OXIDATIVE STRESS AND THE VIABILITY OF OSTEOBLASTS AND CEREBELLAR GRANULE NEURONES

by

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ABSTRACT

L-Glutamate is the major excitatory neurotransmitter in the CNS, where it activates both its ionotropic and metabotropic receptors. Excessive levels of glutamate lead to increased intracellular free calcium concentrations, which activate calcium-dependent events resulting in the production of reactive oxygen species (ROS) such as nitric oxide, superoxide and hydrogen peroxide. Glutamate-induced neuronal damage in stroke and neurodegenerative diseases may be mediated by these ROS, which are known to promote oxidative stress. Glutamate receptors are also expressed in non-neuronal peripheral tissues such as bone, where osteoblasts actively secrete glutamate and express glutamate receptors showing striking electrophysiological, biochemical and molecular similarities to those found in the CNS. The responses of osteoblastic and neuronal cultures to culture conditions, glutamate and ROS-generated oxidative stress were therefore the focus of this study. Murine MC3T3-E1 osteoblast cells were used as a suitable *in vitro* model of bone-forming osteoblasts, compared with cerebellar granule neurones (CGNs) from 8-day old Sprague - Dawley rat pups. The major inducer of oxidative stress employed was hydrogen peroxide (H_2O_2), although other sources have also been examined to a limited extent. Osteoblast cultures were used mostly at 10 div, a time when they are sufficiently differentiated to express functional glutamate receptors. MC3T3-E1 cell maturation was enhanced by the addition of ascorbate and β-glycerol phosphate. Neuronal experiments were carried out at 8 div, except excitotoxicity studies which were conducted at 9 div. Cytosine arabinoside (10μM) was added to the CGN cultures 20 h after plating to eliminate non-neuronal population of cells (mainly glial). The purity of the neuronal cultures was confirmed by immunostaining for the beta-III isoform of tubulin, which is specific for neurones. Cell viability in the presence or absence of

experimental agents was determined with the alamar blue (AB) reduction assay, which relies on metabolic activity. Morphological effects of experimental agents were also monitored using both light and phase-contrast microscopy.

Addition of ascorbate and β-glycerol phosphate at 3 div (switching) significantly increased the proliferation of MC3T3-E1 cells, and their differentiation as measured by ALP activity. Switch and non-switch cells survived equally well in medium containing 10% Foetal Calf Serum (FCS), while serum withdrawal for up to 3 days prior to 10 div resulted in less than 18% reduction in viability. A high potassium ion (K^+) concentration (50mM) was potentially toxic to these cells. In the case of CGNs, serum withdrawal for 1 h at 8 div had no effect on survival, but the absence of serum for 6 h caused more than 32% reduction in viability. Similarly, withdrawal of high K^+ for 2 days also reduced viability >31%.

Glutamate (10μM - 1 mM) applied for 1 h caused concentration-dependent damage to CGNs when the cells were allowed a recovery period of 6 h. This effect was more pronounced following the longer recovery period of 24 h. The more specific agonist NMDA also produced concentration-dependent damaging effects, but was less potent than glutamate. Glutamate or NMDA neurotoxicity was blocked by the non-competitive NMDA receptor antagonist dizocilpine (MK-801), the competitive antagonist D-AP5, and the NMDA glycine-site blocker kynurenic acid, but glutamate toxicity was not prevented by the non-NMDA (AMPA/kainate) receptor antagonist CNQX, confirming the effects were mediated mainly by the NMDA receptor. Mechanisms of glutamate neurotoxicity involved activation of both apoptotic and necrotic pathways. The caspase-3 inhibitor, Z-DEVD-fmk, even at 40μM, and the poly (ADP-ribose) polymerase-1 inhibitors, DPQ (10μM) and nicotinamide (1mM), prevented or attenuated the effect of glutamate. The membrane permeability transition pore blocker,

cyclosporin A (CsA) (0.5 - 10μM), also completely protected neurones against glutamate. Using the osteoblast cells, glutamate and NMDA were not toxic but significantly trophic. Further experiments focussed on ROS, especially H_2O_2, which produced concentration- and time-dependent viability reductions in the two cell types that correlated well with morphological damage, although the osteoblasts were less sensitive to its effect. The more differentiated (switch) osteoblast cultures proved more sensitive to peroxide damage than the less differentiated (non-switch) cultures. Prolonging the recovery period to 24 h after peroxide treatment resulted in further neuronal damage, while in osteoblasts, variable effects were observed at a low H_2O_2 concentration (depending on the treatment duration), but with no effect at a high concentration. The effects of H_2O_2 were prevented by the antioxidant enzyme catalase (CAT), but not by superoxide dismutase (SOD) or the COX inhibitor aspirin. In the presence of Cu^{2+}, the effect of H_2O_2 was potentiated, presumably through the Fenton reaction generation of the hydroxyl radical ($^{\cdot}OH$), although the $^{\cdot}OH$ scavenger mannitol paradoxically worsened, rather than protected, against this damage. Mechanisms of damage by peroxide in MC3T3-E1 osteoblasts and CGNs involved both apoptotic and necrotic pathways. The free radical-generating system of xanthine (X) and xanthine oxidase (XO) elicited a trophic influence on osteoblast cells but caused pronounced damage to the CGNs. Xanthine oxidase alone was also toxic to the CGNs, an effect prevented by the inhibitor allopurinol. In the presence of CAT, but not SOD, the neurotoxic effects of either X or X/XO were abolished, indicative of mediation by H_2O_2 rather than superoxide. 3-hydroxykynurenine was profoundly neurotoxic even at 10μM (6 h), but only reduced osteoblast viability at 1mM (1 or 6 h). An inducer of nitrosative stress, S-nitroso-*N*-acetylpenicillamine (SNAP), had no significant damaging effect on osteoblasts until when applied at 5mM for 24 h, but produced a

concentration- and time-dependent reduction in CGN viability (significant even at 100μM for 6 h and blocked by the nitric oxide synthase inhibitor L-NAME, but not by the soluble guanylate cyclase inhibitor ODQ). An attempt to probe whether mitochondrial impairment underlies the differences in sensitivities of MC3T3-E1 and CGN cultures to oxidative damage revealed that the integrity of the osteoblasts was not significantly compromised in the presence of the mitochondrial poisons: potassium cyanide (KCN) and 3-nitropropionic acid (3-NPA) (except for 24 h application of 3-NPA at 100μM and 1mM). The neurones "succumbed" to lower concentrations and shorter exposures to the poisons. The neurotoxic effects of the poisons were mediated by the NMDA receptor, since they were blocked by the antagonist D-AP5. Thus, while neurones depend critically on mitochondrial oxidative phosphorylation for energy production (ATP synthesis), the osteoblasts might follow preferentially the glycolytic pathway.

The stable adenosine analogue, 2-chloroadenosine (2-ClA), and the A_1 and A_{2A} receptor agonists, CPA and CGS21680, respectively, each conferred partial but significant protection against H_2O_2 damage to osteoblasts, while the respective A_1 and A_{2A} antagonists, DPCPX and ZM241385 had no effect. Glutamate- and H_2O_2-induced damage to CGNs were significantly prevented by CPA and ZM241385, but not by DPCPX or CGS21680.

Therefore, while osteoblasts and neurones may be similar in terms of glutamate receptor expression, they exhibit differences in their manner of responses and the degree of sensitivity to toxic agents. Further elucidation of underlying mechanisms may be furnished through molecular biology-based approaches.

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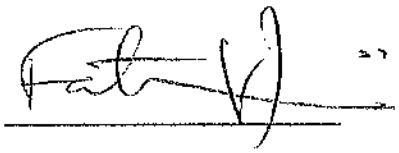
DECLARATION

I, Amos Akintayo Fatokun, declare that this thesis was composed by myself, and also that the experiments described therein were performed by myself, except where referenced.



Amos A. Fatokun

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ABBREVIATIONS

AA	Arachidonic acid
AAT	Aspartate aminotransferase
AB	Alamar blue
AC	Adenylate cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADP	Adenosine diphosphate
AIF	Apoptosis-inducing factor
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocator
AP	Allopurinol
AP-1	Activator protein-1
Apaf-1	Apoptotic protease-activating factor-1
Ara C	Cytosine β-D-arabinofuranoside
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
BAD	Bcl-X_L/Bcl-2-associated death promoter
BBB	Blood-brain barrier
Bcl	B-cell lymphoma
BDNF	Brain-derived neurotrophic factor
BGP	Beta-glycerol phosphate
BMU	Basic multicellular unit
BSA	Bovine serum albumin
BSP	Bone sialoprotein

BSU	Bone structural unit
CAD	Caspase-activated DNase
cAMP	cyclic adenosine monophosphate
CAT	Catalase
Cat. No.	Catalogue number
Cbfa1	Core binding factor alpha1
CBP	CREB-binding protein
CCPA	2-chloro-N^6-cyclopentyladenosine
Cdk	Cyclin-dependent kinase
CFM-2	1-(4-aminophenyl)-3,5-dihydro-7,8-dimethoxy-4H-2,3-benzodiazepin-4-one
CREB	cAMP/calcium response element-binding protein
CRF	Central Research Facility
CGN	Cerebellar granule neurones
CGS15943	9-chloro-2- (2-furanyl)[1, 2, 4] triazolo [1,5-c] quinazolin-5-amine
CGS21680	2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamidoadenosine hydrochloride
2-ClA	2-chloroadenosine
2-Cl-IB-MECA	2-chloro- N^6-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CO	Carbon monoxide
CO_2	Carbon dioxide
COLL-1	Collagen type-1
COX	Cyclooxygenase
CPA	N^6-cyclopentyladenosine
CRMP	Collapsin response mediator protein
CsA	Cyclosporin A
$CuSO_4$	Copper sulphate
CuZnSOD	Copper-zinc superoxide dismutase
DAG	Diacylglycerol

DAO	D-amino acid oxidase
D-AP5	D-2-amino-5-phosphonopentanoic acid
DEPC	Diethylpyrocarbonate
div	day(s) *in vitro*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DPQ	3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone
EAA	Excitatory amino acid
EAAC	Excitatory amino acid carrier
EAAT	Excitatory amino acid transporter
ECACC	European Community of Animal Cell Culture
ECF	Extracellular fluid
EDRF	Endothelial-Derived Relaxing Factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FADH	Flavin adenine dinucleotide reduced
FCS	Foetal calf serum
Fig.	Figure
FMN	Flavin mononucleotide
FMNH	Flavin mononucleotide reduced
GABA	Gamma-amino butyric acid
G_i	Inhibitory G protein
GLAST	Glutamate aspartate transporter
GLT	Glutamate transporter
Glu	Glutamate

GMS	Glycine modulatory site
GPx	Glutathione peroxidase
GRIP	Glutamate receptor interacting protein
G_s	Stimulatory G protein
GSH	Gluthathione (reduced)
GSNO	S-nitrosoglutathione
GSSG	Gluthathione (oxidized)
3-HAA	3-hydroxyanthranilic acid
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HD	Huntington's disease
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
3-HK	3-hydroxykynurenine
4-HNE	4-hydroxynonenal
HO-1	Haem oxygenase-1
H_2O_2	Hydrogen peroxide
5-HT	5-hydroxytryptamine
HW	Higher wavelength
IB-MECA	N^6-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide
ICC	Immunocytochemistry
IFN	Interferon
iGluRs	ionotropic glutamate receptors
IL	Interleukin
IMP	Inosine monophosphate
IMS	Intermembrane space
IP_3	Inositol-1, 4, 5-triphosphate
iNOS	inducible nitric oxide synthase
Jnk-3	c-Jun N-terminal kinase
KA	Kainate
K_{ATP}	ATP-sensitive potassium channels
KCl	Potassium chloride

KCN	Potassium cyanide
KYA	Kynurenic acid
LDH	Lactate dehydrogenase
L-NAME	N_{ω}-Nitro-L-arginine methyl ester hydrochloride
LT	Lymphotoxin
LTP	Long-term potentiation
LW	Lower wavelength
ψ_m	Mitochondrial membrane potential
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MC3T3-E1	Murine calvariae 3T3-E1
MDA	Malondialdehyde
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
mGluRs	metabotropic glutamate receptors
$MgSO_4$	Magnesium sulphate
(+)-MK-801	Dizocilpine / (5S, 10R)-(+)-5-Methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine maleate
MnSOD	Manganese superoxide dismutase
MPP^+	1-methyl-4-phenylpyridinium ion
MPT	Membrane permeability transition
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
$NaHCO_3$	Sodium bicarbonate
NAm	Nicotinamide

NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
NGF	Nerve Growth Factor
NMDA	*N*-methyl-D-aspartate
NMDAR, NR	*N*-methyl-D-aspartate receptor
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox1	NADPH-oxidase 1
3-NPA	3-nitropropionic acid
ns	non-significant
NSAID	Non-steroidal anti-inflammatory drug
NTG	Nitroglycerine
O_2	Oxygen
ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PAR	Poly (ADP-ribose)
PARG	Poly (ADP-ribose) glycohydrolase
PARP	Poly (ADP-ribose) polymerase
PD	Parkinson's disease
8-PT	8-phenyltheophylline
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PDL	poly-D-lysine
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol-4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C

Prx	Peroxiredoxins
P/S	Penicillin-streptomycin
PSD	Postsynaptic density
PTP	Permeability transition pore
PTX	Pertussis toxin
QA	Quinolinic acid
RIPA	Radioimmunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase - polymerase chain reaction
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBTI	Soybean trypsin inhibitor
SCH58261	5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-e]-1,2,4-triazolo [1,5-c] pyrimidine
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
S-ENBA	(2S)-N^6-[2-endo-norbornyl]adenosine
sGC	soluble guanylate cyclase
SIN-1	3-morpholinosydnonimine
SNAC	S-nitroso-*N*-acetylcysteine
SNAP	S-nitroso-*N*-acetylpenicillamine
SNARE	Soluble *N*-ethyl maleimide-sensitive factor (NSF) attachment protein (SNAP) receptor
SNK	Student-Newman-Keuls
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
SOR	Superoxide reductase
SRF	Serum response factor

TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
TRIS-HCl	TRIS (hydroxymethyl) aminomethane hydrochloride
Trk	Tyrosine receptor kinase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
UDP	Uridine diphosphate
VDCC	Voltage-dependent calcium channels
VGLUT	Vesicular glutamate transporter
X	Xanthine
XO	Xanthine oxidase
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide
Z-DEVD-fink	Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone
ZM241385	4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol

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PUBLICATIONS

Papers

Fatokun, A.A., Stone, T.W. & Smith, R.A. (2006). Hydrogen peroxide-induced oxidative stress in MC3T3-E1 cells: The effects of glutamate and protection by purines. *Bone.* In press [Epub ahead of print; April 5, 2006].

