demonstrated neuroprotection, CGCs were fixed overnight in 4% paraformaldehyde and permeabilized with 2% Triton X-100 (TX-100) in Tris buffered saline (TBS; 50mM Tris, 0.9% NaCl; pH 7.6). Cultures were subsequently washed in TBS and blocked overnight at 4°C in solution with 10% normal goat serum and 0.1% TX-100 in TBS and then incubated with TdT reaction mixture as previously described (Chapter Two) for 3h at 37°C. Digoxigenin labeled dUTP was detected by anti-DIG alkaline phosphatase (AP; 1:1000 dilution) in solution with 2% normal goat serum and 0.1% TX-100 and TBS. TUNEL-positive cells were detected using AP substrate solution (170mM 5-bromo-4-chloro-3-indolyl-phosphate; 60mM nitroblue tetrazolium chloride, 100mM Tris-HCl. 0.5mM MgCl$_2$; pH 9). Control cultures included the above treatment with the omission of TdT. Cells were visualized under bright field microscopy and random and representative fields were photographed. Random cell counts for the kinase inhibitors olomoucine, roscovitine, SB203580, PD98059 and U0126, were taken from 2-6 fields of view and unstandarized data were expressed as percentage of the total number of cells.

5.2.7 Data Analysis

Data are given as mean ± S.E.M. from at least quadruplicate experiments across 2-6 independent cultures and concentration-response curves were generated by non-linear regression using computer-assisted curve fitting (GraphPad Prism™). Statistical significance ($p < 0.05$) of data was examined by one- or two-way ANOVA with a Bonferroni post-hoc test to compare individual treatments.

5.3 RESULTS

5.3.1 KA neurotoxicity: Preliminary Observations

Over a period of 4h exposure to KA, CGCs underwent cellular injury which was concentration-dependent [$F(4,67) = 9.18$, $p < 0.05$]. KA-mediated toxicity ($EC_{50} = 43\mu M \pm 6.7$) was completely attenuated by the non-NMDA receptor antagonist CNQX (50μM; Figure 5.1; [$F(1,67) = 14.88$, $p < 0.0001$]) indicative of a KA-receptor mediated mechanism as AMPA receptors are not functional in CGCs when cultured under the present conditions (Chapter Two). Morphological changes induced by KA were not evident 1-2h after stimulation, in particular cellular swelling was not present, implying negligible necrosis (data not shown). Examination of the cultures by phase contrast microscopy 20-24h after KA exposure revealed morphological characteristics consistent with apoptosis, with cellular shrinkage and neurite blebbing evident (Figure 5.2) as previously observed (Chapter Two). However, longer exposure times to some of the CDK and MAPK inhibitors resulted in cellular loss and therefore a 4h exposure time was employed, where the kinase inhibitors alone produced no significant neuronal loss, and KA
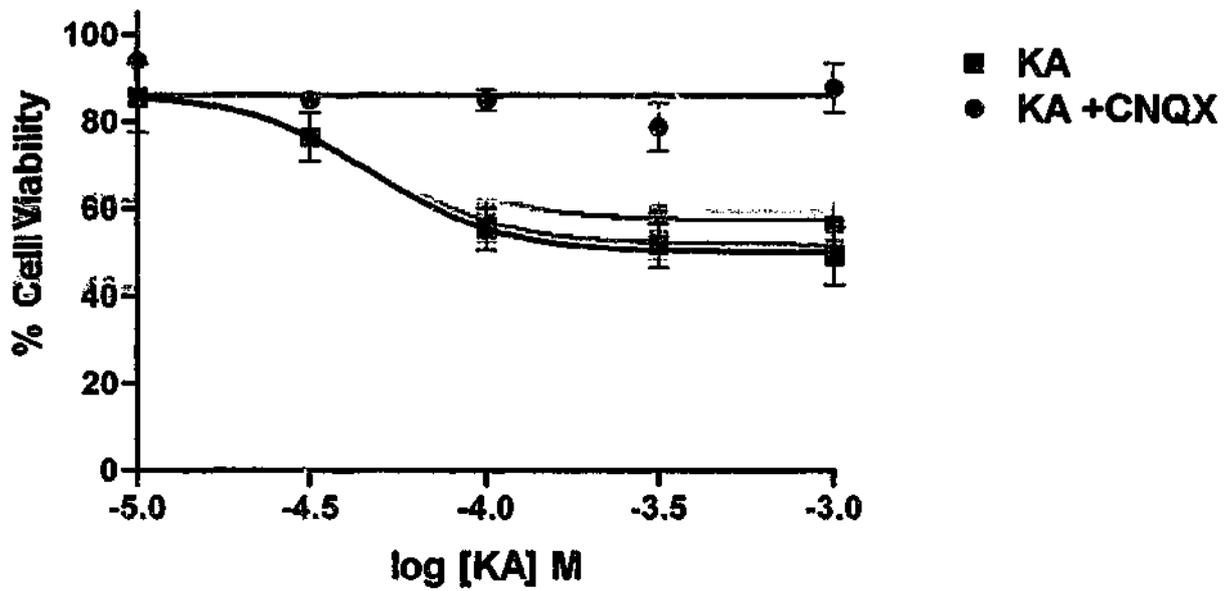
Figure 5.1 KA receptor-mediated neurotoxicity

KA receptor mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At div 8 cultures were exposed for 4h to KA (10-1000μM). Cell injury was found to be concentration-dependent and completely attenuated by CNQX (50μM). Data were standardized relative to vehicle treated (100% cell viability) and low K^+ treated cultures (100% apoptotic cell death), and are plotted as mean ± SEM and were from 6-8 replicate cultures.
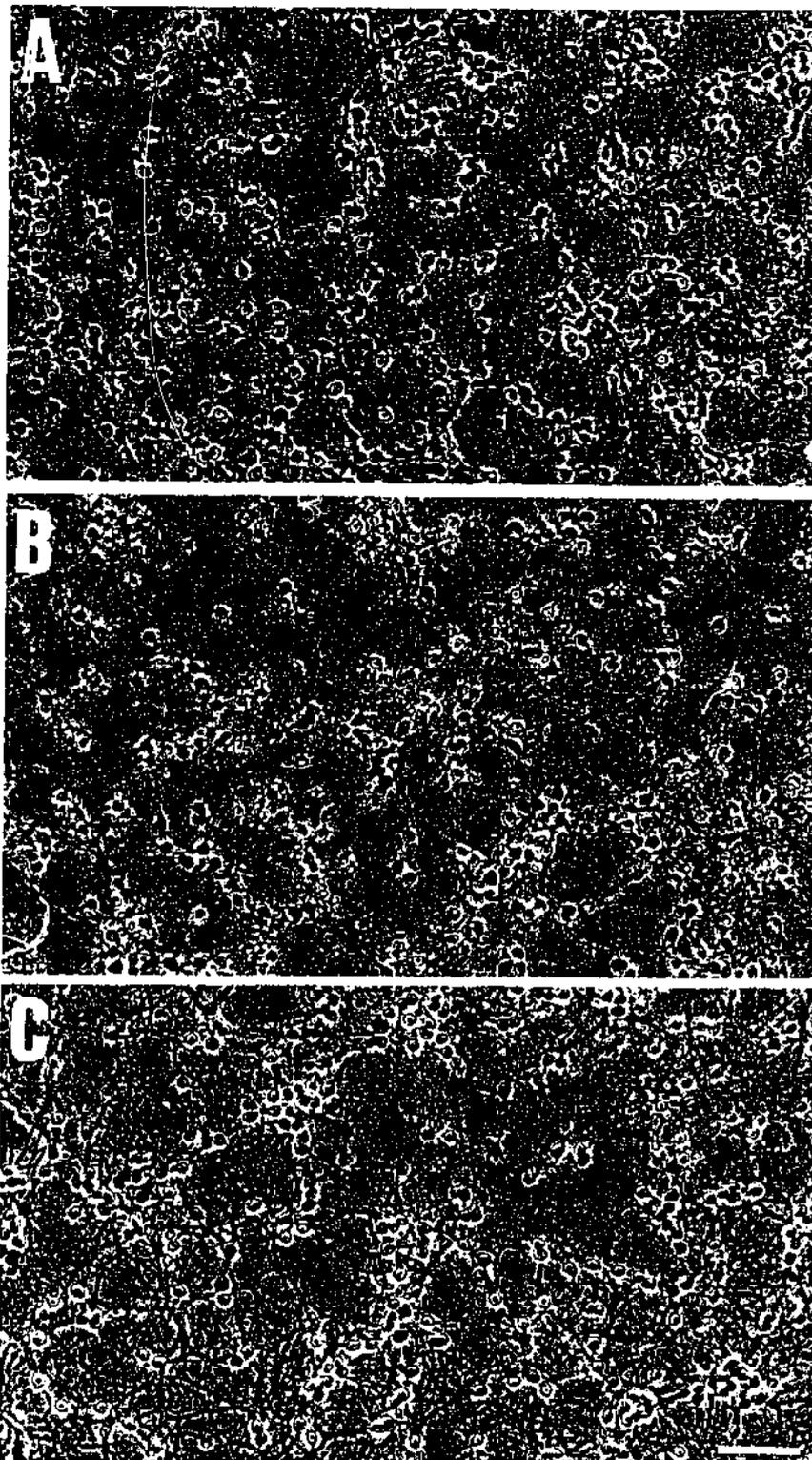
Figure 5.2 Morphology of KA receptor-mediated neurotoxicity

Cerebellar granule cells were exposed to KA for 4h in N2 medium, before the drug-containing media being aspirated and left in drug-free media overnight. KA exposure resulted in neuronal injury that was similar to that found for the longer exposure times used in Chapter Two (24h), with cells demonstrating a shrunken appearance and neurite blebbing evident (100µM; B), when compared to vehicle control (A). CNQX (50µM; C) completely attenuated neuronal injury mediated by KA. Scale bar represents 10µm.

exposure resulted in a reduction in cellular viability with the characteristics of apoptosis. Therefore for routine experimentation, cultures were exposed to KA for 4h in the absence and presence of kinase inhibitors, before the medium containing the drugs was completely aspirated, and left in drug free N2 medium overnight.

5.3.2 Kinase Inhibitors and Inherent Effects on Cellular Viability: Morphological Observations

While the majority of kinase inhibitors had little or no effect on the morphology of the cultures some had adverse effects, particularly after longer exposure times ($> 4h$). Figure 5.3 demonstrates motphological changes induced by the kinase inhibitors in the absence of KA. While morphological changes were not evident in cultures treated with olcmoucine (600µM; Figure 5.3B), roscovitine (100µM; Figure 5.3C), PD98059 (100µM; Figure 5.3D), SB203580 (100µM; Figure 5.3E), flavopiridol (1µM; Figure 5.3F), rapamycin (data not shown), DFO (1mM; data not shown) or mimosine (600µM; Figure 5.3G) some toxicity was evident after exposure to U0126 (100µM; Figure 5.3H), silymarin (500µM; Figure 5.3I), and ciclopirox (500µM; Figure 5.3J), when compared to vehicle treated cultures (Figure 5.3A). Toxicity induced by the kinase inhibitors, when evident, was concentration-dependent as determined by MTT (data not shown) and was largely necrotic in nature, as determined by the loss in cellular density and the early swelling of cells. Some apoptotic cells were evident in higher concentrations of U0126, however the intensity of the insult was largely reduced by the shorter exposure time (4h).
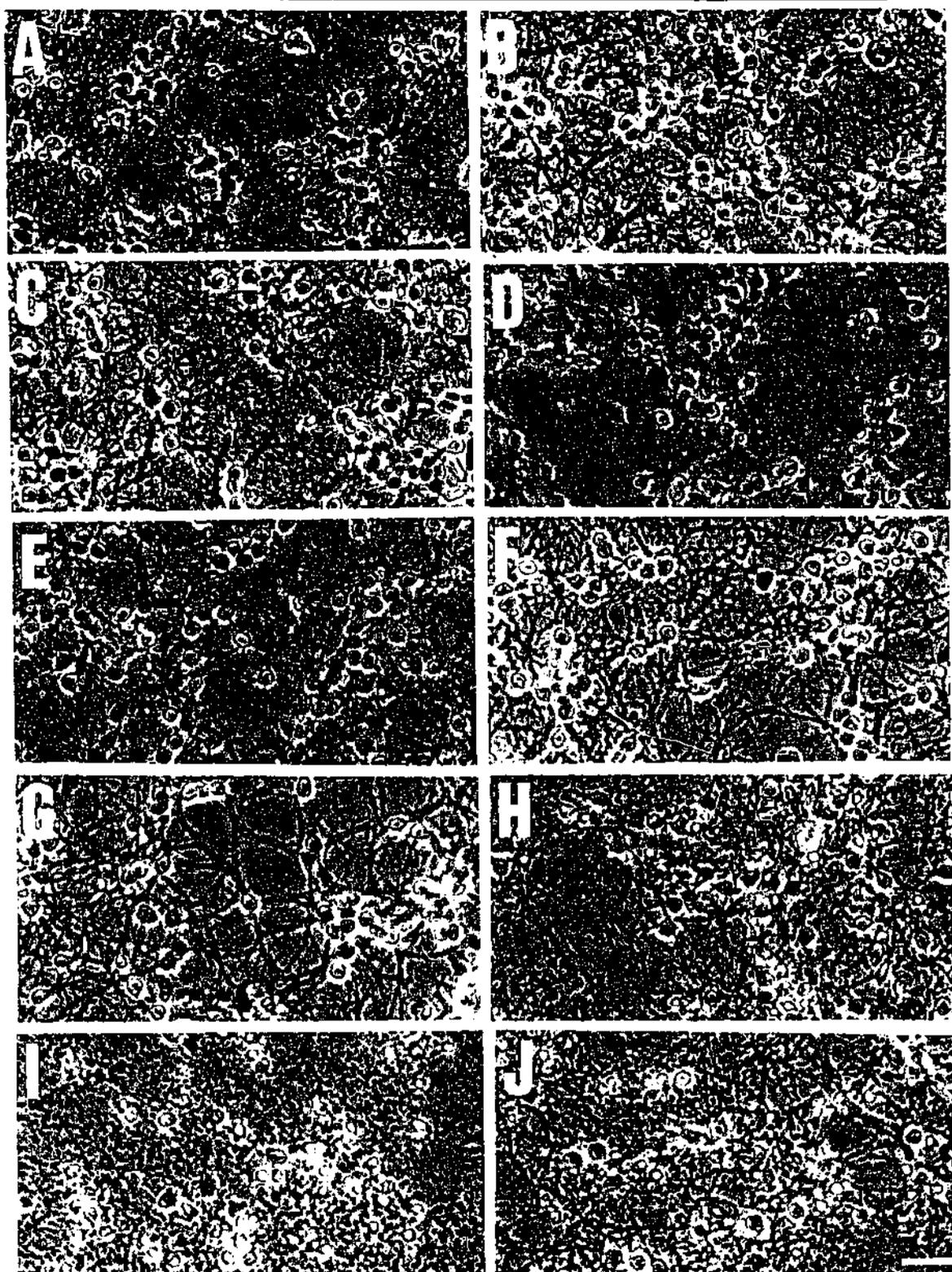
Figure 5.3 Morphological changes induced by kinase and cell cycle inhibitors
Primary cultures of CGCs were exposed to kinase inhibitors on day 8 *in vitro*. Phase contrast photomicrographs, presented here in the absence of KA, demonstrate any inherent neuroprotective or neurotoxic properties of the compounds. While no morphological changes are evident in cultures treated with olomoucine (600μM; B), roscovitine (100μM; C), PD98059 (100μM; D), SB203580 (100μM; E), flavopiridol (1μM; F) or mimosine (600μM; G) some toxicity is evident after exposure to U0126 (100μM; H), silymarin (500μM; I), and ciclopirox (500μM; J), compared to control cultures (A). Scale bar represents 5μm.

5.3.3 Kinase inhibitors and KA Neurotoxicity: Cellular Viability Studies

In biochemical investigations, olomoucine produced a trend towards increasing cellular viability in cells not treated with KA, although this action was not statistically significant [$F_{(5,43)} = 0.165$, $p > 0.05$]. This action of olomoucine, likely to be due to decreased background cell death, was confirmed by observations in the presence of KA where data for cell viability were consistently above vehicle control values (Figure 5.4A). Olomoucine (200-600μM) exerted a concentration-dependent neuroprotection against KA-mediated neurotoxicity [$F_{(5,246)} = 27.56$, $p < 0.0001$], with cellular viability returning to 100% and higher, and with the lower concentrations of olomoucine only partially attenuating neurotoxicity. Iso-olomoucine, the inactive form of olomoucine, had no significant effect on KA-mediated toxicity ([$F_{(5,128)} = 4.10$, $p = 0.539$]; Figure 5.4B). Roscovitine partially, but significantly attenuated KA-induced neurotoxicity [$F_{(5,426)} = 4.21$, $p < 0.05$], but not as effectively as olomoucine, and was completely ineffective at 100μM (Figure 5.5). SB203580 significantly attenuated KA receptor-mediated neurotoxicity, particularly at lower concentrations of KA [$F_{(5,276)} = 5.85$, $p < 0.0001$], and induced no toxicity of its own, even at 100μM (Figure 5.6). The MEK inhibitor PD98509 effectively attenuated KA receptor-mediated toxicity [$F_{(5,276)} = 7.24$, $p < 0.0001$], and was concentration-dependent however, most attenuation of KA receptor-mediated toxicity occurred at lower concentrations of KA (Figure 5.7A). The pharmacological profile of U0126 was the most complex and cellular viability showed more variability when compared to those of the other MAP kinase inhibitors. U0126 was effective at attenuating KA receptor-mediated neurotoxicity at concentrations 1, 5 and
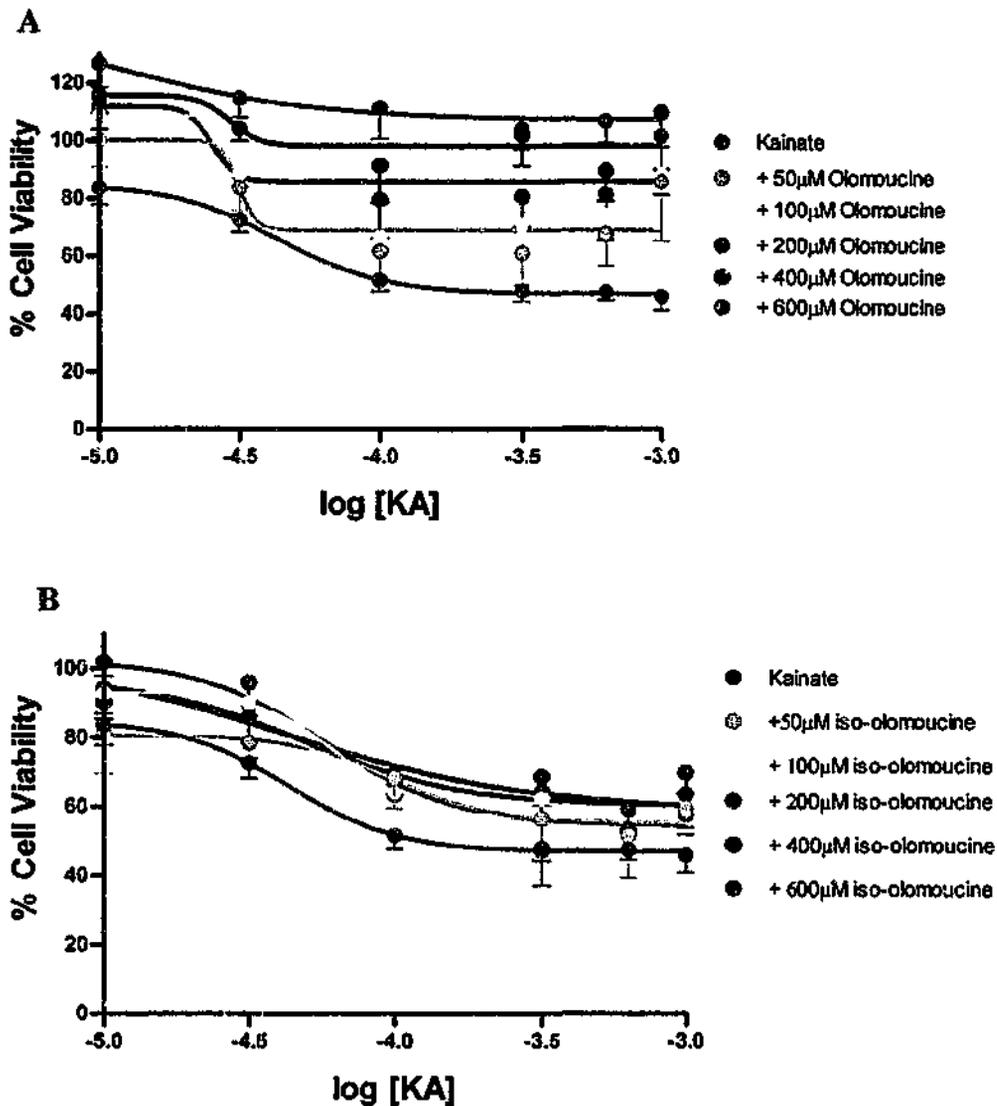
Figure 5.4 The effect of olomoucine on KA receptor-mediated toxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000µM) in the presence of olomoucine (50-600µM; A) or the inactive form iso-olomoucine (50-600µM; B). Olomoucine exerted a marked neuroprotection against KA-mediated neurotoxicity $[F(5,246) = 27.56, p < 0.0001]$. Iso-olomoucine had no significant effect on KA-mediated toxicity $[F(5,128) = 4.10, p = 0.539]$.
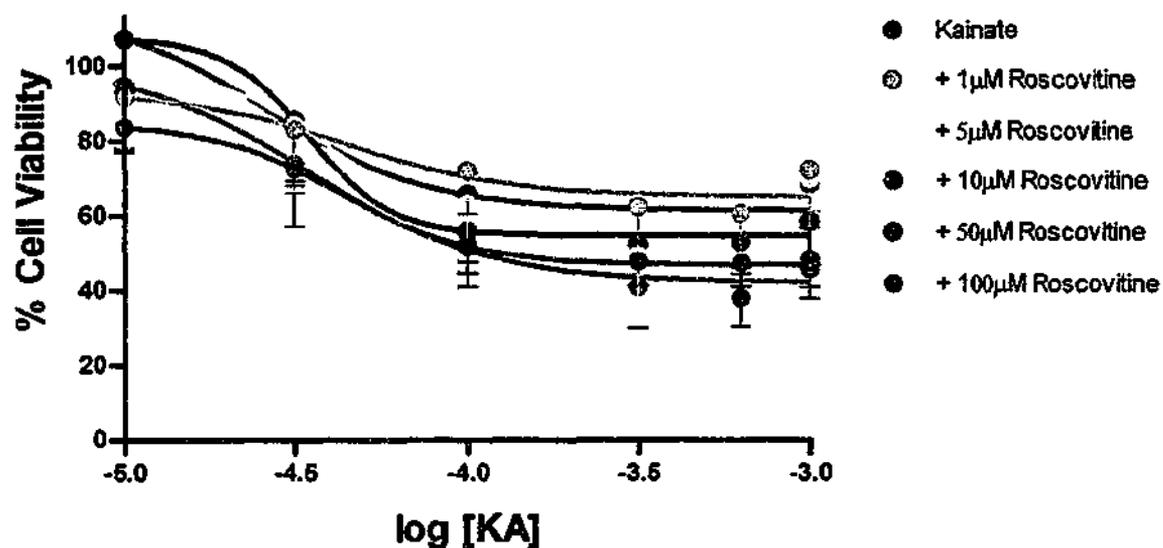
Figure 5.5 The effect of roscovitine on KA-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells and the effect of roscovitine (1-100μM) was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000μM) in the presence of roscovitine. Roscovitine partially, but significantly, attenuated KA-induced neurotoxicity [$F_{(5,426)}$ = 4.21, $p < 0.05$] and was ineffective at 100μM.
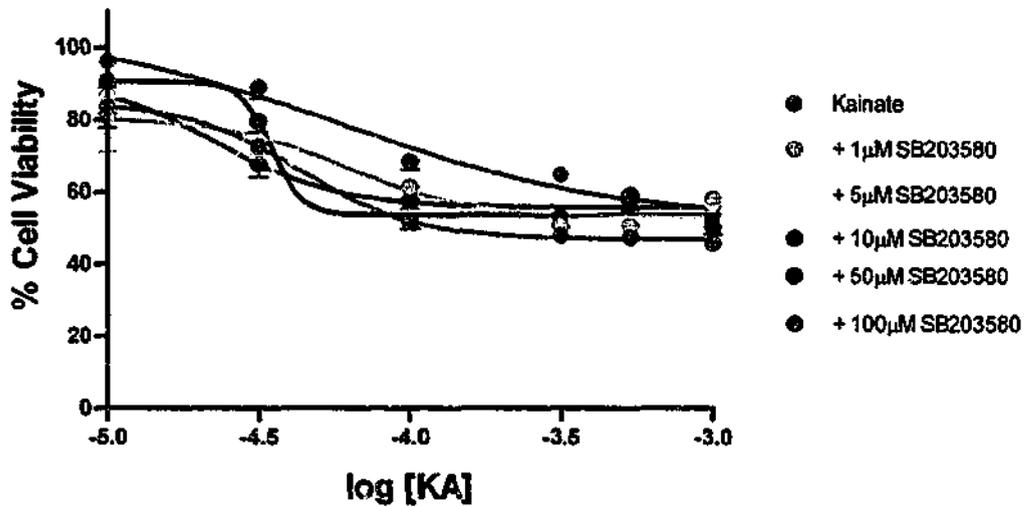
Figure 5.6 The effect of SB203580 on KA-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells and the effect of SB203580 (1-100μM) was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000μM) in the presence of SB203580. SB203580 partially, but significantly, attenuated KA-induced neurotoxicity [$F(5,276) = 5.85$, $p < 0.0001$], particularly at lower concentrations of KA.
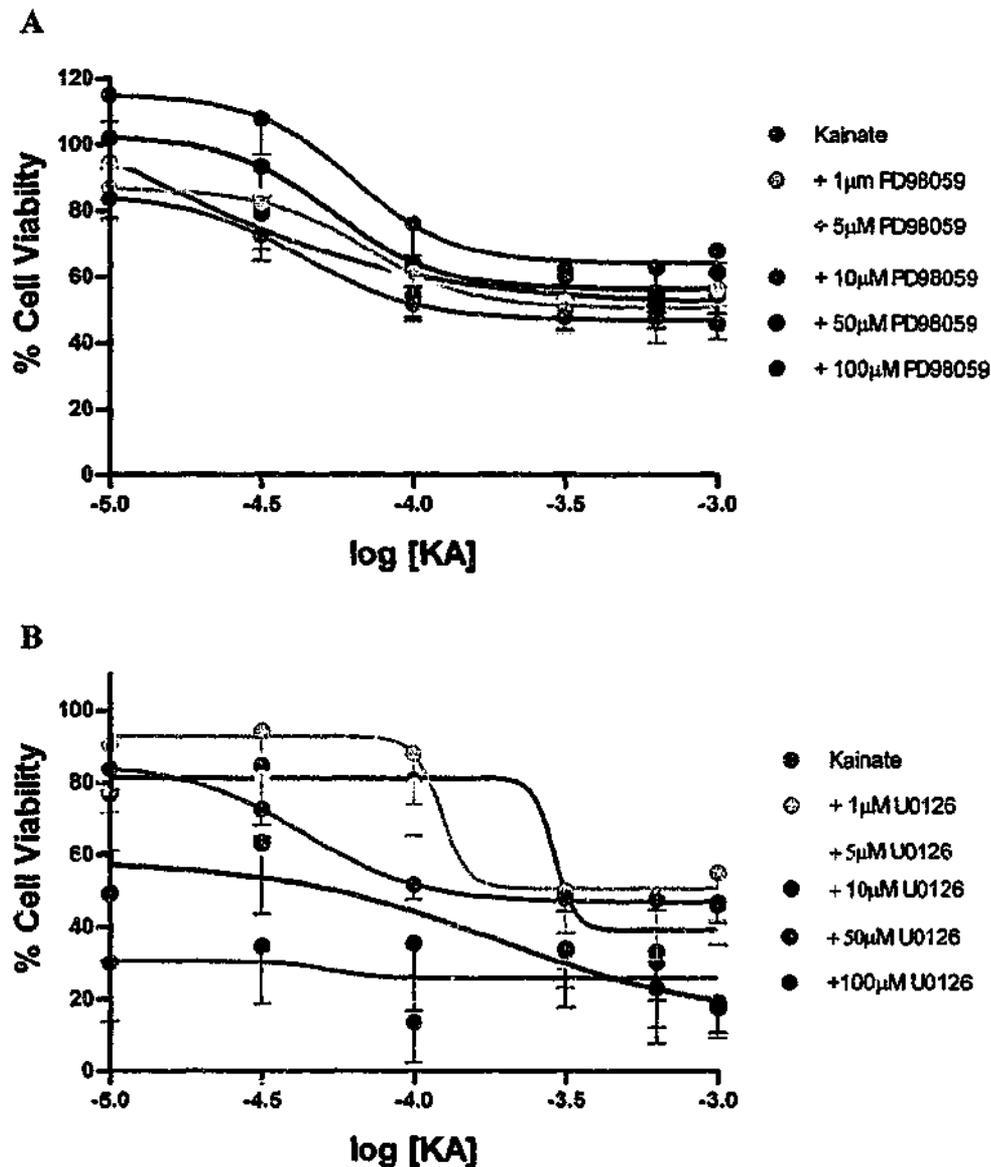
Figure 5.7 The effect of MEK inhibitors on KA receptor-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000µM) in the presence of the MEK inhibitors PD98059 (1-100µM; A) and U0126 (1-100µM; B). PD98059 effectively attenuated KA receptor-mediated toxicity [$F(5,276) = 7.24$, $p < 0.0001$], although its inhibitory action was not concentration-dependent, with most attenuation occurring at lower concentrations of KA. The pharmacological profile of U0126 was the most complex and cellular viability showed more variability. U0126 was effective at attenuating KA receptor-mediated neurotoxicity at concentrations 1, 5 and 10µM [$F(5,206) = 17.1$, $p < 0.0001$]. Treatment with 50 and 100µM U0126 resulted in a marked reduction in cellular viability, exacerbating KA-induced toxicity.