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Abstracts

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Papers in preparation

Fatokun, A.A., Stone, T.W. & Smith, R.A. Mechanisms of glutamate-induced neurotoxicity in cerebellar granule neurones and protection by purines.

Fatokun, A.A., Stone, T.W. & Smith, R.A. Apoptotic and necrotic neuronal cell death induced by hydrogen peroxide: protection by adenosine receptors.

Fatokun, A.A., Stone, T.W. & Smith, R.A. Responses of MC3T3-E1 osteoblast cells to culture conditions and ROS-generating systems.

Fatokun, A.A., Stone, T.W. & Smith, R.A. Effects of the free radical-generating system of xanthine and xanthine oxidase in cerebellar granule neurones.

1. INTRODUCTION

The work presented in this thesis is a study of factors that affect the viability of cells from two different tissues. The work has examined the responses of the osteoblast-like cell line of MC3T3-E1 cells, and cerebellar granule cells as a neuronal cell population. The effects on the viability of these two cell types of agonists and antagonists at glutamate and adenosine receptors have been examined, as well as the effects of oxidative stress induced by hydrogen peroxide (H_2O_2), other reactive oxygen species (ROS)-generating systems and some kynurenines. The mechanisms of damage by ROS-induced oxidative stress and the role of glutamate and adenosine receptors in protection have also been investigated. This introduction will provide background information, firstly on the receptors that have been studied - for glutamate and adenosine, then on reactive oxygen species and oxidative stress, and finally on mechanisms of cell death and the two types of cell tested.

1.1. Glutamate and its receptors in the CNS and bone

Glutamate (Glu) is the major and most important excitatory amino acid (EAA) neurotransmitter in the mammalian central nervous system (CNS), where its effects are exerted by the activation of ionotropic receptors (iGluRs) containing cation-specific ion channels and the G protein-coupled metabotropic receptors (Hollman and Heinemann, 1994). Glutamate (the most abundant amino acid in the diet) and related EAAs, such as L-aspartate, are released by an estimated 40% of all synapses (Meldrum, 2000; Fonnum, 1984). There is also now compelling evidence for the presence of glutamate and its receptors in non-neuronal peripheral tissues such as bone (Skerry & Genever, 2001; Skerry & Taylor, 2001). The discovery of the excitatory action of glutamate dates back to more than four decades ago, and a role for glutamate has been demonstrated in most aspects of normal brain functioning including cognition, learning and memory (Headley & Grillner, 1990). In addition, glutamate has been shown to be involved in synaptic development and plasticity (Komuro & Rakic, 1993). In fact, EAA agonists like quinolinic acid (QA) and *N*-methyl-D-aspartate (NMDA) have been shown to promote biochemical differentiation of cultured neurones- including the cerebellar granule neurones (CGNs)- at early developmental stages (Balazs et al., 1989; Hunt & Patel, 1990) and endogenous glutamate could stimulate neurite outgrowth from CGNs through activation of NMDA receptors (Pearce et al., 1987). However, besides these physiological roles, the pathological significance of glutamate has also been well reported. Thus, excessive glutamate levels in

the extracellular space or the overactivation of glutamate receptors leads to the death of central neurones (Stone & Addae, 2002) such as occurs during stress to the brain induced by ischaemia, hypoxia, hypoglycaemia, head trauma and epileptic seizure. Almost five decades ago, degeneration of neural retina of neonatal mice by systemic administration of glutamate or aspartate was demonstrated (Lucas & Newhouse, 1957). However, the term "excitotoxicity" through massive efflux of glutamate from neurones and glia was coined by Olney (1969), who, alongside co-workers, later described a correlation between the neurotoxic effects and the neuroexcitatory potency of a number of peripherally administered acidic amino acids in the arcuate nucleus of the hypothalamus (Olney et al., 1971). Subsequently, the finding that kainic acid could cause an axon-sparing lesion of the striatum (Coyle & Schwarcz, 1976; McGeer & McGeer, 1976) has inspired intensive research leading to massive evidence that glutamate neurotoxicity is involved in the pathology of neurological conditions such as stroke (Coyle & Puttfarcken, 1993) and various neurodegenerative diseases, such as Alzheimer's (AD), Parkinson's (PD), and Huntington's diseases and amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) (Choi & Rothman, 1990). It is therefore not surprising that, due to its neurotoxic potential, the concentration of glutamate in the extracellular space is kept quite low, with cerebrospinal fluid concentrations of approximately 0.3μM and average brain parenchyma concentration of 3mM, representing a 10,000-fold gradient (Coyle & Puttfarcken, 1993). It is now widely known that the neuronal damage following stroke is largely attributable, not to the immediate hypoxia or ischaemia, but to the massive release of glutamate from neurones and glia which then activates its receptors resulting in a cascade of events leading up to neuronal death (Obrenovitch & Urenjak, 1997). It has been reported in cultured cortical neurones, however, that the development of EAA receptors mediating release responses (e.g., GABA release) precedes the maturation of intracellular mechanisms involved in excitotoxic neuronal injury (Cai & Erdo, 1992). Glutamate is also now known to exhibit glial toxicity through its receptors (Follet et al., 2004).

Receptors for glutamate are classified into the ionotropic (ion channel-forming) receptors and the metabotropic (G protein-coupled) receptors, based on their differential intracellular signal transduction mechanisms and molecular homologies (Hollmann et al., 1989).

1.1.1. Ionotropic glutamate receptors (iGluRs)

Glutamate activates, at least, three types of ionotropic receptors, sensitive, respectively, to *N*-methyl-D-aspartate (NMDA), kainate and α-amino-3-hydroxy-5-methyl-4-isoxazole

propionic acid (AMPA) (Harris, 1995), all of which could increase intracellular calcium levels and thus lead to the generation of nitric oxide (NO) and other reactive oxygen species (ROS), the end result of which is cell death (Ogura et al., 1988). The AMPA and kainate receptors are sometimes collectively termed the non-NMDA receptors. The orphan receptors delta1 and delta2 (GluRδ1 and GluRδ2) have also been proposed, the functions of which are not yet known (Skerry & Genever, 2001), although it is believed that delta2 receptors play important roles in the cerebellum (Yuzaki, 2003) and delta1 receptors in the auditory and vestibular system (Safieddine & Wenthold, 1997). While most excitatory postsynaptic potentials (EPSPs) are generated through activation of both AMPA and NMDA receptors (McBain & Mayer, 1994), it is the AMPA and kainate receptors that mediate fast excitatory postsynaptic potentials (fast EPSPs) whereas the NMDA component is much slower and smaller. Permeability to Ca^{2+} in non-NMDA receptors is about 100 times less than for NMDA receptors, except for particular subtypes of the NMDA receptor (Ogura et al., 1990; Iino et al., 1990). The non-NMDA currents also show little voltage dependency and can be blocked by the selective antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX). Biophysical studies revealed that ion channels gated by non-NMDA glutamate receptors are highly selective for Na^+ and K^+ (Jahr & Stevens, 1987; Macdermott et al., 1986), while the NMDA receptor channel is permeable to Ca^{2+} as well as Na^+ and K^+ (Jahr & Stevens, 1987; Mayer & Westbrook, 1987).

1.1.1.1. NMDA receptors

The NMDA receptor, like other ligand-gated ion channels, is thought to be a heteroligomeric protein (Yamakura & Shimoji, 1999), existing in multiple forms, probably as pentameric assemblies, with different physiological and pharmacological properties and distributions throughout the brain. It is composed of an NR1 (NMDAR1) subunit and at least one isoform of the NR2 (NMDAR2) subunit and possibly NR3 subunits. So far, two major subunits, NR1 and NR2, have been cloned from the rat CNS, with alternative splicing revealing eight functional isoforms for the NR1 subfamily (NR1A-NR1H). The NR2 subfamily consists of four individual subunits, NR2A-NR2D (Collingridge & Watkins, 1994) while there are three NR3 subunits (Hollmann, 1999). The NR2C subunit is expressed in high levels only in the CGNs (Cull-Candy et al., 2001). NR3 subunits are a dominant-negative family that suppresses opening of functional NMDA receptor channels (Das et al., 1998). Glutamate recognition site is thought to be located on the NR2 subunit. Well-established ligands at the NMDA receptors include simple structural derivatives of

glutamate, such as 2-amino-5-phosphono-pentanoic acid (Perkins et al., 1981; Davies et al., 1981) whose applicability as CNS neuroprotectants has been precluded by their inability to cross the blood-brain barrier (BBB) owing to lipophobicity. Selfotel is a related compound with improved ability to cross the BBB (Stone & Addae, 2002). Non-competitive NMDA receptor antagonists include dizocilpine (MK-801), ketamine, dextromethorphan, memantine and phencyclidine, which all block the NMDA channel in the open site (Collingridge & Watkins, 1994). A variety of allosteric modulatory sites exist on the NMDA receptor-channel complex that are amenable to pharmacological modifications, e.g., the polyamine site (at which spermine and spermidine are possible endogenous ligands, with biphasic effects), the zinc-binding site, the phencyclidine-binding site, and the redox site (Watkins et al., 1990; Stone & Addae, 2002). The redox site is also known as the target site where agents with oxidising or reducing activity can modulate the NMDA receptor in the same manner as they would other ligand-gated ion channel receptors. The redox site could therefore switch the receptor between the less active (oxidized) state and the more efficacious (reduced) state. This site is now believed to be the target for agents that are known to suppress NMDA receptor activation such as reactive oxygen species like the superoxide and hydroxyl radicals (Aizenman et al., 1990) and possibly nitric oxide (Lei et al., 1992), though it is possible that there may be several different redox sites and that nitric oxide and other free radicals act largely at different ones (Fagni et al., 1995). These intrinsic redox properties of the NMDA receptor can explain the developmentally regulated expression of excitotoxicity *in vitro*. NMDA receptors at earlier developmental ages prefer a more oxidized state and neurotoxicity would therefore be produced in the presence of a reducing agent in normally resistant younger neurones (Sinor et al., 1997). However, Marks et al. (2005) have also demonstrated that mitochondrial nitric oxide synthase (NOS) mediates much of the decreased vulnerability of hippocampal neurones to NMDA while cytosolic NOS contributes to NMDA toxicity in mature neurones.

In 1987, Johnson and Ascher described the essential co-factor role for glycine, which acts at an allosteric site located on the NR1 subunit of the NMDA receptor while kynurenic acid, a metabolite along the kynurenine pathway of tryptophan metabolism, was shown by Perkins and Stone (1982) to be an antagonist at this site, having an especially high affinity there (Birch et al., 1988; Stone, 1993), though it could also be an antagonist at the glutamate-binding site. Each receptor unit appears to have two glycine and two glutamate binding sites (Laube et al., 1998). Because it is the most salient modulatory site, the

glycine modulatory site (GMS) has been referred to as the *glycine B receptor*, which must be occupied before glutamate can open the NMDA receptor channel (Schell, 2004). Kynurenic acid is also able to antagonize actions mediated by the non-NMDA glutamate receptors (Perkins & Stone, 1982). However, most interest has been devoted to the more potent activity of kynurenic acid at the strychnine-resistant glycine site on the NMDA receptor because, under physiological conditions or at resting potentials, the ion channels associated with the NMDA receptor are blocked by the voltage-dependent Mg^{2+} ions and only after initial depolarization, for example, by AMPA or kainate receptors, is the Mg^{2+} block relieved enough to allow activation of the NMDA receptor (Stone & Addae, 2002). These conditions (blockade in a use and voltage-dependent manner) are noticed in pathological circumstances like hypoxia, ischaemia, epilepsy, and traumatic brain injury, each of which is associated with raised levels of extracellular glutamate (Stone & Simmonds, 1991; Latini & Pedata, 2001). It is therefore reasonable that NMDA receptor antagonists would interfere less with normal fast glutamatergic transmission, essential in the control of many autonomic functions, e.g., cardiovascular and respiratory mechanisms and movement control by the basal ganglia and cognitive functions, than antagonists at kainate and AMPA receptors (Stone & Addae, 2002).

Interestingly, a neuromodulatory role in NMDA receptor activation has also been found for the putative glial neurotransmitter D-serine. It is synthesized from L-serine by a novel pyridoxal-5'-phosphate (B(6))-requiring enzyme, serine racemase, in astrocytic glia ensheathing synapses, especially in regions of the brain abundant in NMDA receptors, although significant amounts of serine racemase and D-serine have recently been reported in primary neuronal cultures and neurones *in vivo* (Kartvelishvily et al., 2006). D-serine seems to be released physiologically in response to activation by glutamate of AMPA receptors on D-serine-containing glia, causing the glutamate receptor-interacting protein, which binds serine racemase, to induce enzyme activity and D-serine release. Matsui et al. (1995) reported that D-serine *in vivo* plays a role in the forebrain while glycine is most significant in the cerebellum. However, it has now been postulated that this amino acid is more likely to be the endogenous ligand for the glycine site of the NMDA receptor than glycine in many, if not most, parts of the brain, as it activates the glycine site of the NMDA receptor with a potency at least three times that of glycine and its localizations resemble that of NMDA receptors more closely than glycine (Schell et al., 1997). It is interesting to note that D-serine also plays a role in neural development by being released from Bergmann glia thereby chemokinetically enhancing the migration of granule cell

cerebellar neurones from the external to the internal granule layer (Mustafa et al., 2004). The catabolism of D-serine occurs via the peroxisomal enzyme D-amino acid oxidase (DAO) (Mustafa et al., 2004; Schell, 2004). This glial source of the neuromodulator perhaps further underpins the importance and active participation of glial cells in the CNS (the macroglia, comprising astrocytes and oligodendrocytes, and the microglia) in the modulation of neurotransmission. Glial cells outnumber neurones and are in intimate contact with neuronal cell bodies, axons, and synaptic terminals (Gallo & Russell, 1995). About 65% of rat or mouse brain cells are glial, whereas the human brain is almost 95% of glia. However, because they are smaller than neurones, glia contribute about 50% of the volume of the human brain. Evidence now mounts to disprove the initial preconception of glia as mere bystanders in the event of neurotransmission (the neurocentric approach), whose sole responsibility is to provide a nutritive microenvironment for the effective survival and functioning of neurones (Ullian et al., 2004). Most of the known neurotransmitter/neuromodulator receptors are expressed by astroglia in primary culture and regulate a variety of second messenger systems (Enkvist et al., 1989) and ion channels (Barres et al., 1990). Though glia cells lack the excitability usually associated with neurones (Barres, 1991), astrocytes in the brain, which constitute almost 50% of cells in the CNS (Hu et al., 2004), form an intimately associated network with neurones and respond to neuronal activity and synaptically released glutamate by raising intracellular calcium concentration (Dani et al., 1992). This could represent the start of back-signalling to neurones (Nedergaard, 1994). It is now known that a bidirectional signalling occurs between astrocytes and neurones (Carmignoto, 2000), which translates to a major involvement of the former in neurotransmission, with glial cells being able to release glutamate through a calcium-dependent mechanism that resembles the synaptic release of neurotransmitters at the nerve terminal (Innocenti et al., 2000; Parpura & Haydon, 2000). Physiological astrocytic calcium levels have been reported to stimulate glutamate release to modulate adjacent neurones (Parpura & Haydon, 2000). Also, astrocytes have been shown to regulate NMDA receptor subunit composition, with the effect of increasing neuronal sensitivity to excitotoxicity (Daniels & Brown, 2001). In fact, the availability of modulators of the NMDA receptor such as D-serine, glycine, and kynurenines including kynurenic acid, 3-hydroxykynurenine and quinolinic acid, is determined, not by neurones, but by astrocytes and microglia (Schousboe, 2003; Coyle & Schwarcz, 2000; Coyle, 2006). The multiple roles of glia include the regulation of external potassium released by neurones into the extracellular space.

The channels to which the NMDA receptors are coupled exhibit high conductance and are permeable to K^+, Na^+ and Ca^{2+}. The opening of the NMDA receptor mediates the entry of a considerable amount of Ca^{2+} into the intracellular space, much more than does any of the other ionotropic (AMPA/kainate) receptors (Ogura et al., 1990; Iino et al., 1990). This elevated level of free cytosolic calcium is critical to NMDA neurotoxicity, although a recent report by Lin et al. (2005) showed that inhibition of P2 receptor may directly modulate NMDA receptor-mediated neurotoxicity through a Ca^{2+}-independent mechanism. In support of the critical role of calcium in NMDA-induced excitotoxicity, cortical neurones containing calcium-binding proteins such as calbindin, parvalbumin, and calretinin are selectively resistant to calcium overload and excitotoxicity *in vitro* (Lukas & Jones, 1994). Much evidence suggests the co-localization of AMPA and NMDA receptors and therefore their co-activation by glutamate released into the synaptic cleft, but it seems that the AMPA receptor has low affinity, thus becoming unbound quickly after the clearance of the transmitter, whereas the NMDA receptor has a greater affinity that favours long binding (Ozawa et al., 1998). The peculiar properties of the NMDA receptor have made it to be a subject of intense investigation in relation to various phenomena such as neuronal plasticity and excitotoxicity. However, as mentioned earlier, immature neurones are resistant to glutamate neurotoxicity and this may be related to maturation of the NMDA receptor complex (Peterson et al., 1989).

1.1.1.2. AMPA and kainate receptors

Due to the inability of agonists or antagonists to clearly distinguish between α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors, they are often collectively referred to as non-NMDA receptors. The AMPA receptors were initially called quisqualate receptors but were renamed AMPA receptors because quisqualate was found to additionally act on mGluRs while AMPA affected the ionotropic receptors more specifically (Krogsgaard-Larsen et al., 1980).

Mammals express four AMPA receptor subunits, GluR1-GluR4, distributed throughout the CNS (Danysz et al., 1995). The AMPA receptor has, at least, three separate binding sites at which agonists or antagonists can act: glutamate-binding, desensitization and intra-ion channel-binding sites. AMPA receptor channels are permeable only to Na^+ and K^+ and almost impermeable to Ca^{2+} in central neurones. They display a slight outward rectification and low permeability to Ca^{2+} (Ozawa et al., 1998), although it has also been found that AMPA receptors display a substantial permeability to Ca^{2+} and a strong inward

rectification in cultured rat hippocampal neurones. There are now several claims of cell death induced by AMPA receptor-mediated Ca^{2+} influx. The Ca^{2+} influx is largely determined by the GluR2 subunit. Most AMPA receptors contain the GluR2 subunit and are therefore impermeable to calcium while those without this subunit pass calcium which can mediate synaptic plasticity and, if in excess, neurotoxicity (Petralia et al., 2004). It is possible to induce inactivation of the NMDA receptor by the activation of the AMPA receptor (Bai et al., 2002) and the co-localization of AMPA and NMDA receptors occurs at synapses in different parts of the CNS (Bekkers & Stevens, 1989).

Kainate activates a distinct class of iGluRs called the kainate-preferring receptors, although it is also a potent agonist at the AMPA receptor. A family of kainate (KA) receptors has been cloned and the five subunits already identified are GluR5, GluR6, GluR7, KA1 and KA2. The modulatory actions of kainate receptors may vary from synapse to synapse (Ozawa et al., 1998).

1.1.2. Metabotropic glutamate receptors (mGluRs)

Metabotropic glutamate receptors, in contrast to ionotropic receptors, mediate their action through intermediary G proteins (Miller, 1991). These G protein receptors can control ion channels or intracellular messenger systems. In general, the action of glutamate on the metabotropic receptors produces changes in cellular function and membrane conductance on a slower time scale than the ionotropic receptors (Stone & Addae, 2002). Activation of presynaptic mGluRs causes a widespread reduction of glutamatergic transmission in the CNS. It is also now becoming clear that mGluRs regulate different neurotransmitters in a number of brain structures. They are highly enriched in the prefrontal cortex, a brain region critically involved in the regulation of cognition and emotion (Tyszkiewicz et al., 2004).

At the moment, cloning has identified eight mGluRs and they have been subdivided into three groups based on the homology of their amino acid sequences, the coupled signal transduction mechanisms, and their pharmacological properties investigated in transfected mammalian cell lines (Conn & Pin, 1997; Nakanishi, 1994). Group I mGluRs comprises the mGluR1 and the mGluR5 subtypes, which are positively coupled primarily to phospholipase C (PLC), resulting in increased phosphoinositide turnover, the subsequent activation of protein kinase C, and the release of Ca^{2-} from intracellular stores (e.g., the endoplasmic reticulum). On the other hand, groups II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, and mGluR8) subtypes are all negatively coupled to adenylyl cyclase, resulting in the reduction of cAMP formation in the cell (Baskys, 1992;

Conn & Pin, 1997). Some of the important roles of the mGluRs include the regulation of synaptic transmission via their modulation of ion channels and ionotropic glutamate receptors in diverse neuronal cell types (Pin & Duvoisin, 1995; Conn & Pin, 1997) and modulation of different subtypes of potassium channels in several types of neuronal cells (Conn & Pin, 1997). They have also been observed to have a role in various forms of synaptic plasticity (Bashir et al., 1993; Riedel et al., 1995; Aiba et al., 1994; Linden & Connor, 1993; Hartell, 1994). The mGluRs can exist on both postsynaptic and presynaptic sites (Pin & Duvoisin, 1995). However, groups II and III appear primarily localized to the presynaptic terminals and Group I to the postsynaptic sites (Shigemoto et. al, 1997). The presynaptic sites are mainly involved in glutamate release. Several mGluRs have been shown to modify Ca^{2+} levels, either at presynaptic or postsynaptic sites (Stone & Addae, 2002).

Due to the various identified effects of metabotropic receptors, they are now widely believed to modulate neuronal cell death. Activation of group I receptors promotes neuronal damage and antagonists are therefore neuroprotective (Pellegrini-Giampietro et al., 1999; Bruno et al., 1999). However, it has been reported that sustained (> 24 h) antagonist treatment can render mGlu1 receptors in CGNs supersensitive to agonists, with a simultaneous decrease in the effectiveness of antagonists (Lavreysen et al., 2005). Group II receptor agonists protect against NMDA-induced cell damage (Battaglia et al., 1998; Bond et al., 2000; Colwell & Levine, 1999), perhaps by a process involving the synthesis of a new protein and the generation of transforming growth factor-β (TGF-β) (Bruno et al., 1998). NMDA is the primary cause of insults that has been examined by most workers. However, it was recently shown that Group II receptor activation protects against damage caused by traumatic brain injury (Zwienenberg et al., 2001). The group III receptors have been given less attention, though their agonists appear neuroprotective (Gasparini et al., 1999; Pizzi et al., 2000). Group III agonists are not protective in mGluR4 (-/-) knockout mice, suggesting this receptor is primarily responsible for the neuroprotection (Bruno et al., 2000). The mGluR5 receptors are involved in the regulation of striatal glutamate release and this may imply an involvement of adenosine A_{2A} receptors in mGluR5-mediated effects (Pintor et al., 2000). The mGluR2/3 play a protective role against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) toxicity, but the efficacy of the agonist, LY379268, critically depends on the extent of the nigro-striatal lesion (Battaglia et al., 2003). Glutamate receptors communicate with Na^+/K^+-ATPase in rat cerebellar granule cells, according to a study that demonstrated the differences in the actions of several

metabotropic and ionotropic glutamate agonists on intracellular reactive oxygen species and the sodium pump (Boldyrev et al., 2003).

1.1.3. Glutamate production and metabolism

No evidence exists for any extracellular enzyme responsible for the metabolism of glutamate. Cellular uptake (the rapid route) and simple diffusion are the substantial means by which glutamate is removed from the extracellular space. Glutamate is mainly taken up into astrocytes. Glutamate transporters are important for the termination of signal transduction mediated by it and also for the prevention of its neurotoxicity (see Hinoi et al., 2004). These Na^+-dependent transporters are classified into five different subtypes: GLAST (glutamate aspartate transporter) or EAAT1 (excitatory amino acid transporter 1); GLT-1 (glutamate transporter-1 or EAAT2); EAAC1 (excitatory amino acid carrier 1) or EAAT3; EAAT4, and EAAT5. GLAST is known to predominate in the cerebellum. On the other hand, there are three vesicular glutamate transporters (VGLUT): VGLUT1, VGLUT2, and VGLUT3 (Gonzalez & Robinson, 2004; see Hinoi et al., 2004). Reactive oxygen species (ROS) oppose the removal of glutamate from the extracellular space by inhibiting the high-affinity glutamate transporters (Coyle & Puttfarcken, 1993). Uptake of glutamate into astrocytes can be followed by two pathways of metabolism. The first is the ATP-dependent conversion to glutamine for release into the extracellular fluid. The released glutamine is then taken up by neurones and reconverted to glutamate for reuse as transmitter. This is the glutamate-glutamine cycle (Fonnum 1993; Laake et al., 1995; Danbolt, 2001). The second possible metabolic route for glutamate involves its metabolism to α-ketoglutarate either by deamination or transamination. α-ketoglutarate may then be converted to succinate, fumarate and malate in the tricarboxylic acid (TCA) cycle. Malate may undergo further metabolism in the TCA cycle or be decarboxylated to pyruvate and lactate, a possibility already demonstrated *in vitro* (McKenna et al., 1996; Danbolt, 2001). The generated lactate is transported from astrocytes into the extracellular fluid from where it can enter into the neurones.

1.1.4. Glutamate signalling and receptor expression in bone

Research in the past few years has revealed that agents developed for use in neuroscience applications might be useful in the modulation of pathology in non-neuronal peripheral tissues, thus impacting on conditions such as osteoporosis, diabetes and wound healing (Skerry & Genever, 2001). An example of such tissues is bone, in which there is the

existence of glutamate signalling in a way similar to how this signalling occurs in the CNS (see review by Skerry & Taylor, 2001) *(Other aspects of bone biology including the specific characteristics of osteoblasts have been discussed in* ***section 1.3*** *of this thesis).* Interest in identifying sites where glutamate signalling could occur outside the CNS evolved as a result of studies of the distribution of radiolabelled glutamate receptor binding agents *in vivo* (Samnick et al., 1998; Nasstrom et al., 1993). Other non-neuronal peripheral tissues in which recent molecular biological analyses have given support to the expression of particular glutamate signalling molecules include the testis, pancreas, adrenal gland, pituitary gland, pineal gland, taste buds, lung, hepatocyte, thymus, cerebral endothelium, megakaryocytes, keratinocytes, lymphocytes, platelets, and heart (see Hinoi et al., 2004). Osteoblasts are now known to actively secrete glutamate, with several osteoblastic cell types spontaneously releasing between 2-7 nmoles glutamate per mg protein, which is equivalent to or greater than reported levels of glutamate release from depolarized neurones (Genever & Skerry, 2001; Skerry & Taylor, 2001). In MC3T3-E1 osteoblast-like cells, the anticonvulsant riluzole, which inhibits neuronal glutamate release, has been reported to inhibit glutamate release and ALP activity and, at higher concentrations, it induces apoptosis, suggesting a trophic role for glutamate in bone development (Genever & Skerry, 2001). Chenu et al. (1998) have also observed that, glutamate receptors, especially the NMDAR1, are expressed by bone cells and are involved in bone resorption. There is also evidence of expression of NMDAR1 and NMDAR2D subunits by human and rat osteoblasts and osteoclasts, suggesting a novel glutamate signalling pathway in bone (Patton et al., 1998). The same authors also found evidence of the expression of post-synaptic density-95 (PSD-95), the NMDA receptor clustering protein associated with signalling in the CNS, while expression of the non-NMDA clustering protein, glutamate receptor interacting protein (GRIP), has similarly been reported (Skerry & Taylor, 2001). Functional NMDA receptors are also expressed by bone marrow megakaryocytes (Genever et al., 1999). A later development confirmed the expression in rat calvaria and MG63 osteoblastic cells of several NR2 subunits mRNAs, including NR2A, NR2B, and NR2D (Itzstein et al., 2001). A study by Genever and Skerry (2001) found that, in the presence of the NMDA channel antagonist MK-801 (100μM), glutamate release was moderately but significantly inhibited. Through binding studies and electrophysiological recordings from patch-clamped clonal human osteoblast-like cells, it was shown that the NMDA receptor displayed similar but not identical gating characteristics to the same receptors in the CNS (Laketic-Ljubojevic et al., 1999). Subsequently, it was demonstrated that primary rat