$10 \mu M$ [$F(5,206) = 17.1$, $p < 0.0001$]. Treatment with 50 and $100 \mu M$ U0126 resulted in a marked reduction in cellular viability, exacerbating KA-induced toxicity (Figure 5.7B).

While previous studies have demonstrated neuroprotection against a variety of apoptotic insults using the cell cycle inhibitors mimosine, silymarin, ciclopirox and DFO (Park *et al.*, 1997; Farinelli & Greene, 1996;), the present study failed to find any significant neuroprotection against KA-mediated neurotoxicity using these agents. Mimosine exposure (50-600μM; Figure 5.8), resulted in no significant neuroprotection against KA receptor-mediated neurotoxicty [$F(5,210) = 1.26$, $p = 0.4741$], however a trend towards neuroprotection was evident with $600 \mu M$ mimosine at lower concentrations of KA (> $100 \mu M$). Rapamycin (50-500μM; Figure 5.9) produced no significant change in KA receptor mediated toxicity [$F(5,210) = 3.00$, $p = 0.1067$], however some evidence of neuroprotection was evident, even at higher concentrations of KA (< $300 \mu M$). Silymarin (Figure 5.10) caused widespread necrosis at $500 \mu M$, and at other concentration (5-100μM) had no significant effect on KA-receptor mediated neurotoxicity, [$F(4,180) = 2.14$. $p = 0.1226$]. Ciclopirox (10-1000μM; Figure 5.11) significantly reduced cell viability, augmenting KA-mediated toxicity [$F(5,210) = 61.69$. $p < 0.0001$], suggesting ciclopirox was a potent neurotoxin. DFO (50-1000μM; Figure 5.12) a potent iron chelator (Kulp and Vulliet, 1996), did not alter the response to KA, demonstrating no toxicity of its own, nor neuroprotection against KA-induced injury [$F(5,210) = 1.85$, $p = 0.3721$]. Flavopiridol (10-1000nM; Figure 5.13), a novel CDK5 inhibitor, although previously shown to be a potent neuroprotectant (Stefanis *et al.*, 1999; Park *et al.*, 1996a), only slightly improved cell viability in cultures treated with KA [$F(5,246) = 3.37$, $p < 0.05$].
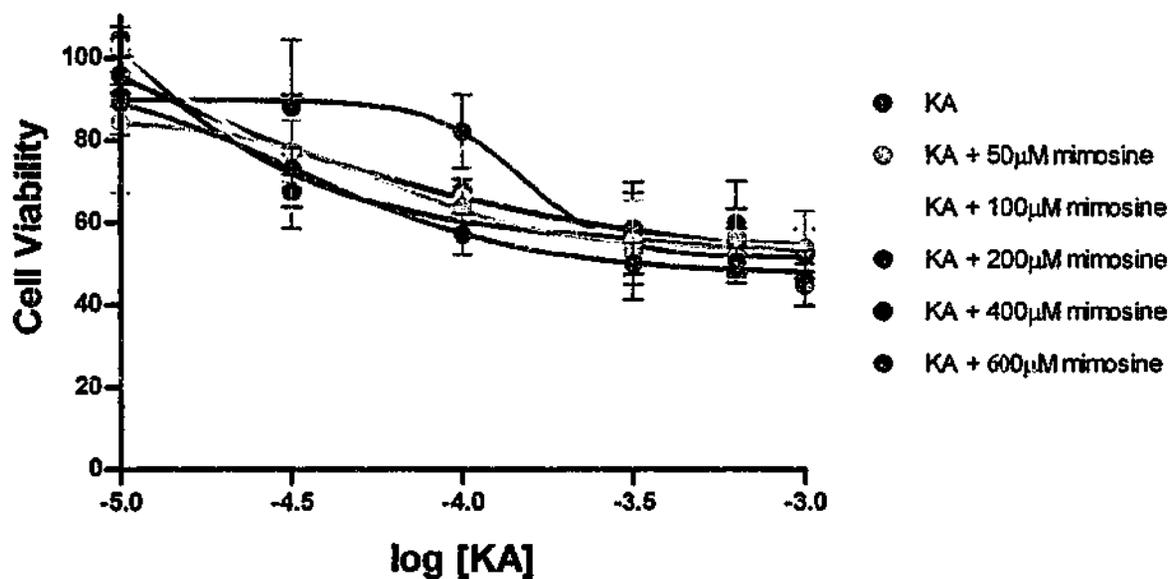
Figure 5.8 The effect of mimosine on KA receptor-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000μM) in the presence of mimosine (50-600μM). Mimosine exposure (50-600μM) resulted in no significant neuroprotection against KA receptor-mediated neurotoxicity [$F(5,210) = 1.26$, $p = 0.4741$], however a trend towards neuroprotection is evident with 600μM mimosine at lower concentrations of KA (> 100μM).
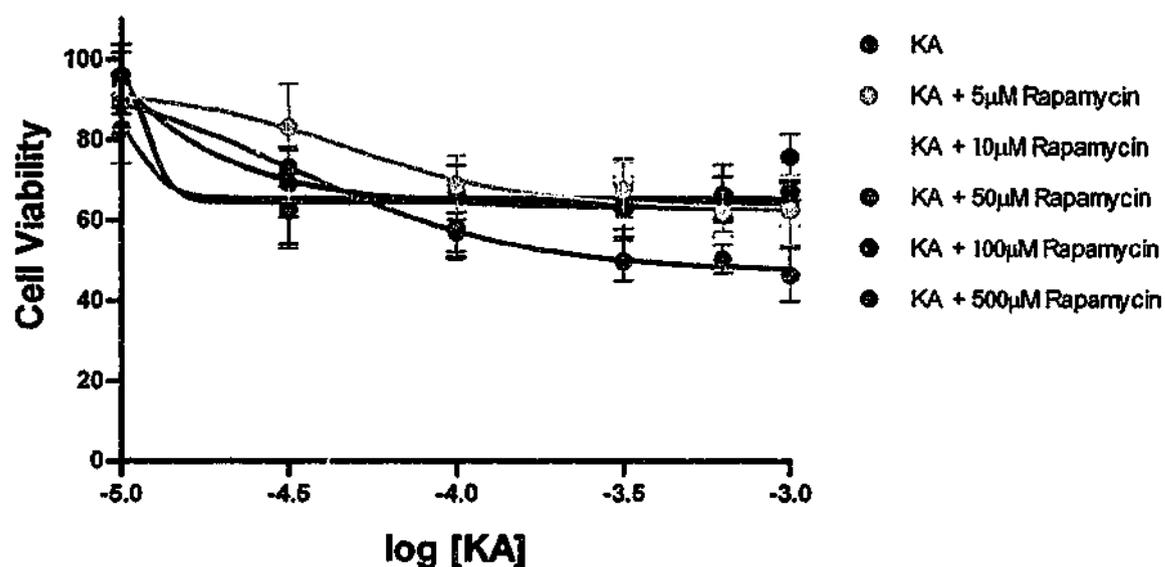
Figure 5.9 The effect of rapamycin on KA receptor-mediated neurotoxicity

KA receptor mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000µM) in the presence of rapamycin (50-500µM). Rapamycin produced no significant change in KA receptor mediated toxicity [$F(5,210) = 3.00$, $p = 0.1067$], however a trend towards neuroprotection was evident, even at higher concentrations of KA.
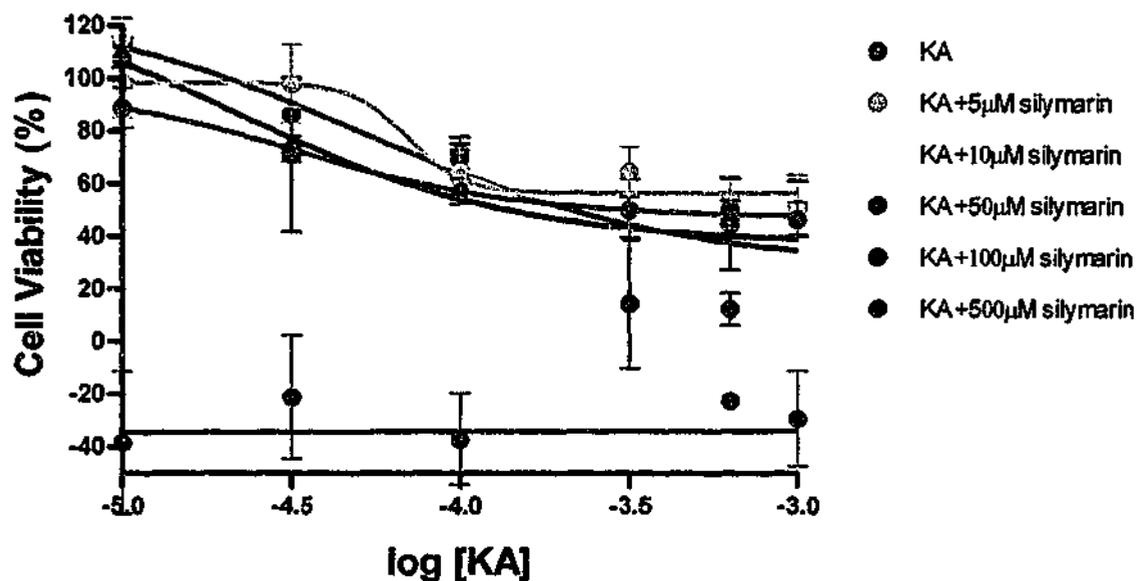
Figure 5.10 The effect of silymarin on KA receptor-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000µM) in the presence of silymarin (5-500µM). Silymarin produced no significant change in KA receptor mediated toxicity [$F_{(4,180)} = 2.14$, $p = 0.1226$], however a trend towards neuroprotection is evident at the milder insults (< 100µM KA). Treatment with 500µM silymarin, produced a widespread neuronal loss.
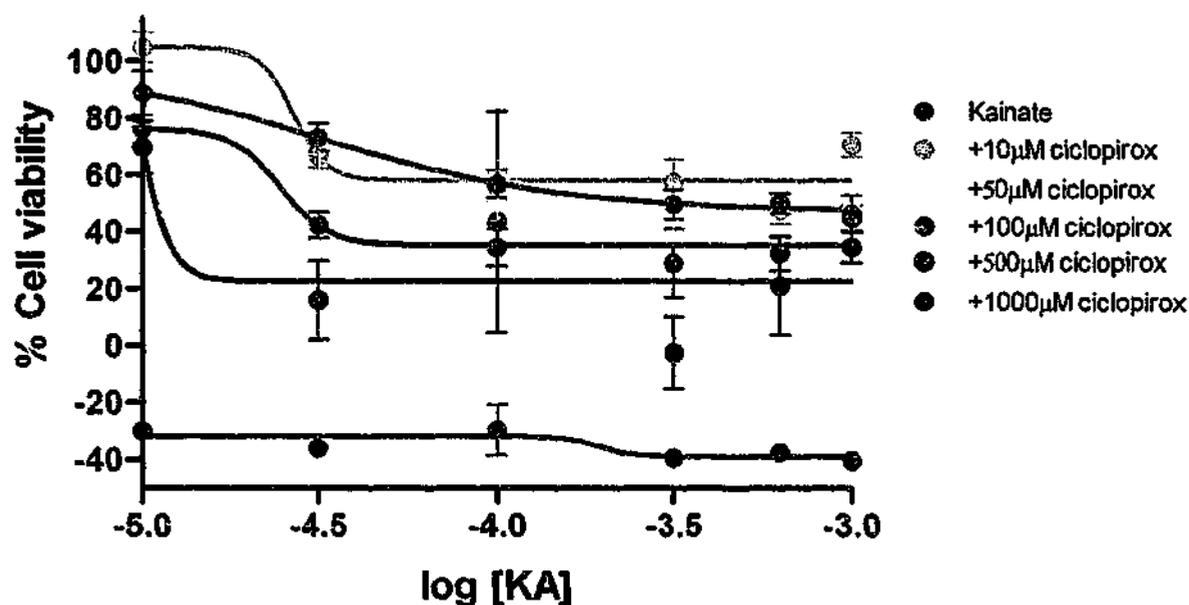
Figure 5.11 The effect of ciclopirox on KA receptor-mediated neurotoxicity