osteoblasts exhibited much closer electrophysiological characteristics to neuronal cells, and that the responses were regulated by metabotropic receptor function (Gu & Publicover, 2000). Gu et al. (2002) showed that the NMDA type glutamate receptors expressed by primary rat osteoblasts have the same electrophysiological characteristics as neuronal receptors. However, currents in the human osteoblastic MG63 cells, unlike those of mammalian neurones, are blocked by Mg^{2+} in a voltage-insensitive manner. Osteoblasts are also known to constitutively express mRNA for non-NMDA receptors such as the GluR3 subunit of AMPA receptors and KA1 and KA2 subunits of kainate receptors (Hinoi et al., 2002). The identification of glutamate receptors in bone cells points to a novel means of paracrine communication in the skeleton (Laketic-Ljubojevic et al., 1999). As further evidence, Mason et al. (1997) reported the mechanically regulated expression of a neural glutamate transporter in bone, implicating a role for excitatory amino acids as osteotropic agents. In fact, it has been proposed that, in bone, a mechanism similar or identical to long term potentiation (LTP) in the CNS regulates long-term osteogenic responses resulting from brief periods of mechanical loading (Spencer et al., 2001). In addition, osteoblasts have been shown to express a functional glutamate transporter GLAST (EAAT1), which is responsible for cellular glutamate uptake, and many SNARE (soluble *N*-ethyl maleimide-sensitive factor (NSF) attachment protein (SNAP) receptor) and related vesicular proteins required for regulated transmitter release, which colocalize with immunoreactive glutamate (Mason et al., 1997; Bhangu et al., 2001). Another glutamate transporter, GLT-1, has also been shown to reside in bone (Huggett et al., 2000). In addition to glutamate transporters, osteoblasts express the exocytotic machinery needed for complete glutamate release, with their glutamate release appearing to be dependent on an AMPA-type glutamate autoreceptor (Genever & Skerry, 2001). These authors found that blockade of AMPA-type glutamate receptors with the highly selective non-competitive antagonist CFM-2 resulted in a significant, dose-dependent inhibition of glutamate release in MG63 cells at 5-50μM concentrations but the antagonist did not affect depolarization-induced inhibition of glutamate release. Bhangu et al. (2001) also reported evidence for targeted vesicular glutamate exocytosis in osteoblasts (mimicking presynaptic glutamatergic neurones) and demonstrated that osteoblastic cells actively release glutamate in a differentiation-dependent manner. It has been suggested that L-glutamic acid may, at least in part, play a role in mechanisms associated with cellular proliferation and/or differentiation through group III mGluRs functionally expressed in rat calvarial osteoblastic cells (Hinoi et al., 2001). By reverse transcriptase polymerase chain reaction (RT-PCR), expression of

mGluR1b (but not mGluR1a, 2, 3, 4, 5, or 6) has been detected in rat femoral osteoblasts and this shows that complex glutamatergic signalling can occur in bone tissue (Gu & Publicover, 2000). It is therefore now understood that osteoblasts express the whole range of glutamate receptors which display the same electrophysiological characteristics as their neuronal counterparts. Since bone cells need to undergo changes in cell mass in response to exercise, for example, it has been hinted that glutamatergic transmission could play a vital role in these skeletal adaptations, not only as a trophic factor but also perhaps by inducing a long term potentiation-like process (Skerry & Taylor, 2001). The need for a tissue like bone - that is slow to respond to changes in circumstances - to have a system capable of responding to rapid changes in the millisecond scale could be conceived in the light of a requirement for bones to be capable of discriminating the difference between the same force applied quickly or slowly (Skerry & Taylor, 2001). Thus, glutamate receptor agonists and antagonists may play a role in the treatment of bone disease and manipulation of bone cell function by moderators of glutamate action could lead to novel therapies for disorders of bone such as osteoporosis (Mason et al., 1997). However, more *in vivo* studies are being undertaken to specifically delineate the physiological roles of glutamate in bone (Skerry & Taylor, 2001). One major challenge in this arena is that the use of antagonists that cross the blood-brain barrier (BBB) in *in vivo* bone studies could cause alterations in the CNS and thus make interpretations difficult. Some efforts are being made to get round this by the use of quinoxalinediones, which do not cross the BBB. These agents have been shown to potentiate the effects of mechanical loading, resulting in more new bone formed than in the limbs of animals treated with vehicle alone (Skerry & Taylor, 2001).

1.2. Adenosine and its receptors

Because adenosine is well established as neuroprotective against glutamate-induced neurotoxicity in the CNS, we would now consider some aspects of the biological functions of this purine nucleoside and its receptors, both in health and in disease states.

1.2.1. Physiological roles

Adenosine is now widely accepted to be a major non-peptide neuromodulator that acts through specific ectocellular receptors, having a multitude of effects on many different organs (Stone & Simmonds, 1991). This purine nucleoside has been observed to act as both a homeostatic modulator and a neuromodulator at the synaptic level and is known as a substantial metabolite in neurones and other mammalian cell types, participating in the

synthesis of nucleic acids, amino acids metabolism and modulation of cellular metabolic status (Stone, 1985). In relation to its homeostatic role in the control of cellular metabolism, adenosine has been called a "local hormone" (Arch & Newsholme, 1978), and in terms of its role in stressful conditions has been termed a "retaliatory metabolite" (Newby, 1984). It also fulfils the criteria of an autacoid (Klinger et al., 2002). There are circumstances in which the intracellular concentration of adenosine increases from the nanomolar range to micromolar concentrations, and it is released to the extracellular medium to maintain the metabolism of neighbouring cells (Meghji, 1991).

In the CNS, adenosine has profound effects, including major behavioural (e.g., sedation and anti-seizure activity) (Snyder, 1985) and neuroprotective effects (Dragunow & Faull, 1988). In fact, in various conditions like hypoxia, ischaemia and brain injury, the extracellular levels of adenosine are increased. Many experimental and pathological conditions are known in which there is increased release of glutamate from cells. In these conditions, increase in the extracellular levels of adenosine has also been observed (Stone & Simmonds, 1991; Latini & Pedata, 2001). The neuroprotective potential of adenosine exploits the mechanisms of blocking the presynaptic release of glutamate after excessive stimulation of glutamate receptors and blocking the postsynaptic sensitization of NMDA receptors (Nishizawa, 2001). This is relevant to most pathological conditions including the ischaemic stroke caused by transient or permanent reduction of cerebral blood flow, which is a leading cause of death and disability in humans (Nishizawa, 2001).

The effects of adenosine are widespread and pleiotropic (Klinger et al., 2002). In the periphery, extracellular adenosine modulates many physiological events in several organ systems by activating A_1 receptors. These include the inhibition of lipolysis and stimulation of glucose uptake in adipocytes and the cardiovascular effects of negative inotropy and chronotropy (Olsson & Pearson, 1990; Pelleg, 1993). On the other hand, in vascular beds, A_{2A} receptor activation causes smooth muscle relaxation, leading to vasodilatation and reduction in blood pressure (Olsson & Pearson, 1990), while in platelet membranes, A_{2A} receptor activation increases intracellular cAMP levels, with the resultant effect of inhibiting platelet aggregation (Palmer & Stiles, 1995). Neutrophil activation could be inhibited by adenosine, leading to decreased neutrophil adhesion and prevention of capillary plugging (Miller & Hsu, 1992). A number of aspects of renal and respiratory physiology could also be affected by exogenous adenosine.

1.2.2. Formation, release, transport and metabolism

Adenosine can be synthesised either intracellularly *de novo* or retrieved by salvage and transported across the membrane, or it can result from the metabolism of ATP.

The final stage of the *de novo* pathway of adenosine production involves the dephosphorylation of adenosine monophosphate (AMP) to adenosine by cytosolic 5'-nucleotidase (Stone & Simmonds, 1991; Meghji, 1991), while the salvage pathway occurs through the transmethylation of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH), which is metabolised further by SAH-hydrolase to adenosine and homocysteine (Ueland, 1982). Another salvage route is the recycling of hypoxanthine to inosine monophosphate (IMP) by the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) (Stone & Simmonds, 1991).

Adenosine is released in a calcium-dependent manner from nerve terminals following nerve stimulation (Meghji, 1991), although there is no evidence of its storage in synaptic vesicles and release from nerve terminals like other neurotransmitters (White & Hoehn, 1991). Much of the released adenosine arises from the intracellular adenosine that can pass bidirectionally via a facilitated diffusional transporter. The accumulation of adenosine during conditions such as ischaemia, hypoxia, head injury, and seizures (Hagberg et al., 1987; Phillis, 1990; Bell et al., 1998) is due to increased adenosine formation resulting from the imbalance between energy supply and demand (Bardenheuer & Schrader, 1986). Therefore, adenosine levels can be regulated by the balance of energy supply and demand (Stone et al., 1991). Potassium, veratridine and electrical stimulation have been shown to release adenosine both *in vivo* and *in vitro* from various brain regions (White & Hoehn, 1991; Sciotti et al., 1993). The adenosine released during EAA activation could provide much protection against EAA-mediated excitotoxicity in the CNS. Some of the adenosine is produced from the extracellular metabolism of adenine nucleotides, in particular ATP (Wieraszko et al., 1989; Cunha et. al., 1996), released into the extracellular space by a non-synaptic mechanism (Stone et al., 1991; Brundege & Dunwiddie, 1997). ATP is co-stored with either noradrenaline in the sympathetic nerves or acetylcholine in the cholinergic synaptosomes (Morel & Meunier, 1981; White, 1988; Silinsky, 1975; Lew & White, 1987; von Kügelgen & Strake, 1991a; 1991b; Zimmermann, 1994). This ATP is subsequently converted to adenosine by ectonucleotidases.

Adenosine is taken up into neurones and neighbouring cells and appears to be converted back into ATP intracellularly (Bender et al., 1981). Its uptake occurs via nucleoside transporters, which operate bidirectionally by a facilitated diffusion mechanism. However,

this process of adenosine uptake is regulated by the concentration gradient for adenosine, implying that uptake into cells is only possible if the intracellular concentration of adenosine is kept below the extracellular concentration. Adenosine becomes inactivated either via the breakdown to inosine by cytosolic adenosine deaminase and subsequently hypoxanthine and xanthine, or via conversion to adenosine monophosphate through phosphorylation by cytosolic adenosine kinase (Arch & Newsholme, 1978; Meghji, 1991). Because of the presence of adenosine deaminase in an extracellular membrane-associated form, adenosine can also be inactivated extracellularly without any need to be taken up (Meghji, 1991). It seems that phosphorylation occurs at low adenosine concentrations whereas the deamination pathway is important at higher adenosine concentrations (Arch & Newsholme, 1978b; Meghji, 1991).

1.2.3. Adenosine receptors

To date, four adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 (all of them members of the P1 purinoceptor) have been cloned and pharmacologically characterized. All of them belong to the family of G protein-coupled receptors acting through intracellular second messengers and have the seven transmembrane helices common to G protein-associated receptors. They are most closely related to the receptors for biogenic amines (Jacobson & Gao, 2006). Among the human adenosine receptors, the most similar are the A_1 and the A_3 adenosine receptors (49% sequence similarity) and the A_{2A} and the A_{2B} adenosine receptors (59% similarity) (Jacobson & Gao, 2006).

Information about the distribution of adenosine receptors has come from the studies of biochemical and physiological roles of adenosine as well as radioligand binding studies. A_1 receptors predominantly exist in the molecular layer of the cerebellum and in the CA1 and CA3 regions of the hippocampus (Goodman & Snyder, 1982; Lee & Reddington, 1986), while moderate A_1 receptor levels are found in the thalamus, caudate-putamen, septum and cerebral cortex. Northern blot analysis of mRNA from various tissues has shown that A_1 receptor message is also found in kidney, heart, testis, and epididymal fat (Stehle et al., 1992). On the other hand, while A_{2A} receptor expression is limited to the striatum, nucleus accumbens and olfactory tubercle, A_{2B} receptor mRNA is distributed throughout the brain (Stehle et al., 1992). The more sensitive RT-PCR technique has shown that A_{2A} receptor mRNA could be detected in all brain regions (Dixon et al., 1996). High levels of A_{2A} receptor exist in striatum and nucleus accumbens but lower levels are found in the cortex, amygdala, olfactory tubercles, hippocampus, hypothalamus, thalamus, and cerebellum. Co-

localization of A_{2A} receptors with other binding sites for other neurotransmitters has been found in some brain areas. For example, A_{2A} receptors and dopamine D_2 receptors have been found co-localized on striatopallidal neurones on the same cell and they are known to influence each other's function (Johansson et al., 1997). Peripherally, the use of Northern blotting experiments has shown reasonable expression of the A_{2A} receptors in the heart, lung, thymus, and epididymal fat and has also demonstrated the A_{2A} receptor message in the caecum, lung, intestine, and urinary bladder, though with lower distribution in the lung (Stehle et al., 1992). In fact, in neutrophils, adenosine A_{2A} receptor activation delays apoptosis (Walker, et al., 1997), while theophylline induces apoptosis of neutrophils through adenosine A_{2A} receptor antagonism (Yasui et al., 2000).

Interactions between adenosine A_1 and A_{2A} receptors have been reported. It is known that activation of adenosine A_{2A} receptor inhibits A_1 receptor activation (Cunha, 2001). The A_{2A} receptor agonist, 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680), attenuates neuronal sensitivity to the A_1 agonist CPA (O'Kane & Stone, 1998) and also induces a low-affinity receptor site for another A_1 agonist, 2-chloropentyladenosine (CCPA) (Dixon et al., 1997), possibly through protein kinase C (Stone, 2005). However, upon agonist stimulation, the A_{2A} receptor response rapidly desensitizes, though reversibly, within a time frame of less than an hour (Klinger et al., 2002). According to Cassada et al. (2002), "adenosine A_{2A} agonists may exert neuroprotective effects by binding to inducible neuronal A_{2A} receptors that are upregulated during spinal cord reperfusion and reduced in response to administration of an A_{2A} receptor-specific agonist".

The A_3 receptor subtype was cloned and characterised in the early 1990s (Meyerhof et al., 1991; Zhou et al., 1992) and its activation mediates the inhibition of adenylate cyclase (Abbracchio et al., 1998).

1.2.3.1. Agonists and antagonist ligands

Known agonists for the adenosine receptor are analogues of adenosine modified in either the purine or ribose portions of the molecule (Williams, 1991). Adenosine analogues have the potential to interact with nucleoside transporter sites as well as to function as adenosine potentiators in addition to acting at extracellular receptor sites (Engler, 1987).

A_1-selective agonists include N^6-cyclohexyladenosine (CHA) and R-N^6-phenylisopropyl adenosine (R-PIA) (Williams, 1987), but N^6-cyclopentyladenosine (CPA), (2S)-N^6-[2-endo-norbornyl]adenosine (*S*-ENBA) and CCPA are the most potent and selective (Miller

& Hsu, 1992). The most selective A_{2A} receptor agonist is CGS21680, which has high affinity and selectivity (140-fold) for A_{2A} over A_1 receptors. CGS21680 and similar radioligands can directly label the A_{2A} receptor in rat brain without the need to block the binding activity at the A_1 receptor (Jarvis et al., 1989). However, a number of reports have indicated that CGS21680 could also be quite potent at rat, and particularly at human, A_3 receptors (Muller, 2000). To date, no useful radioligands for the A_{2B} receptors have been developed. N^6-(3-iodobenzyl)-adenosine-5'-*N*-methylcarboxamide (IB-MECA) was the first highly potent and selective A_3 receptor agonist both *in vivo* and *in vitro* (von Lubitz et al., 1994; Stambaugh et al., 1997; Jacobson et al., 1997). 2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-*N*-methylcarboxamide (2Cl-IB-MECA) is also very potent at the A_3 receptor (Jacobson, 1998).

The 8-phenylxanthines were developed as potent A_1-selective antagonists by the replacement of the phenyl on the 8-position of xanthine with cyclopentyl, resulting in compounds with improved affinity, aqueous solubility and selectivity for A_1 receptors. 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX) is a highly selective A_1 antagonist (Linden, 1991). KFM19 is a keto derivative of DPCPX, reported to have increased aqueous solubility (Ad Ijzerman & van Galen, 1990). The most commonly used A_{2A} antagonists are 9-chloro-2- (2-furanyl)[1, 2, 4] triazolo [1,5-c] quinazolin-5-amine (CGS15943), a nonxanthine antagonist; 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol (ZM241385), and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-e]-1,2,4-triazolo [1,5-c] pyrimidine (SCH58261). A study by Ongini et al. (1999) showed ZM241385 and in particular SCH58261 as having good A_{2A} selectivity. ZM241385 is 80-fold selective for A_{2A} versus A_{2B} receptors, and 500- to 1000-fold selective for A_{2A} versus A_1 receptors (Palmer et al., 1995). No selective antagonists have been reported for the A_{2B} receptor (Jacobson et al., 1993). In fact, the availability of specific agonist and antagonist ligands has greatly facilitated studies of the distribution of A_1 adenosine receptors while the characterization of A_2 receptors has proceeded somewhat more slowly due to a lack of specific ligands (Reddington & Lee, 1991). Jacobson et al. (1997) reported putative A_3 receptor antagonists belonging to three diverse chemical classes, namely the flavonoid MRS 1067, the 6-phenyl-1, 4-dihydropyridines MRS 1097 and MRS 1191, and the triazoloquinazoline MRS 1220. Natural phenolic derivatives and other compounds such as triazolonaphthyridine, thiazolopyrimidine and a derivative of the triazoloquinazoline, CGS15943 (9-Chloro-2-(2-furanyl)-[1,2,4]triazolo[1,5-c]quinazolin-5-amine), have also been reported as high-affinity A_3 receptor-selective antagonists

(Jacobson et al., 1996; Kim et al., 1996). Generally speaking, studies using adenosine antagonists must be aware that long-term (e.g., 24 h) treatment with these antagonist compounds can have effects that resemble the acute (e.g., 1 h) effects of adenosine receptor agonists, and vice-versa (Jacobson et al., 1996).

1.2.3.2. G-protein coupling

Adenosine receptors are linked to many effectors via various classes of G proteins (Fredholm et al., 1989). It does appear that the postsynaptic effects of adenosine are mediated by a pertussis toxin (PTX)-sensitive G protein (Mynlieff & Beam, 1994), as it has been shown that these effects could be blocked by pertussis toxin *in vivo* (causing adenylate cyclase inhibition and increased K^+ conductance), while presynaptic effects have not been affected by PTX (Gilman, 1987). A_1 receptor-mediated responses are coupled via pertussis toxin-sensitive G proteins to different effectors including adenylate cyclase, phospholipase C, Ca^{2+} channels, Cl^- channels and K^+ channels (Dascal, 2001). Adenylate cyclase (AC), the enzyme that converts ATP to cAMP, is regulated by hormones, neurotransmitters and other regulatory molecules through its interaction with G proteins and receptors. A total of nine isoforms of adenylate cyclase has been cloned in mammals, numbered AC1-AC9 (Hanoune et al., 1997). Sattin & Rall (1970) showed that adenosine increases cyclic AMP accumulation in cortical slices. Conversely, in cultured rat forebrain neurones, elevation of intracellular cAMP evokes activity-dependent release of adenosine (Lu et al., 2004). A biphasic effect of adenosine on cAMP accumulation has been reported, as low micromolar concentration stimulates adenylate cyclase while higher micromolar concentration inhibits it (Haslam & Lynham, 1972). These biphasic concentration-dependent effects of adenosine are suppressed by A_1 and A_2 antagonists such as caffeine and other methylxanthines, i.e., A_1 and A_2 adenosine receptor antagonists block both the stimulatory and the inhibitory actions of adenosine on adenylate cyclase (Sattin & Rall, 1970; Hanoune, et al., 1997). In general, A_2 receptor agonists increase the rate of ATP metabolism to cAMP by enhancing the activity of adenylate cyclase through its action on stimulatory G protein (G_s), while adenosine A_1 receptor agonists inhibit the rate of ATP metabolism to cAMP by attenuating the activity of adenylate cyclase through its action on inhibitory G protein (G_i).

Another group of intracellular second messengers includes inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG), which are generated from the breakdown of the phospholipid, phosphatidylinositol-4,5-biphosphate (PIP_2), via the action of PLC. These

compounds act as second messengers mediating the effects of many neurotransmitters including acetylcholine (ACh), 5-hydroxytryptamine (5-HT) and noradrenaline and form internalization of the extracellular signal. However, adenosine does not seem to have an effect on phosphatidylinositol turnover, even though there have been some reports that adenosine and its several analogues mediate the regulation of phosphatidylinositol (PI) responses to histamine and neurotransmitters such as noradrenaline (Morgan, 1991).

1.2.4. Adenosine and neuroprotection

It is known that purine nucleosides such as adenosine have the ability to modulate Ca^{2+} activity within cells and especially at the presynaptic sites, where adenosine A_1 receptors decrease the release of several neurotransmitters while adenosine A_{2A} receptors increase the release of some transmitters (Chen & Lambert, 1997; Goncalves et al., 1991; Goncalves & Ribeiro, 1996). The manner of this calcium modulation has yet to be fully elucidated. Under most experimental conditions, the effect of adenosine or adenosine receptor agonists is an inhibition of neuronal activity mediated by A_1 receptor activation. The A_1 receptors are linked to inhibition of the release of the most classical neurotransmitters, including glutamate, gamma-amino butyric acid (GABA), norepinephrine, acetylcholine, 5-hydroxytryptamine and dopamine (Dunwiddie, 1985; Greene & Hass, 1991). The most profound inhibitory effects are generally seen on excitatory glutamatergic systems. Because inhibitory modulation of inhibitory systems (e.g., GABA) is less frequently observed, the net effect of adenosine receptor activation in almost all regions of the brain is to reduce excitability. The inhibitory action of adenosine in the CNS is predominantly due to its presynaptic action (Lupica et al., 1992). Intracellular calcium buffers do not prevent the inhibitory effects of adenosine on acetylcholine release (Silinsky et al., 1989), a study which, alongside others, suggests that adenosine inhibits transmitter release from motor nerves by reducing the responsiveness of some component of the secretory apparatus to Ca^{2+}. Adenosine, by inhibiting phospholipase, could inhibit neurotransmitter release by reducing intraneuronal Ca^{2+} mobilization and by attenuating the activation of PKC. Adenosine could also modulate potassium currents in presynaptic terminals. This is evidenced by the demonstration that blockers of potassium channels such as 4-aminopyridine are able to prevent the inhibitory effects of adenosine on transmitter release in peripheral sympathetic nerves (Stone, 1981) and in glutamate-releasing fibres in the hippocampus (Scholfield & Steel, 1988). As further evidence of neuroprotection, Gupta et al. (2002) observed the protective effect of

adenosine against neuronal injury induced by middle cerebral artery occlusion (MCAO) in rats as evidenced by diffusion-weighted imaging.

In general, the A_1 receptor mediates, at least, three cellular mechanisms, including inhibition of transmitter release (glutamate in particular), hyperpolarization of neurones, and direct inhibition of certain kinds of Ca^{2+} channels. The inhibitory modulation of transmitter release by adenosine depends on a direct inhibition, via G protein, of mainly N-type calcium channels, although this is still a subject of much debate (Wu & Saggau, 1994; Ribeiro, 1995). Further inhibitory mechanisms appear to operate in motor neurones. These mechanisms are capable of reducing excitotoxicity by decreasing Ca^{2+} entry, which is thought to be a key step in excitotoxic damage. Again, by reducing metabolic rate, adenosine helps to preserve ATP stores that are essential for pumping Ca^{2+} out of the cell (Dunwiddie & Masino, 2001). It should be noted, however, that, while activation of A_1 receptors can provide neuroprotection, the protective effect appears to become saturated by high levels of endogenous extracellular adenosine during oxygen-glucose deprivation (Lobner, 2002). Deletion of the A_1 receptor gene in a study by Olsson et al. (2004) did not alter neuronal damage following ischaemia in mice *in vivo* or *in vitro*, suggesting that the effects of the receptor are compensated for in knockout animals. Evidence exists for a direct effect of adenosine on Ca^{2+} fluxes into synaptic terminals, though there are also indications that adenosine might alter the availability of intraterminal Ca^{2+} to the secretory/release process. These adenosine effects on Ca^{2+} activity therefore often oppose the effects induced by a rise in calcium levels that occurs with agents such as glutamate.

Even though adenosine receptors may also enhance neurotransmitter release, these actions are less common than the inhibition of transmitter release (Cunha et al., 1994). In striatal cholinergic nerve terminals, at the neuromuscular junction and in hippocampus GABAergic nerve terminals, modification of neurotransmitter release via A_{2A} receptors is decreased by inhibitors of adenylate cyclase (Cunha & Ribeiro, 2000a; Gubitz et al., 1996). Protein kinase C (PKC) or phospholipase C inhibitors are able to inhibit the effects of A_{2A} receptors, whereas protein kinase A (PKA) inhibitors slightly inhibit or do not change the effects of A_{2A} receptor activation. It has been demonstrated further that, activation of A_{2A} receptors triggers cAMP accumulation but a parallel signalling system via PKC is also activated by presynaptic A_{2A} receptor activation (Cunha & Ribeiro, 2000b). The A_{2A} receptor can also modify neurotransmitter release via P-type calcium channels where its activity seems to be modified by both PKC and PKA. However, it is not clear whether both

pathways can modulate this same target or just P-type calcium channels are the main target for PKC pathway (Cunha, 2001).

The adenosine A_{2A} receptor can preferentially elicit excitatory amino acid release over inhibitory amino acid release from rat cerebrocortical synaptosomes (Marchi et al., 2002) and can also increase glial glutamate efflux (Li et al., 2001). It seems that it is the antagonism of this action that most probably underlies the neuroprotective effect of A_{2A} receptor blockade, which has been demonstrated *in vivo*. Antagonists at adenosine A_{2A} receptors have been shown to be neuroprotective in animal models of ischaemia (Sheardown & Knutsen, 1996; Monopoli et al., 1998; Ongini et al., 1997) and excitotoxicity (Jones et al., 1998a; b; Stone et al., 2001). It has been shown that the A_{2A} receptor can inhibit evoked GABA release in the striatum (Kirk & Richardson, 1995), although Sebastiao & Ribeiro (1996) have suggested that this effect may only be specific to receptors from these brain regions that are already proposed as being atypical, owing to their enhanced G_s coupling. Blockade of striatal adenosine A_{2A} receptor reduces, through a presynaptic mechanism, quinolinic acid-induced excitotoxicity, implying a possible relevance to neuroprotective interventions in neurodegenerative diseases of the striatum (Popoli et al., 2002). Because blockade of A_{2A} receptors has differential effects on striatal cell death *in vivo* depending on its ability to modulate presynaptic over postsynaptic receptor activity, therapeutic use of A_{2A} antagonists in Huntington's as well as in other neurodegenerative diseases could exhibit undesirable biphasic neuroprotective-neurotoxic effects (Blum et al., 2003). The results of studies by Petzer et al. (2003) suggest that monoamine oxidase B (MAO-B) inhibition may contribute to the neuroprotective potential of A_{2A} receptor antagonists such as KW-6002 and open the possibility of designing dual-targeting drugs that may have enhanced therapeutic potential in the treatment of Parkinson's disease.

An interesting conundrum is the observation through *in vivo* data that both adenosine A_{2A} receptor agonists and antagonists are neuroprotective. The neuroprotection that is paradoxically seen with adenosine A_{2A} receptor activation *in vivo* could be due to effects on cerebral blood flow, glucose utilization, platelet aggregation and superoxide generation from neutrophils (Stella et al., 1996; Hourani, 1996; Nehlig et al., 1994; Cronstein et al., 1985). It is possible that adenosine could reduce free radical-associated cell damage that occurs in ischaemia and excitotoxicity (Lafon-Cazal et al., 1993; Reynolds & Hastings, 1995; Patel et al., 1996). In PC12 cells, hypoxia stimulates the expression of the adenosine A_{2A} receptor gene (Kobayashi & Millhorn, 1999) and chronic hypoxia is known to reduce

adenosine A_{2A} receptor-mediated inhibition of calcium current via downregulation of PKA (Kobayashi, et al., 1998). Adenosine A_{2A} receptors could therefore exert neuroprotection *in vivo* via peripheral mechanisms of action while centrally, e.g., in hippocampal synaptosomes, they could enhance evoked GABA release (Cunha & Ribeiro, 2000a), resulting in a general reduction of neuronal excitability and hence limiting cell death during an ischaemic insult. It is also becoming clear that the adenosine A_{2A} receptor has a wide range of intracellular effectors that can influence neuronal excitability and viability, thus exerting a direct neuroprotective effect. These effectors include the PLC / IP_3 / calmodulin and calmodulin kinase II pathway (Wirkner et al., 2000); ATP-sensitive potassium channels (Haynes, 2000), and growth factors, especially nerve growth factor (NGF) (Arslan et al., 1997; Arslan & Fredholm, 2000).