KA receptor mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000µM) in the presence of ciclopirox (10-1000µM). Ciclopirox (1000µM) significantly reduced cell viability, below 0% cell viability, augmenting KA-mediated toxicity [$F_{(5,210)}$ = 61.69. $p < 0.0001$], and suggesting that it was a potent neurotoxin.
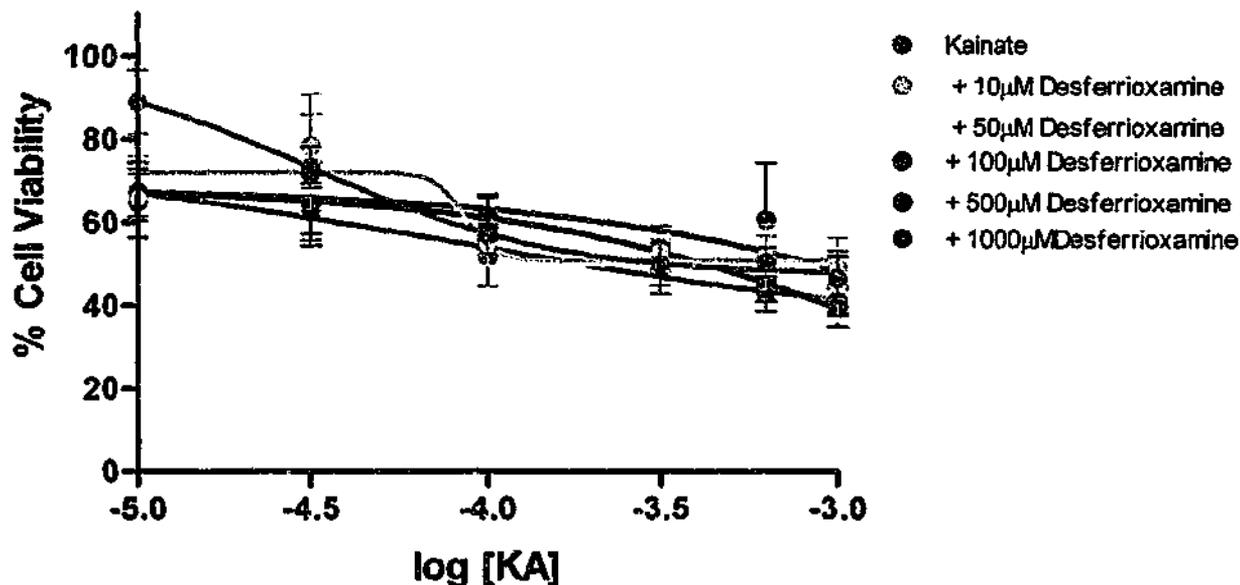
Figure 5.12 The effect of DFO on KA receptor-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000μM) in the presence of desferrioxamine (DFO; 50-1000μM). DFO did not alter the response to KA, nor did it demonstrate any inherent neurotoxicity or neuroprotection [$F(5,210) = 1.85, p = 0.3721$].
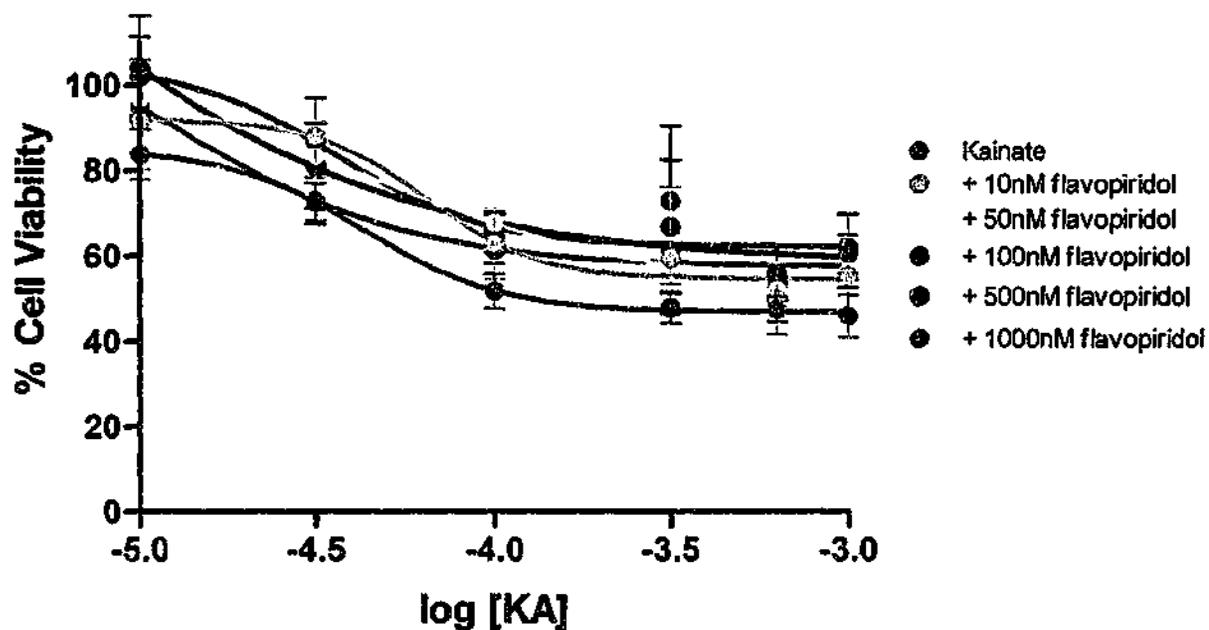
Figure 5.13 The effect of flavopiridol on KA receptor-mediated neurotoxicity

KA receptor-mediated neurotoxicity in cultured cerebellar granule cells was determined using the MTT cell viability assay. At *div* 8 cultures were exposed for 4h to KA (10-1000μM) in the presence of flavopiridol (10-1000nM). Co-exposure with flavopiridol resulted in a significant reduction in loss of cell viability induced by KA, [$F_{(5,246)} = 3.37$, $p < 0.05$], however some toxicity was still evident.

5.3.4 Kinase Inhibitors and KA Neurotoxicity: Morphological Observations

Treatment with olomoucine (400µM; Figure 5.14C) completely attenuated, in an apparently concentration-dependent manner, all morphological changes induced by KA (100µM, Figure 5.14B), which included neurite blebbing and shrunken cell bodies. Iso-olomoucine (400µM; Figure 5.14D), the inactive form of olomoucine (Havlicek *et al.*, 1997), produced no changes when compared to cultures treated with KA alone. The other compounds demonstrated more complex actions on the cultures and observations did not always correlate with the viability data. Roscovitine, surprisingly did not attenuate KA-induced neurotoxicity to the same extent as olomoucine, observations in agreement with biochemical findings. Roscovitine has previously been shown to potently attenuate neuronal apoptosis induced by withdrawal of trophic support (Maas *et al.*, 1998). While the present study showed that neurones treated with roscovitine demonstrated less morphological changes indicative of apoptosis, unlike sister cultures treated with olomoucine, some cellular damage was still evident (50µM; Figure 5.15C). The p38 MAP kinase inhibitor, SB203580 (1-100µM), slightly improved the cellular viability of the cultures, particularly at higher concentrations (50-100µM) and in cultures treated with < 100µM KA (100µM; Figure 5.16C), with some preservation of neurites and cellular shape. Interestingly, the MEK inhibitors PD98509 and U0126, differed in their ability to attenuated KA-mediated neurotoxicity. While PD98509 was effective at attenuating morphological changes induced by KA exposure (10-100µM; Figure 5.17E) without inducing toxicity of its own (50µM; Figure 5.17B), concentrations of U0126 (> 50µM) induced cellular loss (100µM; Figure 5.17C). Lower concentrations of U0126, were
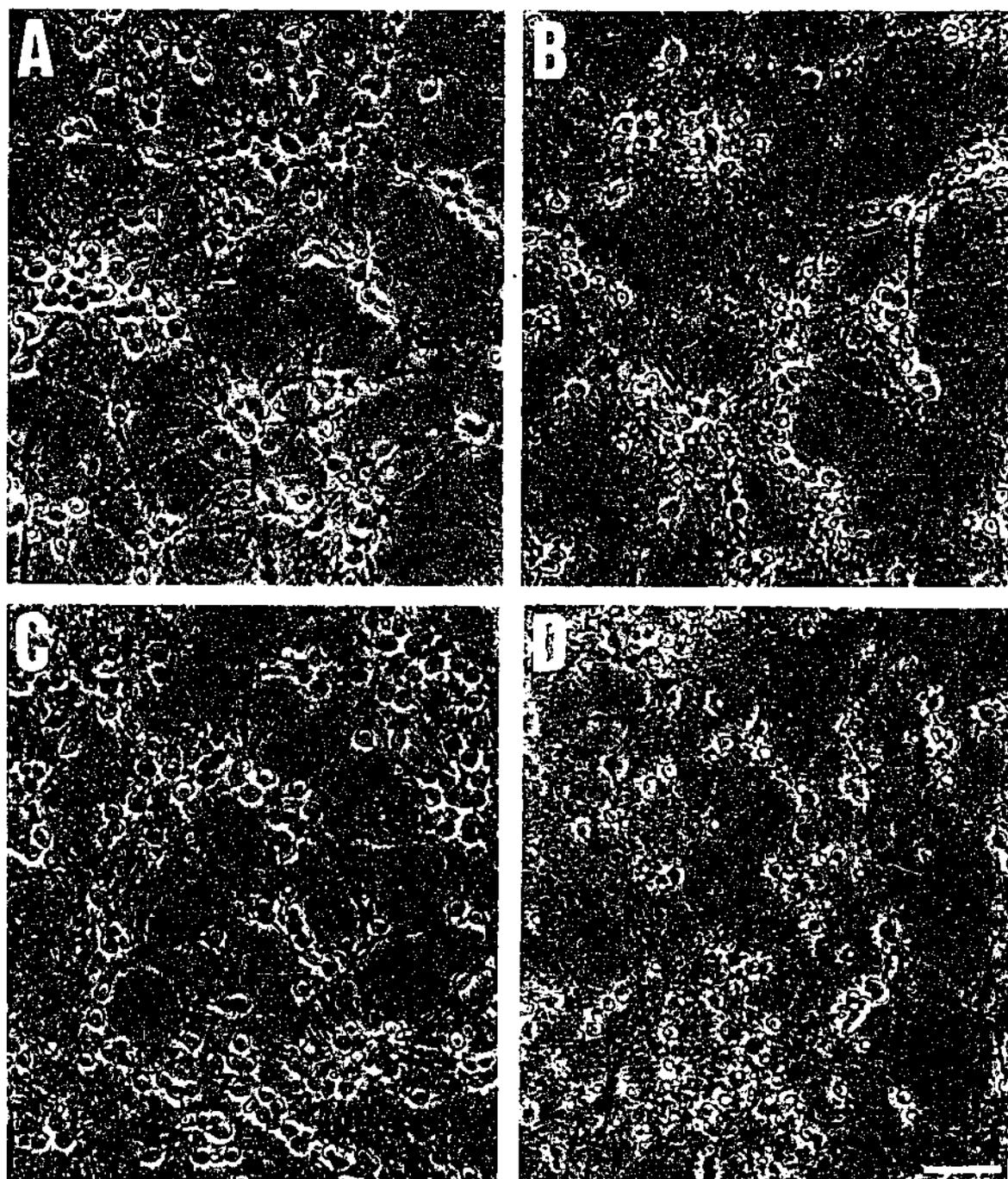
Figure 5.14 The effect of olomoucine on KA receptor-mediated neurotoxicity