A number of interactions at the receptor level have been identified between purines and glutamate (Stone & Addae, 2002), where activation of NMDA receptors or group I metabotropic glutamate receptors could suppress the presynaptic inhibitory effects of adenosine in the hippocampus (Bartrup & Stone, 1988; 1990; Shahraki & Stone, 2003). The converse has also been demonstrated with adenosine and NMDA (de Mendonca et al., 1995; Norenberg et al., 1997). Nitric oxide and superoxide anion, which are generated upon NMDA receptor activation, have been reported to block presynaptic adenosine A_1 receptor responses in rat hippocampus (Shahraki & Stone, 2004). Agonists at other ionotropic glutamate receptors did not reduce responses to adenosine (Stone, 2005). It seems that the interaction between NMDA and adenosine involves the enhancement of responses mediated by A_{2A} receptors, so that A_{2A} receptors can, in turn, suppress A_1 receptor activation (Stone, 2005).

The effects of adenosine A_3 receptor stimulation depend on the differential impact of this receptor on both neuronal and non-neuronal elements of the cerebral tissue, for example, astrocytes, microglia, and vasculature (von Lubitz et al., 2001). Adenosine A_3 receptors, though cardioprotective, proved severely cerebrodestructive when administered prior to global ischaemia in gerbils and the stimulation of the A_3 adenosine receptors appears to reduce the efficacy of some of the neuroprotective actions mediated by adenosine A_1 receptors (von Lubitz, 1999). Data from pyramidal cells of the rat cingulate cortex show that a high level of endogenous adenosine (occurring during hypoxia) activates A_3 receptors, mediating a depression of the synaptic transmission. The A_3 receptors inhibit glutamate release additionally to and also independently of the A_1 receptors. These two distinct mechanisms of synaptic modulation could contribute to the neuroprotective action

of adenosine during hypoxia and therefore offer new approaches for therapeutic strategies (Lewerenz et al., 2003). The A_3 adenosine receptor induces cytoskeleton rearrangement in human astrocytoma cells via a specific action on Rho proteins (Abbracchio et al., 2001).

1.2.5. Adenosine and bone cell development

Alongside growth factors, cytokines and glutamate, adenosine is also a modulator of bone remodelling in the osseous microenvironment. Shimegi (1996) reported that adenosine has a mitogenic effect on MC3T3-E1 cells. Adenosine could enhance MC3T3-E1 cell proliferation by itself and it was shown to also enhance platelet-derived growth factor-induced mitogenicity in them (Shimegi, 1998). Cyclopentylxanthine and pertussis toxin (PTX) partly antagonised this proliferating effect, confirming A_1 receptors partly mediated the action. PTX-sensitive G proteins have been reported to be potent mediators of cell proliferation and alkaline phosphatase (ALP) activity of osteoblast-like cells in response to factors acting through G protein-coupled receptors such as epinephrine (adrenaline) (Suzuki et al., 1998). However, some of the mitogenic effect of adenosine was not xanthine- and PTX-sensitive, meaning that other adenosine receptors played a part in the effect as well. Nevertheless, despite these reports, controversy still surrounds the expression of P1 receptors (adenosine receptors) in osteoblasts, with evidence for (Lerner, et al., 1987) and against (Jones et al., 1997) their expression. There is growing evidence that extracellular nucleotides, signalling through P2 receptors, might also play a significant role in modulating osteoblast and osteoclast function (Hoebertz et al., 2003). Osteoblast responses to nucleotides were recently shown to increase during differentiation (Orriss et al., 2006).

1.3. Aspects of bone biology

This section would introduce the bone tissue in relation to the active process of remodelling and then consider the bone-forming osteoblasts in some detail.

1.3.1. Bone tissue

Traditionally, the skeleton is recognised as a means of locomotion, protection of vital organs and provision of a readily available source of phosphorus and calcium, but only recently was it recognised as a dynamic tissue constantly remodelling itself. Two main cell types carry out this act: osteoclasts and osteoblasts. In order to achieve bone renewal, osteoclasts erode cavities removing old or damaged bone tissue while osteoblasts, upon

recruitment and activation, synthesise new bone matrix (Genever & Skerry, 2001). Bone matrix is composed of type 1 collagen and several noncollagenous proteins (Raynal et al., 1996) and inorganic materials, mainly calcium hydroxyapatite. According to Weinstein & Manolagas (2000), remodelling or turnover is carried out by juxtaposed osteoclasts and osteoblasts comprising temporary anatomical structures known as basic multicellular units (BMUs). An imbalance between osteoblasts and osteoclasts has been implicated in the pathogenesis of certain metabolic diseases including osteoporosis, Paget's disease, and osteopetrosis (Ducy et al., 2000; Teitelbaum, 2000).

Bone turnover involves activation, the conversion of an inactive skeletal surface to a remodelling site. The reconstitution by osteoblasts of previously osteoclast-resorbed cavity with new bone occurs through a process known as coupling. The adult skeleton, containing approximately 35 million bone structural units (BSUs) (one from each previous BMU), is almost completely regenerated every 10 years (Corral et al., 1998). It is known that bone sialoprotein (BSP), which is one of the most prominent of noncollagenous proteins in the bone matrix, stimulates *in vitro* bone resorption (Raynal et al., 1996). Recently, a role was defined for acetylcholinesterase (AChE) as a bone matrix protein. Using Western blot analysis, Inkson et al. (2004) identified expression of two AChE species in osteoblastic cells, a major species of 68 kDa and a less abundant species of about 55 kDa. They also confirmed that AChE secretion levels corresponded with ALP activity and its expression was regulated by mechanical strain both *in vitro* and *in vivo*. As bone formation approaches completion, some osteoblasts differentiate into osteocytes (the primary sensors of strain in bone) and become enveloped by the developing matrix; others become quiescent bone-lining cells, and the remainder most probably undergo apoptosis (Lynch et al., 1998; Jilka et al., 1998; Skerry & Taylor, 2001).

Postmenopausal osteoporosis is one of the prevalent disorders of bone remodelling. It is characterised by low bone mass and microarchitectural deterioration of the skeleton, leading to an increased risk of fracture after minimal trauma. It stands as the most common form of osteoporosis and the most common of all systemic osteopathies. The fractures result from the accelerated loss of bone that occurs in women after natural or surgically-induced menopause. The oestrogen loss that occurs during menopause causes an increase in interleukin-1 (IL-1), IL-6 and tumour necrosis factor-α (TNF-α) production, which mediates an increase in osteoblast and osteoclast numbers and consequently an increased frequency of their activation (Jilka, 1998). As well as changes that occur in the number of

bone cells, deeper-than-normal erosion of cavities also takes place and this increased remodelling alone causes a loss of bone mineral density because bone resorption is faster than bone formation and new BMUs are less dense than older ones. The accelerated loss of bone that occurs after oestrogen deficiency is not only from an increase in osteoclast number but also from an increase in osteoblast and osteocyte apoptosis. However, in osteopetrosis, increases, rather than decreases in bone mass, occur.

In bone, alkaline phosphatase (ALP) is a ubiquitous membrane-bound tetrameric enzyme and is commonly used as a marker of osteogenic development *in vitro* and in the clinic (Henthorn, 1996). Robison (1923) was the first to postulate that alkaline phosphatase plays a role in bone development, concluding that there is an "enzyme present in the ossifying cartilage of young rats and rabbits which rapidly hydrolyzes hexosemonophosphoric acid in yielding free phosphoric acid". This enzyme has come to be known now as alkaline phosphatase, which has been hypothesized to be involved in the mineralization process, even though its precise physiological significance has yet to be elucidated. Its expression is partly transcriptionally regulated but there is little evidence for translational and post-translational control (Henthorn, 1996). This shows ALP as involved in mineralization by MC3T3-E1 cells. There is now evidence for a role of p38 mitogen-activated protein kinase (p38 MAPK) in the expression of ALP during osteoblastic cell differentiation (Suzuki et al., 2002).

1.3.2. The osteoblasts

Osteoblasts are the skeletal cells that synthesize and regulate the deposition and mineralization of the extracellular matrix (Aubin & Liu, 1996). They are thought to arise from mesenchymal stem cell precursors (Franceschi & Iyer, 1992) and are located on the surface of bone. The balance between cell populations in an organism is controlled by the rates of proliferation, differentiation and programmed cell death or apoptosis (Quarles et al., 1992). Bone formation during bone development and remodelling necessitates stringent control of osteoblast proliferation and differentiation (Stein et al., 1996). By responding to systemic hormonal influences and secreting a variety of autocrine and paracrine signalling factors, osteoblasts are important for coupling the activities of osteoclasts and osteoblasts as well as ensuring the maintenance of bone mass (Genever & Skerry, 2001). During the initial period of differentiation *in vivo*, osteoblasts are responsive to physiological requirements for expression of genes that support competency for proliferation, cell cycle progression and extracellular matrix biosynthesis as well as those that suppress expression

of post-proliferative bone phenotypic genes (Stein et al., 1996). In the mature phenotype is found the ability of the cells to synthesize collagen type 1 (COLL-1) and a wide variety of noncollagenous proteins such as osteocalcin, bone sialoprotein (BSP), osteopontin, proteoglycans and hormone as well as growth factor receptors. However, different osteoblasts may express just a subset of molecules from the potential osteoblast repertoire (Aubin & Liu, 1996). Collagen constitutes approximately 90% of bone matrix proteins (Harada et al., 1991). Expression of the bone/liver/kidney (tissue non-specific) isoform of ALP is positively correlated with bone formation. As the specific activity of the enzyme in a population of bone cells increases, there is a corresponding shift to a more differentiated state (Aubin & Liu, 1996). An essential transcription factor for osteoblast differentiation and bone formation is Core binding factor alpha1 (Cbfa1) (Harada et al., 1999) and the suppression of osteoblast function by the cAMP pathway occurs through proteolytic degradation of Cbaf1 involving the ubiquitin/proteasome-dependent mechanism (Tintut et al., 1999).

1.4. Reactive oxygen species (ROS)

One of the major sources of reactive oxygen species is the overactivation of glutamate receptors. In fact, these ROS are now widely believed to mediate glutamate-induced neurotoxicity in a variety of conditions, leading to neuronal death. This section therefore endeavours to introduce the nature and effects of the ROS, their sources, the natural antioxidant defence systems in the body, and ROS production by glutamate receptor activation and kynurenines.

1.4.1. Chemical nature (reactivity) and sources in biological systems

Intracellular free radicals (free low molecular weight molecules with an unpaired electron) are often ROS and vice versa and the two terms are usually seen as equivalents (Nordberg & Arnér, 2001), although some ROS such as hydrogen peroxide (H_2O_2) are, strictly speaking, not free radicals. According to Olanow (1993), free radicals are partially reduced and contain an orbital with an unpaired electron. Therefore, in order to gain stability, as they are chemically reactive, the radicals "steal" electrons (hence are called electron lovers) or hydrogen atoms from their neighbours, turning these molecules also to radicals (Phillis, 1994) and thereby instigating a continuous chain of reactions. Free radicals or ROS are involved in several normal (Rice-Evans, 1994) or aberrant (Coyle & Puttfarcken, 1993) biological (metabolic) processes *in vivo*. It is estimated that 2-4% of the oxygen

consumed during oxidative phosphorylation in the mitochondria is converted to ROS. Apart from the mitochondria, NADPH-oxidase 1 (Nox 1) also generates a significant amount of ROS in the cell (Desouki et al., 2005). NADPH oxidase is a multi-component, membrane-associated enzyme that catalyzes the one-electron reduction of oxygen to superoxide using NADH or NADPH as the electron donor. ROS include a number of chemically reactive molecules that are obtained from oxygen (Fridovich, 1999; Betteridge, 2000; Halliwell, 1996; 1999). Some of them are very reactive, e.g., the hydroxyl radical, while some are less so, e.g., the superoxide anion and H_2O_2, both of which are established products of the respiratory burst when the plasma membrane NADPH oxidase of neutrophils and macrophages is activated (Burdon, 1995).

Reactive oxygen species (ROS) are produced and degraded by all aerobic organisms, and this leads to either physiological concentrations required for normal cell function, or excessive quantities, a state referred to as oxidative stress (Nordberg & Arnér, 2001). Because of their high reactivity, ROS are prone to cause damage and are thereby potentially toxic, mutagenic, or carcinogenic (Nordberg & Arnér, 2001), their intracellular production threatening the integrity of various biomolecules including proteins (Stadtman & Levine, 2000), lipids, as well as nucleic acids such as DNA (Marnett, 2000; Gilgun-Sherki et al., 2001), as these molecules are oxidized by them (Gilgun-Sherki et al., 2001). The initiated chain reactions generate numerous toxic reactants that rigidify membranes by cross-linking (Coyle & Puttfarcken, 1993). The oxygen radicals can occur as alkyl or peroxyl radicals in lipids (Sies, 1997). In certain disease states such as ischaemia, radicals can be produced at elevated rates. Other pathways leading to free radical production include nitric oxide synthesis and arachidonic acid metabolism. In the process of generating energy during aerobic metabolism, cells reduce oxygen to water and there is transfer of electrons. The result of the leakage of high-energy electrons is the formation of reactive oxygen species (ROS). Several enzymes in the brain including monoamine oxidase (MAO), tyrosine hydroxylase and L-amino oxidase generate H_2O_2 as a normal by-product of their activity while auto-oxidation of endogenous substances such as ascorbic acid and catecholamines also yields H_2O_2. Phospholipase A_2 (PLA_2), when activated in a Ca^{2+}-dependent process, can generate arachidonic acid (AA) from membrane phospholipids and AA could in turn yield superoxide anion when subsequently metabolised through the cyclooxygenase or lipoxygenase pathway that leads to the production of eicosanoids. Similarly, calcium-dependent activation of nitric oxide synthase (NOS) can produce the gaseous free radical nitric oxide (NO), which rapidly reacts with

the superoxide anion producing peroxynitrite ($ONOO^-$), although it can also be converted to other reactive nitrogen species (RNS) such as nitrosonium cation (NO^+) or nitroxyl anion (NO^-), depending on the microenvironment (Stamler et al., 1992). Peroxynitrite has the activity of hydroxyl radical and nitrogen dioxide radical, although it does not readily decompose into these entities (Dawson & Dawson, 2004). It also has the ability to directly nitrate and hydroxylate aromatic rings on amino acid residues and as a potent oxidant reacts readily with sulfhydryls and zinc-thiolate (Ischiropoulos & Beckman, 2003; Dawson & Dawson, 2004). When accumulated hypoxanthine and xanthine are converted by xanthine oxidase to uric acid, superoxide anion is produced. This xanthine oxidase can derive from xanthine dehydrogenase through a calcium-dependent conversion (involving calcium-activated peptidases, such as calpain I) under conditions of energy failure and elevated intracellular calcium levels (Coyle & Puttfarcken, 1993).