Cultures treated with KA in the presence and absence of olomoucine and iso-olomoucine were examined using phase-contrast microscopy. Photomicrographs represented here show treatment with olomoucine (400μM; C) completely attenuated all morphological changes induced by KA (100μM; B), so that cultures looked no different from untreated controls (A). Iso-olomoucine (400μM; D), the inactive form of olomoucine, produced no changes when compared to cultures treated with KA alone. Scale bar represents 10μm.
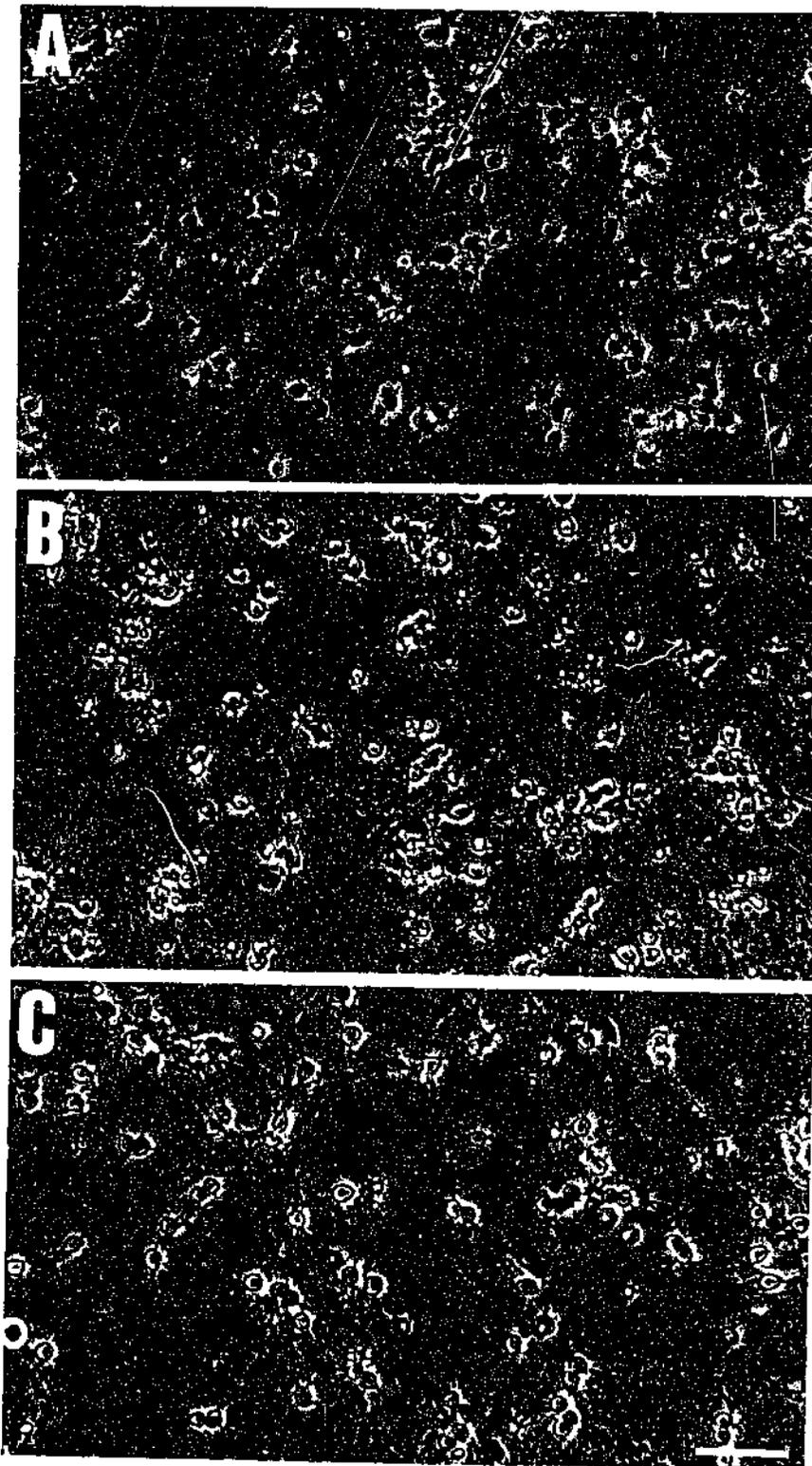
Figure 5.15 The effect of roscovitine on KA receptor-mediated neurotoxicity

Cultures treated with KA in the presence and absence of roscovitine were examined using phase-contrast microscopy. Photomicrographs represented here demonstrate a slight reduction in neuronal injury in the presence of roscovitine (50μM; C) compared to KA treatment alone (100μM; B), however cultures still demonstrate neuronal injury when compared to control (A). Scale bar represents 10μm.
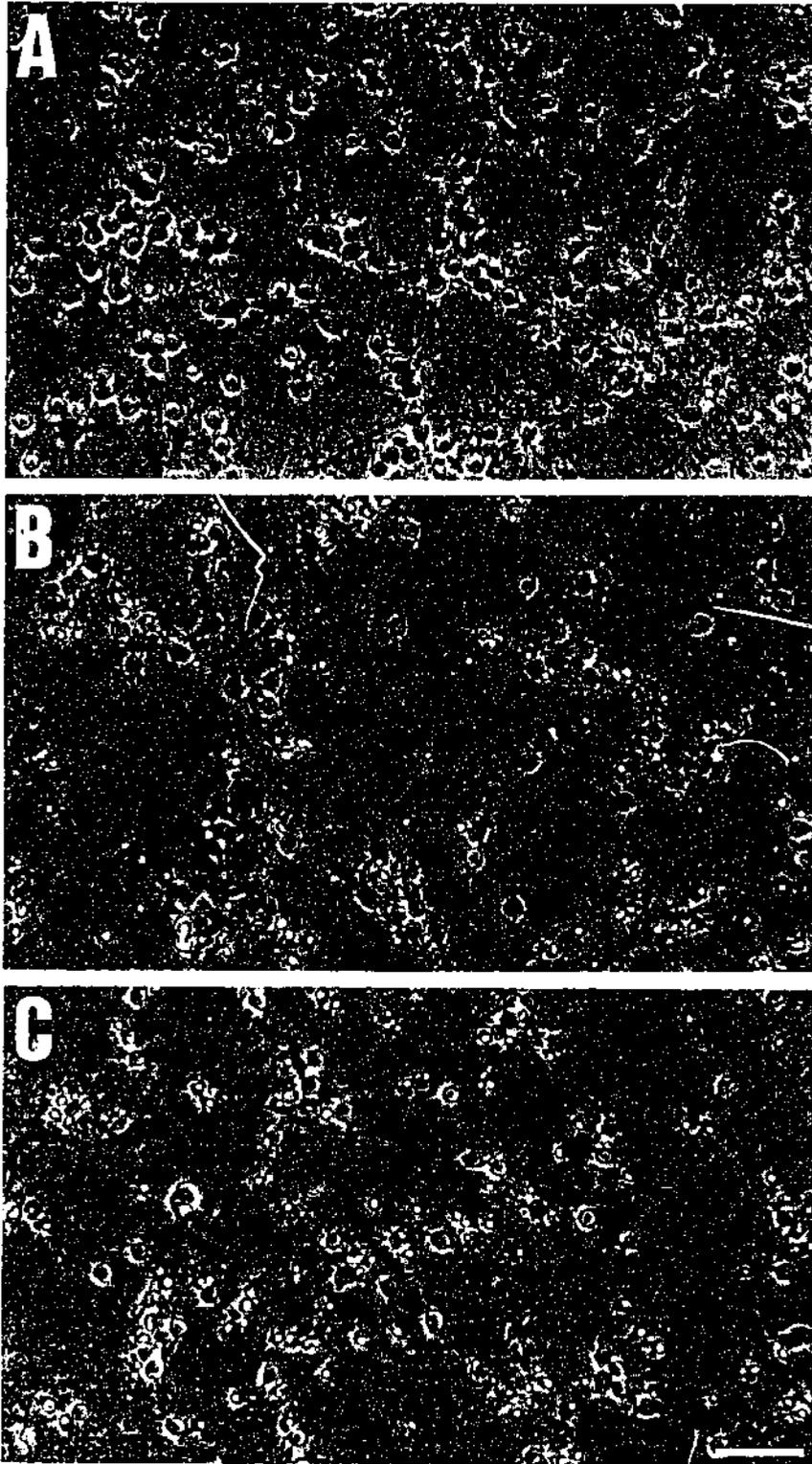
Figure 5.16 The effect of SB203580 on KA receptor-mediated neurotoxicity

Cultures treated with KA in the presence and absence of SB203580 were examined using phase-contrast microscopy. Photomicrographs represented here demonstrate a slight reduction of neuronal injury in KA treated cells co-exposed to the p38 MAP kinase inhibitor, SB203580 (100μM; C), compared to KA treated alone (B) and control (A). Scale bar represents 5μm.
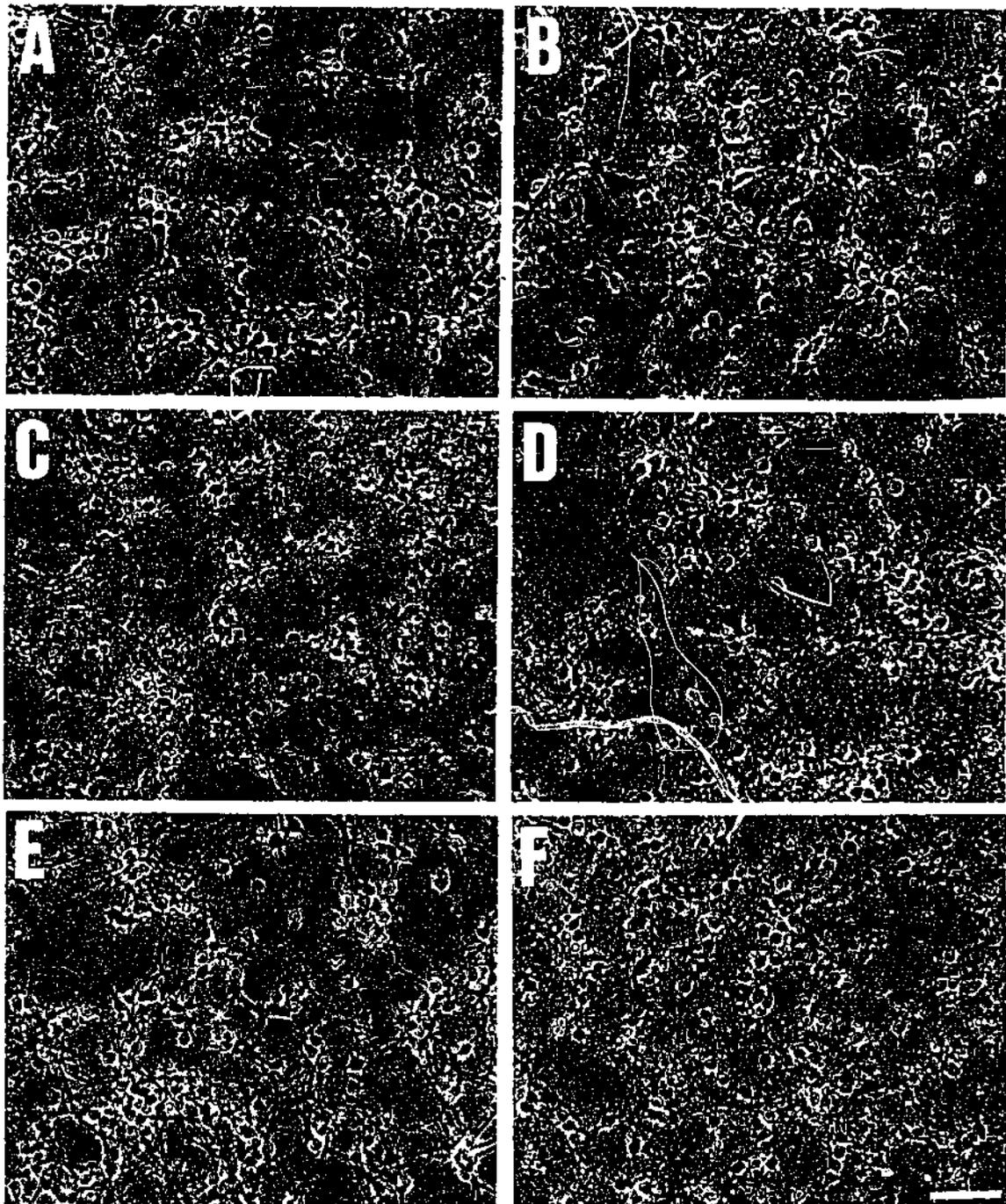
Figure 5.17 The effect of PD98059 and U0126 on KA receptor-mediated neurotoxicity
Cultures treated with KA in the presence and absence of the MEK inhibitors were examined using phase-contrast microscopy. The MEK inhibitors PD98509 and U0126, differed in their ability to attenuated KA-mediated neurotoxicity. While PD98509 (50μM; E) was effective at attenuating morphological changes induced by KA exposure (100μM; D) without inducing toxicity of its own (50μM; B), compared to untreated cultures (A), higher concentrations of U0126 (100μM; C) induced some cellular loss. However, lower concentrations of U0126 (10μM), were effective at attenuating cellular shrinkage and neurite blebbing associated with KA-mediated cell death (F). Scale bar represents 10μm.

effective at attenuating cellular shrinkage and neurite blebbing associated with KA-mediated cell death (100μM; Figure 5.17F). Detailed morphological analysis of the actions of the other compounds were not undertaken because of their lack of effect as determined by MTT. Exposure of CGCs to the vehicles, either 1% DMSO or 2% ethanol. had little effect on the morphological integrity of the cultures.

5.3.5 *Kinase Inhibitors and KA Receptor-mediated Apoptosis*

KA receptor-mediated neurotoxicity under the present experimental conditions occurs almost exclusively by apoptosis (Cheung, *et al.*, 1998a; Chapter Two) and to determine whether the protective effects of the kinase inhibitors, as determined by the cell viability assay, were reflected by attenuation of apoptosis TUNEL labeling was employed. Data from the use of this molecular revealed extensive TUNEL-positive profiles amongst KA treated cells (100μM; Figure 5.18B) when compared to vehicle treated cultures (Figure 5.18A), consistent with observations made under phase-contrast microscopy. Treatment with olomoucine (200μM; Figure 5.18C) completely attenuated apoptotic labeling, while the inactive form iso-olomoucine did not reduce TUNEL labeling (Figure 5.18D). Roscovitine treatment in the presence of KA resulted in some decrease of the incidence of TUNEL labeling (Figure 5.18E), however when compared to control some apoptotic labeling was still evident. SB203580 (50μM; Figure 5.18F) partially attenuated apoptotic labeling, except at the higher concentrations of KA (1mM; data not shown). PD98509, like SB203580, decreased KA-mediated TUNEL labeling, particularly at the lower concentrations of KA, but not to control values (Figure 5.18G). U0126, while decreasing TUNEL labeling at certain concentrations (1-10μM), failed to exert a major effect on KA-mediated apoptosis and many TUNEL-positive cells were still evident (Figure 5.18H).
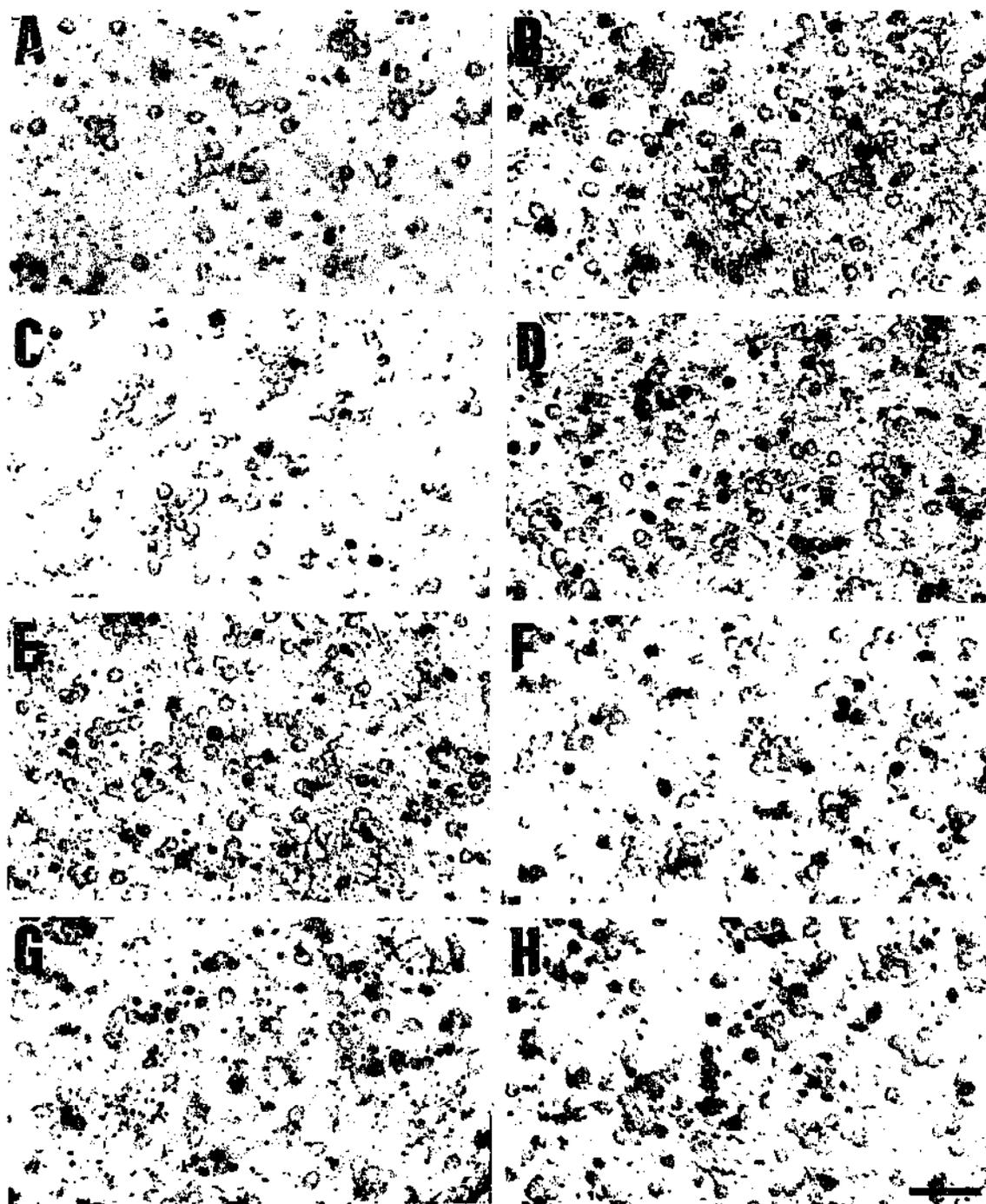
Figure 5.18 The effect of kinase inhibitors on KA receptor-mediated apoptosis
The mode of cell death induced by KA primary cultures of cerebellar granule cells was determined using the TUNEL technique. Bright field photomicrographs from representative fields of neurones exposed to KA (300μM) revealed a marked increase in TUNEL positive profiles (B) when compared to vehicle treated controls (A). Olomoucine (400μM) attenuated TUNEL labeling induced by KA treatment (C), however iso-olomoucine (400μM) was ineffective (D). The other kinase inhibitors only partially attenuated KA-mediated TUNEL labeling. While some apoptotic cells were still evident, fewer TUNEL positive cells are present when compared to KA treated alone cultures, including roscovitine (100μM; E), SB203580 (100μM; F), PD98059 (100μM; G) and U0126 (50μM; H). Scale bar represents 10μm.

Cell counts for TUNEL were conducted for those inhibitors that demonstrated significant, or a strong trend towards, neuroprotection in the MTT cell viability assays and by morphological examination. Cell counts for TUNEL labeling generally followed the trends seen in MTT data for selected kinase inhibitors. The best correlation among the various kinase inhibitors was seen with olomoucine, which produced the greatest attenuation of TUNEL-positive staining induced by KA $[F_{(5,83)} = 85.48, p < 0.0001]$ and attenuated KA-mediated apoptosis even at low concentrations (Figure 5.19). While roscovitine significantly attenuated TUNEL-labeling, it was less effective than olomoucine $[F_{(5,83)} = 13.52, p < 0.0001]$ (Figure 5.20). SB203580 significantly decreased KA-mediated TUNEL labelling $[F_{(5,77)} = 10.106, p < 0.001)$, however closer examination of individual KA treated groups revealed no concentrations of SB203580 were statistically significant when compared to cultures treated with KA alone (Figure 5.21). PD98509 and U0126 significantly reduced TUNEL labeling $[F_{(5,65)} = 10.875, p < 0.0001]$ and $[F_{(5,72)} = 7.39, p < 0.0001]$, respectively (Figure 5.22 and Figure 5.23, respectively). TUNEL counts did not entirely correlate with MTT data, with no significant attenuation at 100μM of KA in the presence of the MEK inhibitors observed for TUNEL counts, despite an increase in cell viability as determined by MTT.

5.4 DISCUSSION

The present findings using cultures of primary murine CGCs and pharmacological inhibitors of the cell cycle, CDK and MAPK, demonstrated for the first time an involvement of these intracellular signaling cascades in KA-receptor mediated apoptosis. KA treated CGCs demonstrated the morphological characteristics of apoptosis, with shrunken cell bodies and the degeneration of neurites. TUNEL analysis revealed a marked
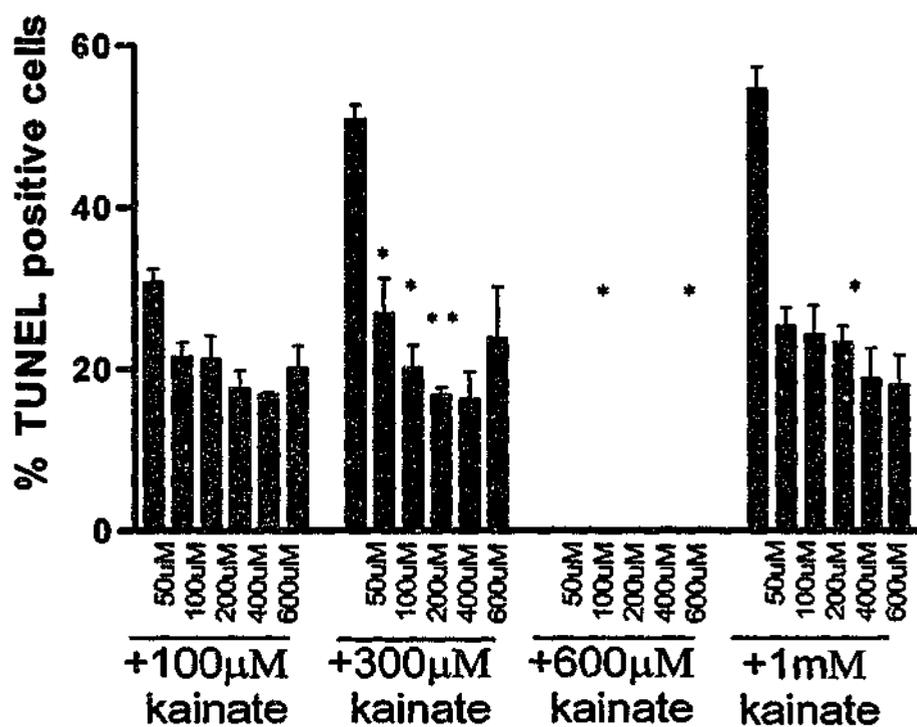
Figure 5.19 The effect of olomoucine on KA receptor mediated apoptosis

Primary cultures of cerebellar granule cells were exposed to KA on *div* 8. Apoptosis was assessed using the TUNEL technique, which labels the nick end DNA fragments. Increasing concentrations of KA increased the number of TUNEL positive cells, implying KA concentration-dependently induced apoptosis. Olomoucine (50-600µM) significantly attenuated KA-mediated TUNEL labeling $[F(5,83) = 85.48, p < 0.0001]$. Data are unstandardized and are plotted as mean ± SEM and were from 2-3 independent cultures. * represents statistical significance, $p < 0.05$, one-way ANOVA.
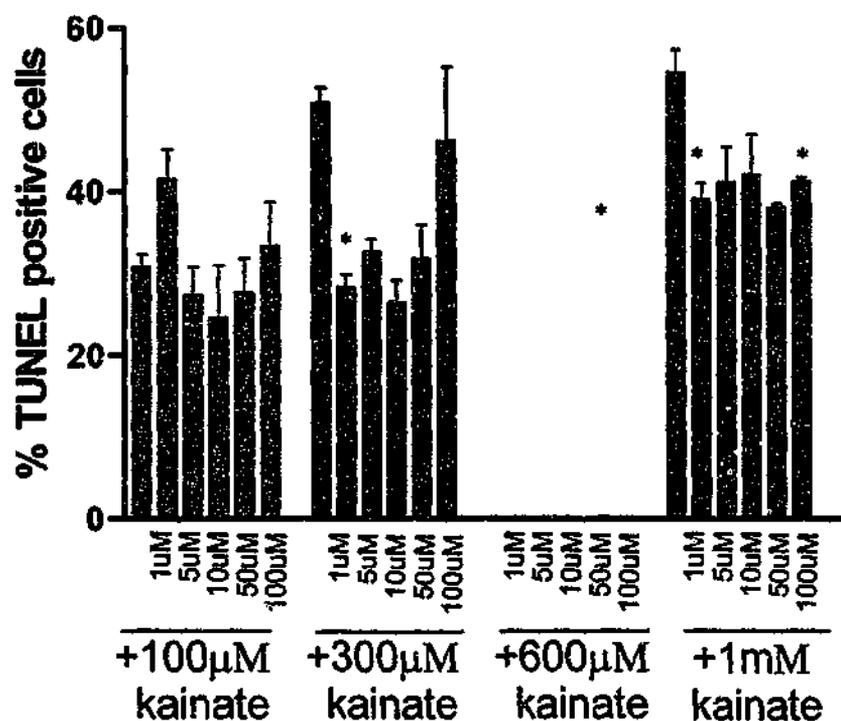
Figure 5.20 The effect of roscovitine on KA receptor apoptosis

Primary cultures of cerebellar granule cells were exposed to KA on *div* 8. Apoptosis was assessed using the TUNEL technique, which labels the nick end DNA fragments. Increasing concentrations of KA increased the number of TUNEL positive cells, implying KA concentration-dependently induced apoptosis. Roscovitine (1-100μM) produced an overall neuroprotection [$F(5,83) = 13.52$, $p < 0.0001$], however not all concentrations were statistically significant. Data are unstandardized and are plotted as mean ± SEM and were from 2-3 independent cultures. * represents statistical significance, $p < 0.05$, one-way ANOVA.
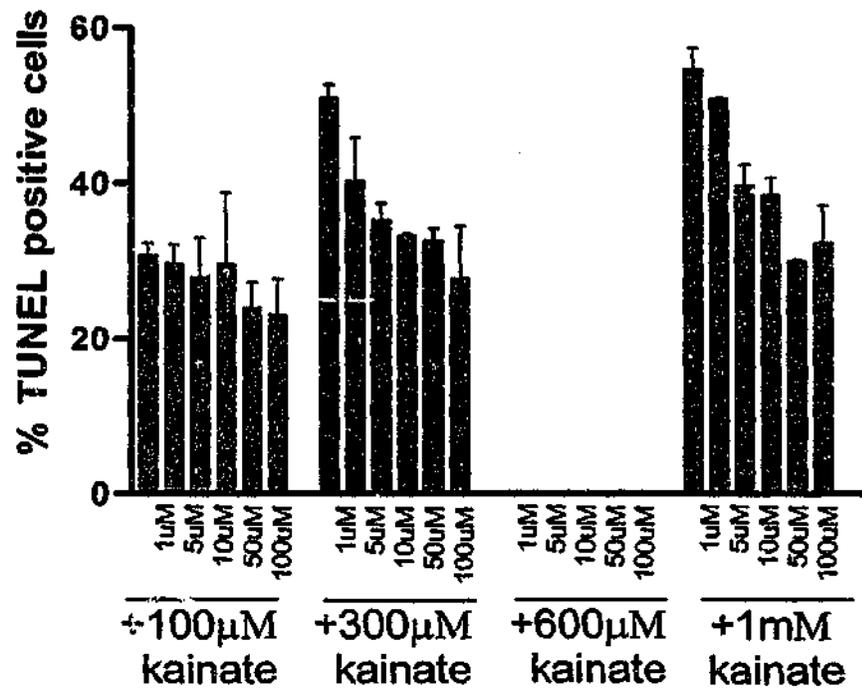
Figure 5.21 The effect of SB203580 on KA receptor-mediated apoptosis