1.4.2. ROS-induced cell proliferation

Reactive oxygen species signal cascades are involved in cell growth, cell death, mitogenesis, angiogenesis and carcinogenesis (Desouki et al., 2005). Growth responses have been seen to be elicited by H_2O_2 in mouse osteoblastic cells (MC3T3) (Nose et al., 1991), even though its cytotoxic effects are well known and have been studied by a number of groups (Burdon, 1994). Exogenously added superoxide and H_2O_2 as active oxygen species stimulate the expression of early growth-regulated genes such as c-*fos* and c-*jun* and such observations have led to suggestions that superoxide and H_2O_2 might function as mitogenic stimuli through biochemical processes common to natural growth factors (Burdon, 1994). The observation that superoxide (or its dismutation product, hydrogen peroxide) are released by cells, either constitutively in the case of tumour cells, or following cytokine stimulation, has led to the speculation that they might possibly serve as a sort of "autocrine" growth stimulation system or a means of "intercellular communication" (Burdon, 1995). Therefore, in mammalian cells, low concentrations of H_2O_2 are known to stimulate growth while high concentrations are damaging, leading to cell death (Burdon, 1994; 1995).

1.4.3. Induction of oxidative stress

Oxidative stress is toxic free radical damage arising from an imbalance between radical generating and radical scavenging systems in the body, in favour of the former, which can occur when the amount of free radicals is increased or when the antioxidant enzymes levels

are decreased (Harris, 1992; Sies, 1984; Sies & de Groot, 1992; Stogner & Payne, 1992). This is, of course, an important pathway leading to neuronal degeneration and is implicated in many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and Huntington's disease. The brain is at risk from oxidative damage due to high oxygen consumption (20% of the total body basal O_2 consumption), critically high levels of both iron and ascorbate, relatively low levels of antioxidant protective agents and tendency to accumulate metals (Gaeta & Hider, 2005). Neurodegeneration increases with aging, particularly of the CNS, and this is related to damage inflicted by free radicals, as there are regions of increased iron (which catalyzes free radical formation) within the brain and neural tissue which are rich in polyunsaturated fatty acids and are therefore susceptible to attack by free radicals (Reiter, 1995). Also, decrease in defence mechanisms with age causes increased oxidative damage and free radical generation. It has been proposed that oxidative stress is involved in the aging process both by inducing damage to mitochondrial DNA and by other mechanisms (Cadenas & Davies, 2000; Finkel & Holbrook, 2000). Oxidative events implicated in toxic oxidative stress also include alterations in mitochondrial lipids (e.g., cardiolipin) and mitochondrial proteins (e.g., aconitase and uncoupling protein 2) (Fariss et al., 2005). It is known that a major consequence of oxidative stress is lipid peroxidation, which results from an interaction of ROS with polyunsaturated lipids in cell membranes (Callaway et al., 1998). Subsequently, changes occur in structure, function and permeability of the membrane, leading ultimately to cell death. Lipid peroxidation is, therefore, an important cause of neuronal damage in neurodegenerative diseases. The accumulation of lipid peroxidation products like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) has been demonstrated in affected regions in brains of Alzheimer's disease patients (Neely et al., 1999). Oxidative damage is caused by ROS including superoxide anion radical, hydrogen peroxide, and the hydroxyl radical, which is the most toxic and reactive. The one, two and three electron reductions of O_2 generate, respectively, the $O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$ (Reiter et al., 2002). The superoxide anion ($O_2^{\cdot-}$), obtained from molecular oxygen by the univalent reduction (addition of an electron) of triplet-state oxygen ($^3O_2$) (Droge, 2002), is not highly reactive, even though it is a free radical. This is because it lacks the ability to penetrate lipid membranes and is therefore enclosed in the compartment where it is produced (Nordberg & Arnér, 2001). However, it can combine with nitric oxide to produce the highly toxic radical, peroxynitrite (Droge, 2002). On the other hand, H_2O_2 is not a free radical, but because it is stable (with a long half-life), produced by almost all tissue types

where its concentrations can rise, and has the ability to penetrate biological membranes by diffusing within and across cells, it is highly important (Reiter et al., 2002). Hydrogen peroxide is a mediator of damage in certain pathological conditions and has been shown to induce lipid peroxidation in rat brain homogenates (Garcia et al., 2000). Increased production of H_2O_2 in the CNS has been implicated in the pathogenesis of several neurodegenerative diseases, including PD and AD, and in ischaemic reperfusion and stroke (Mazzio & Soliman, 2003). In Parkinson's disease, H_2O_2 generated from presynaptic Lewy body α-synuclein may be associated with neurodegeneration of nigral cell bodies in the substantia nigra and destruction to the nigrostriatal tract (Turnbull et al., 2001). In Alzheimer's disease, β-amyloid plaque builds up in the brain and causes intracellular accumulation of hydrogen peroxide (Behl et al., 1995). The use of H_2O_2 as an inducer of damage is, therefore, a potentially clinically important model of oxidative stress. Nevertheless, a number of reports have indicated that the modulatory and pathological consequences of H_2O_2 are often mediated by the hydroxyl radical and not by H_2O_2 per se (Halliwell, 1992; Avshalumov et al., 2000).

The hydroxyl radical is possibly capable of doing more damage to biological systems than any other ROS, owing to its strong reactivity with biomolecules (Nordberg & Arnér, 2001). It has been theorized that in excess of 50% of the free radical-mediated molecular destruction of cells is a direct consequence of the hydroxyl radical, although its absolute significance to cellular malfunction, diseases and aging has been difficult to define unequivocally (Reiter et al., 2002). It is not generated directly by any known enzymatic reaction, but can be produced by H_2O_2 through slow decomposition, a process profoundly accelerated in the presence of Fe^{2+} by the Fenton reaction (Coyle & Puttfarcken, 1993). It is agreed that iron is known to be critically vital to biological reactions in living cells and in the brain is required for sustenance of brain's high respiratory activity, myelinogenesis, and for the production of many neurotransmitters including dopamine, noradrenaline, and serotonin and for generation of GABAergic activity (Moos & Morgan, 2004). However, the divalent state of iron makes it very reactive and therefore extremely toxic if its intracellular concentrations are not tightly regulated (Lee et al., 2006). Iron overload in the early stages of life has been reported to induce cognitive impairment, possibly by inducing oxidative damage in the brain (de Lima et al., 2005). This is why proteins such as ferritin and transferrin, which sequester transition metal ions such as iron, have neuroprotective properties. While iron has been clearly identified with the pathology of Parkinson's disease, iron, copper and zinc have all been associated with the progression of Alzheimer's

disease (Gaeta & Hider, 2005). Unlike superoxide anion and H_2O_2 whose cellular levels are regulated by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), there are no analogous enzymes for regulating the highly reactive ·OH. Its management therefore depends on the endogenous antioxidants ascorbate and reduced gluthathione (GSH) (Cohen, 1994; Rice, 2000).

Another example of reactive species is nitric oxide. Nitric oxide (NO), formerly identified as Endothelial-Derived Relaxing Factor (EDRF), is rather an odd member of the free radical family with a multiplicity of roles in the CNS and the periphery, including neurotransmitter functions and mediation of both proliferation and death, depending on tissue types and hence has been referred to as a Janus molecule. Studies of NO now mainly span the cardiovascular, nervous and immune systems (Moncada & Higgs, 2006). It is produced through the metabolic action of nitric oxide synthase (NOS) on L-arginine (oxidation of its guanidine group) with a stoichiometric formation of citrulline, though direct reduction of nitrite to NO has also been reported in the ischaemic heart (Zweier et al., 1995; Zhang & Snyder, 1995). NO is known to be the mediator of tumoricidal and bactericidal actions of macrophages and is a likely transmitter of nonadrenergic, noncholinergic neurones (Zhang & Snyder, 1995). There are three isoforms of NOS: the calcium/calmodulin-dependent neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3), and the calcium-independent, inducible NOS (iNOS or NOS2). nNOS and eNOS are the constitutive nitric oxide synthases. The inducible form (iNOS) is now known to bind, S-nitrosylate and activate cyclooxygenase-2 (Kim et al., 2005). Physiologically, NO activates soluble guanylate cyclase (sGC), leading to increase in cGMP, although it is now also known to inhibit mitochondrial cytochrome c oxidase. According to Nordberg & Arnér (2001), NO is similar to superoxide in many aspects in that it does not readily react with most biomolecules, despite its having an unpaired electron, but easily reacts with other free radicals (e.g., peroxyl and alkyl radicals), generating mainly less reactive molecules and thus functioning somewhat as a free radical scavenger. In fact, it has been shown that nitric oxide inhibits peroxide-mediated endothelial toxicity (Degnim et al., 1998). On the other hand, it readily reacts with the superoxide radical, forming the extremely toxic peroxynitrite. The rate of this reaction is about three times faster than the rate at which superoxide dismutase (SOD) catalyzes the dismutation of superoxide radical to hydrogen peroxide.

1.4.4. Generation of ROS by glutamate receptor activation

Glutamate is the major effector of oxidative stress in the brain where excessive stimulation of glutamate receptors results in excitotoxicity (Coyle & Puttfarcken, 1993) and has already been shown to induce oxidative stress *in vitro* (Behl et al., 1995). Glutamate-induced oxidative stress alone or combined with other glutamate-independent sources of ROS (such as the pathological event of reperfusion injury) (Siesjo, 1992) has been implicated in the aetiology of neurodegenerative diseases such as PD, ALS, and HD (Coyle & Puttfarcken, 1993). It is now known that overstimulation of glutamate receptors, especially the NMDA receptors, leads to significant elevation of intracellular calcium concentration. However, though less significant, additional calcium influx is also possible through the voltage-dependent calcium channels (VDCC), the sodium-calcium exchanger, and non-specific membrane leakage (Choi, 1988). Release of calcium from intracellular calcium stores could also contribute to the net pool of free cytosolic calcium (Nicoletti et al., 1986). This results in the activation of calcium-dependent enzymes (lipases, phosphatases, etc.), including neuronal nitric oxide synthase (nNOS), which produces nitric oxide (NO) from the substrate L-arginine. However, this elevated intracellular calcium level also leads to the generation of reactive oxygen species such as superoxide anion and H_2O_2, and all of these ROS are now believed to mediate glutamate neurotoxicity. Some authors have argued that distinct, membrane receptor-specific, neurotoxic signalling pathways transduce calcium-dependent excitotoxicity and the precise relationship, at the molecular level, between Ca^{2+} loading and neurotoxicity has yet to be defined (Sattler & Tymianski, 2000). Free intracellular calcium ($[Ca^{2+}]_i$) is maintained around 100nM compared to approximately 1mM found in the extracellular space ($[Ca^{2+}]_e$) (Pringle, 2004). An increased production of superoxide has been shown to mediate glutamate-induced neuronal death (Luetjens et al, 2000). Nitric oxide is able to compete with superoxide dismutase (SOD) for the superoxide radical, resulting in the formation of the very toxic product, peroxynitrite. A study by McManus et al. (2004) showed that hypothermia, which has long been known to be an effective neuroprotective strategy against a variety of brain injuries, protected against ischaemic insults in cultured hippocampal slices by reducing free radical production, further strengthening the view that excessive glutamate release, as occurs in ischaemia, could induce substantial oxidative stress mediating neuronal damage. The mitochondria appear to be the major component involved in glutamate-induced formation of ROS, as this process is inhibited by 85-90% in the presence of mitochondrial inhibitors such as rotenone and oligomycin (Brocard et al., 2001).

In this context, quinolinic acid (QA), a potent endogenous excitant and selective agonist at the NMDA receptors (Stone & Perkins, 1981), and whose potential as a neurotoxin was first demonstrated by Lapin (1978), has similarly been shown to induce lipid peroxidation *in vitro* by a number of authors, e.g., Rios & Santamaria (1991). It seems that quinolinic acid may act by mechanisms additional to activation of NMDA receptors, perhaps involving lipid peroxidation and ROS formation (Stone et al., 2000). In fact, some reports have argued that quinolinate does not have a direct neurotoxic effect, but modulates lipid peroxidation via its interaction with iron (Stipek et al., 1997). All cell types, including the NADPH diaphorase-positive neurones, appear to be sensitive to the toxic effect of quinolinic acid (QA) depending on the amount injected (Boegman, et al., 1987), although some authors insist that this neuronal population is spared QA toxicity (Beal et al., 1991). The NADPH diaphorase has been identified as nitric oxide synthase (NOS) (Dawson et al., 1992; Lipton et al., 1993; Behrens et al., 1996). QA is increasingly implicated in neurodegenerative disorders, especially the AIDS-dementia complex and Huntington's disease (Stone, 2001).

There is also now a well-characterized form of glutamate-induced neurotoxicity that results from oxidative stress, mediated, not by glutamate-gated ion channel, but by a cystine transporter to which glutamate binds (Bannai & Kitamura, 1980). The cystine deprivation that occurs through glutamate binding leads to reduced glutathione levels and the intracellular accumulation of oxidants (Coyle & Puttfarcken, 1993).

1.4.5. Kynurenines and ROS production

Even though it has been shown that tryptophan is also metabolised in the gut by bacterial tryptophanases resulting in the formation of indole, which in turn yields a strongly sedative compound oxindole (Vederas et al., 1978; Kawata et al., 1991), the kynurenine pathway metabolizes over 90% of dietary tryptophan that is not used in protein synthesis to generate the essential co-factors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). This metabolic pathway was well investigated in the 1950s and 1960s, with a view to understanding its contribution to the synthesis of NAD and NADP, which are two ubiquitous co-enzymes involved in basic cellular processes (Wolf, 1974). Products of the pathway include quinolinic acid, an endogenous agonist ligand which remains the only known endogenous compound able to activate selectively receptors for NMDA (Stone & Perkins, 1981; Stone, 2001), and kynurenic acid, a broad-spectrum glutamate receptor antagonist that acts at the strychnine-resistant, glycine-

sensitive site on the NMDA receptor (Perkins & Stone, 1982). Three different enzymatic activities, at least, may metabolize kynurenine in mammalian tissues, involving kynurenine hydroxylase (forming 3-hydroxykynurenine), kynurenine aminotransferase (forming kynurenic acid), and kynureninase (forming anthranilic acid). An excellent review of the roles of kynurenines in the CNS and their therapeutic importance has been published (see Stone, 2001).