Primary cultures of cerebellar granule cells were exposed to KA on *div* 8. Apoptosis was assessed using the TUNEL technique, which labels the nick end DNA fragments. Increasing concentrations of KA increased the number of TUNEL positive cells, implying KA concentration-dependently induced apoptosis. SB203580 (1-100μM) significantly decreased KA-mediated TUNEL labelling [$F(5,77) = 10.106$, $p < 0.001$), however closer examination of individual KA treated groups revealed no concentrations of SB203580 were statistically significant when compared to cultures treated with KA alone (one-way ANOVA). Data are unstandardized and are plotted as mean ± SEM and were from 2-3 replicate cultures.
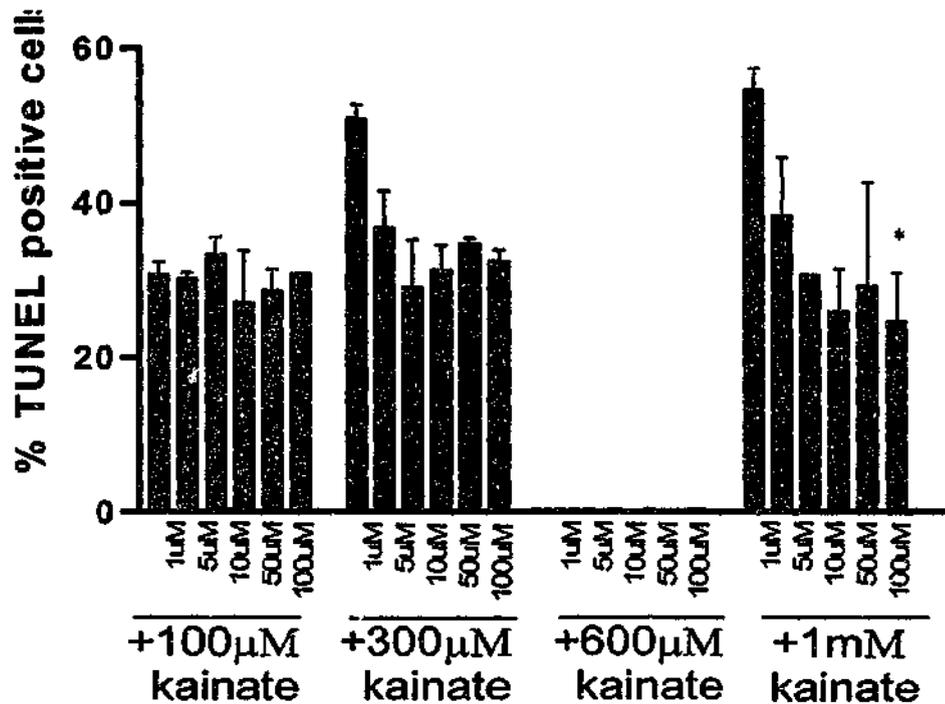
Figure 5.22 The effect of PD98059 on KA receptor-mediated apoptosis

Primary cultures of cerebellar granule cells were exposed to KA on *div* 8. Apoptosis was assessed using the TUNEL technique, which labels the nick end DNA fragments. Increasing concentrations of KA increased the number of TUNEL positive cells, implying KA concentration-dependently induced apoptosis. The MEK inhibitor PD98059 (1-100µM) produced a significant overall effect attenuating apoptotic labeling [$F(5,65)$ = 10.875, $p < 0.0001$], but few individual points were significant when compared to KA alone treatment group (one-way ANOVA). Data are unstandardized and are plotted as mean ± SEM and were from 2-3 replicate cultures. * represents statistical significance, $p <$ 0.05, one-way ANOVA.
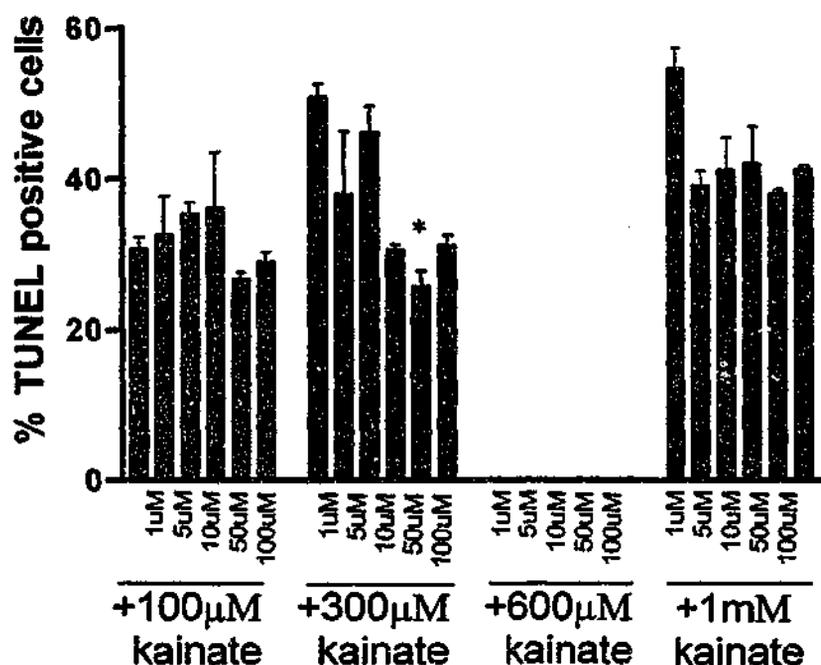
Figure 5.23 The effect of U0126 on KA receptor-mediated apoptosis

Primary cultures of cerebellar granule cells were exposed to KA on *div* 8. Apoptosis was assessed using the TUNEL technique, which labels the nick end DNA fragments. Increasing concentrations of KA increased the number of TUNEL positive cells, implying KA concentration-dependently induced apoptosis. The MEK inhibitor U0126 (1-100µM) produced a significant overall effect attenuating apoptotic labeling [$F(5,72) = 7.39$, $p < 0.0001$], few individual points were significant when compared to KA alone treatment group (one-way ANOVA). Data are unstandardized and are plotted as mean $\pm$ SEM and were from 2-3 independent cultures. * represents statistical significance, $p < 0.05$, one-way ANOVA.

increase in positive-labeled cells in KA-treated CGCs when compared to vehicle control as previously reported (Chapter Two). KA exposure has been shown to activate p42 and p44 MAP kinases in oligodendrocyte progenitors, an action thought to be mediated through the activation of the AMPA receptors (Liu *et al.*, 1999). The current model system has previously been shown not to contain functional AMPA receptors (Chapter Two), implying observations from the present study were mediated through the KA subtype of Glu receptor. Activation of JNK, p38 and the ERK kinases have been reported after NMDA receptor stimulation in hippocampal neuronal cultures (Mukherjee, 1999), but are unlikely to contribute to the results of the present study as growth and culture conditions mitigate against NMDA receptor-mediated toxicity (see Chapter Two). To our knowledge there has been no studies conducted to examine the effects of pharmacologically inhibiting p38, p44 and p42 MAP kinases and CDKs on excitotoxic neuronal death mediated by the KA receptor, without influences from the AMPA receptor, or other cell types such as glia.

Olomoucine was the most effective agent at attenuating KA-receptor mediated apoptosis in the current model system. The concentrations employed in the current study have previously been shown to inhibit CDC2/cyclin B1, CDK2/cyclin A, CDK5/p35, CDK6/cyclin D3, ERK, JNK and p38 kinase activity (Table 5.1). Therefore, to further examine the kinases implicated in KA receptor-mediated apoptosis more selective compounds were used. Roscovitine is a selective CDC2, CDK2 and CDK5 inhibitor (Meijer *et al.*, 1997), and under the present conditions only inhibited some 20% of neuronal death and apoptosis, suggesting that the CDKs only play a minor role in mediating excitotoxic apoptotic death. Roscovitine has previously been shown to be less potent at protecting retinal neurons against serum-withdrawal-mediated injury than olomoucine (Maas *et al.*, 1998), consistent with findings presented here. SB203580 at concentrations used in the present study is a selective p38 MAP kinase inhibitor with little

effects on the CDKs (Cuenda *et al.*, 1995). SB203580 was effective at attenuating KA receptor-mediated neuronal loss, particularly at the lower concentrations of KA (10-100μM), with little effect at 1mM KA. TUNEL labeling was slightly reduced at 300-1000μM KA treatment after SB203580 exposure, however TUNEL counts revealed no significant change at 100μM KA, although a trend towards neuroprotection was evident. Surprisingly, PD98059 and U0126 produced differing levels of protection, as previously reported in studies of cerebral vasospasm (Zhang *et al.*, 2000), implying that while these compounds are directed at the same kinase (Favata *et al.*, 1998), they may possess differing activity on other kinases. PD98059, only slightly improved the cell viability of cultures treated with KA, whereas lower concentrations of U0126 significantly attenuated KA receptor-mediated apoptosis, and seemed to be more effective than PD98059. There seems to be an involvement of p38 and the ERK kinases in KA-receptor-mediated apoptosis, however there is no clear indication that either pathway mediates cellular death. SB203580 and PD98059 inhibited KA receptor neurotoxicity most effectively at higher concentrations, where these compounds may not be acting selectively at their targeted kinases.

Mimosine, ciclopirox, and DFO have all been previously shown to be neuroprotective against DNA-damage induced apoptosis in cortical, PC12 and sympathetic neurones (Park *et al.*, 1997; Farinelli & Greene, 1996). While the mechanism of protection of these agents was associated with their ability to inhibit the cell cycle, these agents also have other activities. DFO and mimosine are both quite potent iron chelators and free radical scavengers, whereas rapamycin affects the stability of the cyclin D1 transcript and protein (Hashemolhosseini *et al.*, 1998). While a marked increase in cyclin D1 activity during KA receptor-mediated apoptosis was described earlier (Chapter Four), it may be only a marker rather than a mediator of apoptosis, thereby explaining the ineffectiveness of

rapamycin against KA-induced apoptosis in the current study. Other explanations as to why agents that have previously been reported to be potent neuroprotectants, but were ineffective in the current model system, could be explained in terms of the insult, as most of the previous studies have used withdrawal of trophic support, rather than a receptor-mediated insult. Naïve PC12 cells are not rescued from withdrawal of trophic-support injury by ciclopirox, DFO or mimosine, in agreement with results presented here (Park *et al.*, 1996a), and together with current findings demonstrate that these agents seem to be only protective in certain cell types and after distinct insult. This "selective" inhibition of apoptosis in different models indicates the manner in which the cell cycle is blocked may be a critical component of determining whether these agents promote survival.

While MTT data and TUNEL cell counts generally followed the same pattern for the CDK and MAP kinase inhibitors, less neuroprotection was noted for the kinase inhibitors as reflected by the number of TUNEL-positive cells than was expected from the MTT data. These differences may have been attributable to technical considerations and possibly the multiple washes of the cultures during the experiments, resulted in a higher background cell death, and hence an increase in the number of TUNEL-positive cells. As indicated in results, data for TUNEL labeling are unstandardized, unlike MTT data, which was compared to untreated controls and 100% cell death. Secondly, it is possible that while these inhibitors are increasing cell viability, they are not attenuating, but rather delaying apoptosis, resulting in an increase in MTT reduction but only a slight reduction of TUNEL labeling. Alternatively, since cellular viability measured by MTT reduction reflects mitochondrial function the diminution seen here may reflect upstream changes that are not totally predictive of the extent of final downstream DNA fragmentation (Budd *et al.*, 2000). The delay in apoptosis may explain the good correlation between the MTT and TUNEL data for olomoucine, as this inhibitor blocks both the CDK and MAP kinases,

perhaps indicating the therapeutic benefits of inhibiting concurrently these two mechanisms for the execution of apoptosis mediated by the KA receptor. Perhaps by inhibiting one specific kinase the apoptotic pathway may proceed down an alternative pathway still allowing cell death. Excitotoxicity has previously been shown to involve the cell cycle (Uberti *et al.*, 2000) and MAP kinases (Murray *et al.*, 1998; Yang *et al.*, 1997), and is therefore likely that not just one pathway is essential for the execution of apoptosis. While many cellular survival/death pathways are intertwined, the activation of either the cell cycle or the MAP kinases may in fact result in neuronal death, through the activation of a common downstream pathway, perhaps involving the pro-apoptotic members of Bcl-2 family and caspases (Ikeuchi *et al.*, 1998). However, if both pathways are inhibited, as demonstrated with olomoucine, apoptotic pathways are completely blocked and apoptosis cannot occur.

While the exact mechanism by which these kinases are in involved neuronal apoptosis and/or neuronal survival is still to be elucidated. our data add to the growing body of evidence suggesting CDK and MAP kinases play central roles in neuronal apoptosis. In addition, the MAP kinases and CDKs seem to be central to a complex mosaic of pathways, influencing various intracellular mediators including nuclear factor $\kappa\beta$, AKT/protein kinase B, and the Bcl-2 family, which have all been implicated in neuronal apoptosis (Thomson *et al.*, 1999; Fukunaga & Miyamoto, 1998). Whilst the present results do support the growing body of evidence involving the cell cycle and MAP kinases in excitotoxicity, since none of kinase inhibitors available commercially is specific for one kinase it is impossible to elucidate whether one kinase *per se* is responsible for the execution of apoptosis. However, agents like olomoucine, which demonstrate no toxicity of their own and inhibit multiple kinases, may be of therapeutic benefit for not only cancer

patients, but also patients suffering neurodegenerative conditions as a result of excitotoxic neuronal death.

CHAPTER SIX

GENERAL DISCUSSION

Glu, the major neurotransmitter of the mammalian CNS, has been implicated in the neuronal loss that occurs in a variety of pathological conditions, including stroke and epilepsy, and neurodegenerative conditions including ALS, Parkinson's disease and Huntington's Chorea (Leist & Nicotera, 1998; Lipton & Rosenberg, 1994). The NMDA subtype of iGluR has been well studied, compared to the AMPA and KA receptors and until recently it has been virtually impossible to distinguish between KA and AMPA responses, largely due to the lack of selective compounds for these receptors. The objective of this thesis was to investigate Glu receptor-mediated toxicity, with particular focus on the KA subtype of receptor, and to examine the involvement of the cell cycle and MAP kinases in mediating KA receptor-mediated apoptosis. The approach employed involved the development of primary cultures of CGCs in which the pharmacology, morphology of injury and molecular biology of cell death was examined following KA exposure. Overall, the results indicate KA to be a potent neurotoxin, with exposure resulting in the majority of cells dying by an apoptotic mechanism.

The current study employed a culture model, utilizing primary cultures of murine CGCs. Culture models of the nervous system are particularly useful, as confounding influences from the intact nervous system, such as non-neuronal cell populations and uptake systems, are more readily controlled. Neurotoxicity studies are readily undertaken in culture models, where morphology and staining with apoptotic or necrotic dyes is readily achieved. In addition, receptor expression is readily determined by immunocytochemistry, with specific antibodies, or *in situ* hybridization. CGCs are a particularly useful model system as they are an essentially homogenous cell preparation, and non-neuronal cell proliferation is readily controlled by mitotic inhibitors such as aphidicolin, which was used in the present studies. Therefore, sensitive analyses, such as

RT-PCR, are readily applied without "false" positive results from non-neuronal cells, a particularly important consideration for the cell cycle gene expression studies.

Chapter Two examined toxicity mediated by the iGluRs in primary neuronal cell cultures of CGCs from neonatal mouse brain. The culture system developed used a serum-free system, which mitigated against non-neuronal cell proliferation (Miller & Johnson, 1996), and allowed the analysis of cell death in a controlled milieu. NMDA and KA, but not the AMPA receptor agonists, AMPA and FW, caused a concentration-dependent cell death. KA and NMDA exposure resulted in a neurotoxicity that was attenuated by CNQX and MK-801, receptor antagonists selective for KA and NMDA, respectively, suggesting receptor-mediated effects. NMDA and KA toxicities were concentration- and time-dependent, with differing sensitivities on various days *in vitro*. Toxicity mediated by the NMDA receptor was largely necrotic in nature, whereas KA exposure predominantly resulted in apoptosis. To further advance the studies presented in Chapter Two, characterization of the iGluR receptor subtypes by RT-PCR, immunocytochemistry and Western blotting would have provided other insights into the action of the iGluR agonists, and indeed whether AMPA receptors are absent or not functional in primary cultures of CGCs.

Recently, a variety of putative low-affinity KA receptor agonists have developed and Chapter Three describes their excitotoxic profiles. As CGCs expressed no functional AMPA receptors (Chapter Two), this model system was particularly useful as only NMDA and KA receptor responses needed discriminating, and this separation was readily achieved with the selective antagonists MK-801 and CNQX. Neuronal cell death was used as a model to study the activity and potency of the low-affinity KA receptor agonists, using concentration-response curves to examine the neurotoxic profiles of the agonists. To examine the way in which neuronal loss was occurring various morphological, molecular

and cellular techniques were employed to determine whether cell death was either apoptotic or necrotic, or a combination of both. This study clearly demonstrated that studies conducted at recombinant receptors (Donevan *et al.*, 1998; Small *et al.*, 1998) cannot be readily extended to describe activities at native receptors. These novel compounds, LY339434, 4MG, IW and ATPA, demonstrated complex neurotoxic profiles, with ATPA and IW likely to exert the majority of their toxicity at the low-affinity KA receptors, while LY339434 appeared to be a potent NMDA receptor agonist. Neurotoxicity mediated by 4MG was not attenuated to any great extent by any of the employed iGluR antagonists, suggesting toxicity may be an action through activity on the Glu transporters, previously reported for 4MG (Aprico *et al.*, 2001; Vandenberg *et al.*, 1997). The neurotoxic profiles of these compounds were complex, with all, except ATPA, demonstrating a continuum of necrosis and apoptosis. To further investigate the actions of these compounds, more selective antagonists, for specific low-affinity KA subunits could have been used. While at this stage only antagonists for GluR6 (NS-102) (Verdoorn *et al.*, 1994) and GluR5 (LY382884, LY294486 and LY293558) (Bleakman & Lodge, 1998) are available, and the selectivity of these agents may be questionable as shown for the novel low-affinity KA receptor agonists presented here. Studies conducted at selective receptor subtypes are important, as emerging evidence suggests some homomeric receptor assemblies render neurones more vulnerable to injury than other subtypes, most likely due to Ca^{2+} permeability (Bennett *et al.*, 1996). In addition, changes in receptor subunit expression have been identified in various neuropathologies (Porter *et al.*, 1997; Paschen *et al.*, 1996; Breese *et al.*, 1995; Nishikawa *et al.*, 1983), suggesting targeting these receptors may alleviate neuronal loss and prevent neuropathologies.

The involvement of the cell cycle in apoptosis was examined, as few studies had reported neuronal proliferation after noxious stimuli (Bossenmeyer-Pourie *et al.*, 1999;

Gould & Tanapat, 1997) and numerous studies are emerging demonstrating an involvement of the cell cycle in neuronal apoptosis (Timsit *et al.*, 1999; Park *et al.*, 1998; Guegan *et al.*, 1997; Farinelli & Greene, 1996; Oka *et al.*, 1996; Park *et al.*, 1996; Herrup & Busser, 1995; Freeman *et al.*, 1994). While it is not likely that neuronal proliferation was present in the model system described herein, most likely due to the lack of the appropriate growth factors and nutrients supplied by glial cells, a clear change in the cell cycle protein cyclin D1 was noted. To examine whether excitotoxic neuronal death involved other components of the cell cycle, the expression of cyclins A, B1, and D1 were investigated after KA receptor-mediated apoptosis. BAX expression was also examined, as a marker of apoptosis, which is known to be activated during cyto c release in the death cascade that involves mitochondrial dysfunction (Desagher & Martinou, 2000; Nicholls & Budd, 2000; Shimizu *et al.*, 1999; Chao & Korsmeyer, 1998; Wolter *et al.*, 1997). There was a concentration- and time-dependent expression of both cyclin D1 and BAX protein after KA exposure, which was attenuated by CNQX, indicating a KA receptor-mediated response. Maximal expression of cyclin D1 was 2h after KA exposure, occurring upstream of BAX expression, which was maximal 12h after KA. No changes in expression of cyclins B1 and A were evident, suggesting that while the neuron may be signaling to enter the cell cycle, through the activation of cyclin D1, it does not enter the S phase, where cycle A expression is involved. The failure of the neurones to progress into the S/G2 phase of the cell cycle is likely to be due to the absence of the appropriate nutrients, growth factors and cellular signals, available in the intact CNS, which comes largely from the glial cell population. To further examine the involvement of the cell cycle in excitotoxic neuronal cell death other members of the cell cycle could have been examined, including the CDKs and their endogenous inhibitory counterparts, and investigations into whether the neurones actually proliferate, using BrdU, may have been examined. This topic is also

worthy of further investigations in *in vivo* models of excitotoxicity, such as KA injury after intrahippocampal administration.

BAX expression has been demonstrated in a number of apoptotic paradigms (Liu & Zhu, 1999; White *et al.*, 1998; Gleichmann *et al.*, 1998; Johnson *et al.*, 1998; Vekrellis *et al.*, 1997), and while not the focus of the current study, the neuronal "death" cascade and its pattern of activation, could have been further examined by including studies using caspase inhibitors and examining other members of the Bcl-2 super family. Similarly, the involvement of caspases in KA receptor-mediated apoptosis was not specifically studied here, but there are various reports of excitotoxic injury being caspase dependent (Masliah *et al.*, 1998; Du *et al.*, 1997) and independent (Johnson *et al.*, 1998; Miller *et al.*, 1997). In fact in the present system, KA injury appeared to be insensitive to inhibitors of caspases 1 and 3 (data not shown), supporting other evidence for a lack of caspase involvement in some apoptotic paradigms (see Chapter One).

Another approach adopted to evaluate the role of the cell cycle in neuronal apoptosis mediated by the KA receptors, employed the use of compounds that inhibit varying stages of the cell cycle. Exposure times to inhibitors and to KA had to be modified for these series of studies, as many of the inhibitors demonstrated inherent neurotoxic properties. Exposure of CGCs to KA for 4h still produced a concentration-dependent cell death that was predominantly apoptotic in nature (approximately 50% of neurones), therefore this time was employed and after 4h culture media containing KA and the inhibitors was aspirated and the cells left overnight in drug-free media. Generally, compounds that inhibited the G1/S transition phase of the cell cycle were most effective. However DFO, an iron chelator with antioxidant properties, that inhibits the G1/S transition, was not protective against KA insult, although oxidative stress is a common mediator of exicitotoxic cell death (Coyle & Puttfarcken, 1993). Other compounds that

inhibit the G1/S transition that were not protective against KA-mediated apoptosis, included ciclopirox, mimosine, silymarin, compounds that demonstrated inherent neurotoxicity, and only non-selectively inhibited the cell cycle. Therefore to further examine the involvement of the cell cycle more selective inhibitors of the cell cycle kinases, the CDKs, were employed. The most potent neuroprotective agent used was the more general kinase inhibitor, olomoucine, which completely attenuated KA receptor-mediated apoptosis and also produced a trend towards increasing cellular viability in untreated control cultures. To analyse the protective nature of olomoucine, more selective agents were then used to dissect which component of olomoucine's actions was protective. Roscovitine, an inhibitor of CDC2, CDK2 and CDK5 (Meijer *et al.*, 1997), produced some protection against KA, but was not as protective as olomoucine. Olomoucine has inhibitory activity on the CDKs and also MAP kinases and therefore inhibitors of these kinases were examined for any effect against KA receptor-mediated apoptosis. The p38 MAPK inhibitor, SB203580, only partially attenuated KA-receptor mediated neurotoxicity, with the majority of protection occurring at the lower concentrations of KA. The ERK inhibitors, U0126 and PD98059, like SB203580, were only partial neuroprotectants, and U0126 demonstrated marked neurotoxicity at higher concentrations. The neuroprotection seen with olomoucine is not likely due the inhibition of one kinase but likely to be due to the simultaneous inhibition of multiple kinases, and therefore the inhibition of multiple intracellular pathways. Additionally, the findings with the selective inhibitors of MAP kinases demonstrate the involvement of cellular cascades involving ERK and p38 MAPK in support of a body of evidence indicating their activation in apoptotic signaling cascades (Maas *et al.*, 1998; Xia *et al.*, 1995). To further support this series of experiments phosphorylation studies may have been conducted on the MAPKs to examine whether any changes accompany KA receptor overstimulation. Phosphorylation studies, using selective

antibodies, would have also allowed the examination of whether these kinase inhibitors were in fact selective, and indeed whether were inhibiting kinase activity at the concentrations employed. Furthermore, studies involving a combination of the kinase inhibitors may have been used to investigate whether KA receptor-mediated apoptosis was indeed dependent upon the inactivation of multiple pathways. Preliminary studies of this nature were made difficult by inherent vehicle-induced neurotoxicity.

Overall, neurotoxicity in CGCs caused by NMDA is largely necrotic in nature, while AMPA receptors seemed to be absent from this model system. KA receptor-mediated neurotoxicity is largely apoptotic in nature, however stimulation with IW can result in widespread necrosis at higher concentrations. KA receptor-mediated apoptosis is accompanied with a selective, time- and concentration-dependent increase in cyclin D1 expression, and is attenuated by MAP kinase and CDK inhibitors, suggesting an involvement of these kinases, and the proteins they influence, in the apoptotic pathways KA activate.

This study has revealed a number of cellular targets that might be exploited to inhibit excitotoxicity that occurs via KA receptors. Whilst blockade of KA receptor *per se* is an approach not likely to be therapeutically beneficial because KA receptors will almost certainly be involved in essential physiological events, classical pharmacological strategies directed at various cellular kinases are likely to be neuroprotective in excitotoxicity. The application of strategies such as differential display, gene arrays or proteomics in the well defined model of injury described herein (KA receptor-mediated apoptosis in cultured CGCs) might yield unique data on new targets downstream of overstimulation of the iGluRs, and give further insight into the intracellular "excitotoxic death pathways".

APPENDIX

Appendix I Composition of HBSS

COMPONENT	CONCENTRATION (mM)
KCl	5
KH_2PO_4	0.3
NaCl	138
Na_2HPO_4	0.3
D-Glucose	5.6
Phenol Red	0.03

Appendix II Composition of B27 components

COMPONENT	CONCENTRATION (μg/ml)
BSA	2500
Biotin	0.1
L-carnitine	2
catalase	2.5
corticosterone	0.02
ethanolamine	1
D(+)-galactose	15
glutathione	1
insulin	4
linoleic acid	1
linolenic acid	1
progesterone	0.063
putrescine	16.1
retinyl acetate	0.1
selenium	0.01
superoxide dismutase	2.5
apo-transferrin	5
triiodothronine	0.02
DL-α-tocopherol	1
DL-α-tocopherol acetate	1

Appendix III Composition of N2 Media

COMPONENT	CONCENTRATION (µg/ml)
Insulin (Bovine)	500
Human Transferrin	10000
Progesterone	0.63
Putrescine	1611
Selenite	0.52

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