Quinolinic acid can be excitotoxic to hippocampal neurones at concentrations not injurious to neurones by itself in the presence of donors of nitrosative and oxidative stress (Behan & Stone, 2002). However, a number of kynurenines are now known to generate substantial levels of ROS. They include 3-hydroxyanthranilic acid (3-HAA) and 3-hydroxykynurenine (3-HK). In fact, 3-HK may be the most toxic of all kynurenines or tryptophan metabolites (Okuda et al., 1998). It is toxic to neurones mainly as a result of conversion to quinonimines and ROS that initiate apoptosis (Eastman & Guilarte, 1990), but its toxicity can also result from its metabolism to 3-HAA, which can undergo auto-oxidation to yield superoxide anions (Stone & Darlington, 2002). Quinolinic acid and 3-HK are known to damage cells by different mechanisms (Chiarugi et al., 2001). These observations have further strengthened the argument that ROS are major contributors to the process of neurodegeneration, since these kynurenines are involved in the pathology of neurodegenerative diseases.

1.4.6. Antioxidant defence mechanisms

Defence mechanisms to prevent and keep the damage caused by free radicals under control are present in the body under normal circumstances (Reiter, 1995). However, these defences are complex and tissues are equipped with different patterns of antioxidant defence based on cell type and function, as well as on physiological states (Sies & Stahl, 1995). The cellular antioxidant defence systems are classified into two groups, enzymatic and non-enzymatic, of which there are indirect and direct acting agents (Gilgun-Sherki et al., 2001). The antioxidant enzyme systems include superoxide dismutases (SOD), superoxide reductases (SOR), catalases, peroxiredoxins (Prx), glutathione peroxidases (GPx) and other glutathione-related enzymes. The low molecular weight antioxidant compounds include vitamin C (a hydrophilic antioxidant) and vitamin E or alpha-tocopherol (a chain-breaking hydrophobic antioxidant), different selenium compounds, lipoic acid, and ubiquinones, all of which interact with the mammalian thioredoxin system, a ubiquitous oxidoreductase system with antioxidant and redox regulatory roles (Coyle &

Puttfarcken, 1993; Nordberg & Arnér, 2001). However, the superoxide dismutases, catalases and glutathione peroxidases are the three main classes of protective enzymes. There are also free radical-induced cytoprotective genes, such as the antioxidant-like stress protein haem oxygenase-1 (HO-1), which catabolises the pro-oxidant haem to generate biliverdin, iron, and the vasodilator carbon monoxide (CO). Elevated HO-1 activity has been shown to be protective against several pathological conditions (Maines, 1997). Biliverdin is subsequently converted by biliverdin reductase to bilirubin, an antioxidant that can scavenge lipid peroxyl radicals (Baranano et al., 2002), while CO has both anti-apoptotic and anti-inflammatory properties (Otterbein et al., 2000).

Superoxide dismutase (SOD) catalyses the formation of hydrogen peroxide from superoxide radical. There are three forms of SOD expressed in the eukaryotic cells, encoded by three separate genes: the copper-zinc SOD (CuZnSOD) or SOD 1 found in the cytosol, the manganese-containing SOD (MnSOD) or SOD 2 localized to the mitochondrial matrix, and the extracellular form of CuZnSOD, which is expressed at low levels in plasma and extracellular fluids where it partially protects nitric oxide by reducing the concentration of superoxide radical (Fridovich, 1989; Nakazono et al., 1991). However, when the activity of SOD is chronically elevated above normal, as occurs in Down syndrome subjects, it can be pro-oxidative, which is why the disease is believed to be, at least in part, a consequence of excessive free radical generation (Busciglio & Yankner, 1995; Reiter et al., 2002). Catalase (a haemoprotein with four haem groups) and glutathione peroxidase (GPx) (containing selenium as a prosthetic group) catalyse the breakdown of hydrogen peroxide to water and oxygen, with glutathione peroxidase being more important in neural tissue (Reiter, 1995), perhaps partly because, relative to GPx which is found in high concentrations in the brain, there is little catalase in both grey and white matter (Coyle & Puttfarcken, 1993). In addition, aside from its ability to eliminate H_2O_2, GPx is also involved in the detoxification of lipid peroxyl radicals (Coyle & Puttfarcken, 1993) or lipid hydroperoxides (Podratz & Windebank, 2005). These lipid hydroperoxides can decompose to alkoxy radicals and aldehydes in the presence of Fe^{2+} (Coyle & Puttfarcken, 1993). Gluthathione peroxidase makes use of reduced gluthathione (GSH), a tripeptide synthesized intracellularly (Coyle & Puttfarcken, 1993), as a substrate that donates hydrogen, thus becoming converted to oxidized gluthathione or gluthathione disulphide (GSSG). Glutathione disulphide (GSSG) can be converted back to gluthathione by gluthathione reductase in an NADPH-consuming process (Droge, 2002). Another cytosolic enzyme, quinone reductase, first noted for its protection against carcinogens, also

catalyzes a two-electron reduction of quinones to hydroquinones, which are more stable and less reactive (Lind et al., 1982).

Wang et al. (2004) reported that overexpression of antioxidant enzymes protects cultured hippocampal and cortical neurones from necrotic insults. 17β-oestradiol has been found to reduce lipid peroxidation induced by quinolinic acid in brain homogenates (Heron & Daya, 2000). Similarly, Rau et al. (2003) showed that oestradiol attenuates programmed cell death after stroke-like injury while vitamin E has been reported to reduce lipid peroxidation induced by nitric oxide in rat brain homogenates (Escames et al., 1997).

1.5. Mechanisms of cell death

Agents (such as ROS and glutamate in the CNS) which induce cell death in biological tissues bring about demise by either apoptosis or necrosis, which are the two classical extreme pathways of cellular death, each with its distinct features. The classification has been primarily based on morphological criteria (Wyllie et al., 1980). However, there is growing evidence that a number of death processes may simultaneously activate the two pathways, thus resulting in a form of "hybrid" death that is neither entirely apoptotic nor entirely necrotic (Cheung et al., 1998; Martin et al., 1998).

1.5.1. Apoptotic mechanisms

Apoptosis, a term first coined in 1972 (Kerr, 1998), is the basis of programmed cell death (PCD), a delayed form of cell death from less severe insults that is energy-dependent and associated with activation of a genetic programme (Kam & Ferch, 2000). Apoptosis is an important mechanism for the selective elimination of mammalian cells distinct from the process of cell death by necrosis (Wyllie et al., 1994). Recent widespread interest in cell death processes has generated controversy over definitions that distinguish apoptosis from other forms of cell death (see Sloviter, 2002). According to Cohen (1997), apoptosis is defined as the process of cell death associated with caspase activation or caspase-mediated cell death, a view that presumes that caspases represent its final common mechanistic pathway. However, this definition may need to be expanded, as a number of caspase-independent apoptotic mechanisms have also been recently reported, mainly involving the apoptosis-inducing factor (AIF). It seems there is no consensus yet on the classification of the different forms of programmed cell death. In their review, Krantic et al. (2005) highlighted a classification that, though more constraining, is currently considered one of

the most accurate. It is based on nuclear morphology and divides PCD into classical apoptosis, apoptosis-like PCD, and necrosis-like PCD, respectively characterized by nuclear condensation that is 'crescent-like,' partial or peripheral, or absent. Classical apoptosis is the best-known phenotypic expression of PCD, resulting in caspase activation. Apoptosis-like PCD is broader and includes caspase-independent mitochondrial pathways. With regard to necrosis-like PCD, cell death programme is triggered by organelles other than mitochondria, such as lysosomes, endoplasmic reticulum (ER) and the nucleus, and by proteases other than caspases, such as cathepsins and calpains originating from lysosomes and the ER, respectively. Calpains play a central role in ischaemic neuronal death by cleaving a variety of substrates such as cytoskeletal proteins (e.g., spectrins) and important regulatory proteins (e.g., cyclin-dependent kinase-5 (Cdk-5)) (Patzke & Tsai, 2002). It has been recently reported that the cleavage by calpains of collapsin response mediator protein-3 (CRMP-3) induced neuronal death after glutamate excitotoxicity and ischaemia (Hou et al., 2006), and inhibitors of calpains such as chlortetracycline and demeclocycline have been shown to protect mouse neurones against glutamate toxicity and cerebral ischaemia (Jiang et al., 2005).

Hallmarks of apoptosis include shrinkage of the cytoplasm, phosphatidylserine translocation, and condensation of nuclear material into "clumps" (Banasiak, et al., 2000; Valencia & Moran, 2004). Subsequently, the nucleus undergoes fragmentation and the endoplasmic reticulum fuses with the plasma membrane forming vesicles and convoluting its surface. The final stages of apoptosis witness cellular fragmentation forming membrane-bound apoptotic bodies that contain intact cytoplasmic organelles and nuclear fragments (Kerr, 1998).

There are two primary modes of apoptosis induction. One is through the death receptors in the plasma membrane, called the extrinsic pathway and the other, the intrinsic pathway, is via mitochondrial dysfunction (Verdaguer et al., 2004). The extrinsic pathway involves cell surface death receptors which are members of the nerve growth factor/TNF superfamily of receptors and include Fas (CD95/APO-1), tumour necrosis factor (TNF) receptor 1 (TNFR1), as well as DR-3, DR-4, and DR-5 (Ashkenazi & Dixit, 1998; Krammer, 1999; Kinloch et al., 1999; Sheikh & Fornance, 2000). They can be activated by both cell surface-bound and soluble ligands such as FasL (CD95L), tumour necrosis factor-α (TNF-α), lymphotoxin-α (LT-α) and TNF-related apoptosis-inducing ligand (TRAIL) (Ashkenazi & Dixit, 1998; Krammer, 1999). These receptors are widely distributed in the body and are often found in cells from the immune system and in many somatic tissues

(Kinloch et al., 1999). Tumour necrosis factor type I receptor (TNFRI) was shown to be required for amyloid-β protein-induced neuronal death (Li et al., 2004). It is also becoming increasingly recognised from a molecular perspective that neuronal PCD consistently shows a unique property of pathological re-initiation of the cell cycle (Krantic et al., 2005).

1.5.1.1. Caspase-dependent mechanisms

It is well recognized that in apoptosis, the effector molecules are the cysteine-dependent, aspartate-directed proteases called caspases (cysteine-aspartate proteases), even though BAD (Bcl-X_L/Bcl-2-associated death promoter) can initiate apoptosis and a reciprocal regulation of Bcl-2 and Bax expression seems to occur in glutamate-induced excitotoxicity (Schelman et al., 2004). The tumour suppressor p53 is able to upregulate Bax transcription and repress Bcl-2 transcription, thereby altering the Bcl-2 : Bax ratio in favour of apoptosis via the mitochondrial pathway (Miyashita & Reed, 1995; Bates & Vousden, 1999), while pro-apoptotic Bcl-2 family proteins can also induce the release of cytochrome c from isolated mitochondria (Fariss et al., 2005), leading to apoptosis. In contrast, anti-apoptotic Bcl-2 members can target the protein kinase Raf-1 to mitochondrial membranes, allowing this kinase to phosphorylate BAD or other protein substrates involved in apoptosis regulation, with the resultant effect of promoting resistance to apoptosis (Wang et al., 1996).

Caspases were discovered over a decade ago with two aspartate cleavage sites, one of which excises the prodomain while the other cleaves the large domain from the small domain. They are distinct from other proteases because of their aspartate specificity, as the only other protease that shares this substrate specificity is granzyme B, though its own active site is serine (Earnshaw et al., 1999; Sarin et al., 1996). It is known that caspases are synthesised and exist mostly in the cytoplasm of viable cells as inactive pro-enzymes. Activation of caspase-zymogens is an early event in the process of apoptosis. Once cytochrome c (a water-soluble, basic, haem-containing protein that binds to the anionic phospholipid cardiolipin, located exclusively on the inner mitochondrial membrane of eukaryotic cells (Tuominen et al., 2002)) is released from the mitochondria, it combines with Apaf-1 (apoptotic protease-activating factor-1) and the duo recruits and activates pro-caspase 9 to form the apoptosome by means of which cleavage and activation of pro-caspase-3 into caspase-3 occur. There are upstream initiator caspases that begin the proteolytic cascade in apoptosis (caspases 8 and 9) and downstream effector caspases that cleave cellular proteins (caspases 3, 6, and 7) (Stepanichev et al., 2005). For the extrinsic

pathway, the binding of members of the death receptor family (e.g. Fas–TNFR-1–TRAIL-R1) and their cognate ligands (Nagata, 1997) results in an oligomerization of receptors and a subsequent activation of procaspase-8 and, depending on the cell type, active caspase-8 either cleaves and activates procaspase-3 directly or it cleaves the proapoptotic Bcl-2 protein Bid to tBid, which then recruits the mitochondrial apoptotic pathway, resulting in the activation of procaspase-3 and other effector caspases (Robertson et al., 2004). It therefore means that the two pathways of apoptosis converge on caspase-3 induction, which is possibly why, out of the 14 caspases identified to date (Los et al., 2001), caspase-3 has been recognised as a central player in mediating apoptosis and hence is the most widely investigated. Both pathways are associated with activation of caspase-activated DNase (CAD) and also with typical internucleosomal DNA fragmentation (Hengartner, 2000). There is, however, a paucity of studies addressing the role of caspase-3 induction in apoptosis in MC3T3-E1 osteoblast-like cells, though staurosporine and hypoxia were reported to induce activation of the protease in these cells (Chae et al., 2000; 2001).

The X-ray crystal structure of caspase-3 reveals the formation of a tetramer having two small and two large subunits, which is believed to be the catalytically active form. Despite its association with apoptotic cell death, a beneficial role has been suggested for caspase-3-mediated mechanisms in neuronal plasticity when Stepanichev et al. (2005) showed that Z-DEVD-fmk, a caspase-3 inhibitor, blocked long-term potentiation in hippocampal slices, thus impairing spatial memory in the water maze. There is also growing evidence that caspases and other apoptosis regulators participate not only in cell death, but also in the control of cell cycle (Los et al., 2001).

1.5.1.2. Caspase-independent mechanisms

It is now clear that a number of apoptotic events could occur independently of caspase activation. The best example of an effector of caspase-independent cell death is the apoptosis-inducing factor (AIF) (Susin et al., 1999; Joza et al., 2001), which is normally localized to the inter-membrane mitochondrial space. AIF is a 67-kDa flavoprotein that is similar to bacterial oxidoreductases (Susin et al., 1999) and is evolutionarily conserved. It also displays NADP(H) oxidase and monodehydroascorbate reductase activities (Miramar et al., 2001). Upon mitochondrial outer membrane permeabilization (following cytotoxic insults), it translocates to the nucleus where it induces peripheral chromatin condensation and large (high-molecular-weight (50kb)) DNA fragmentation. This translocation of AIF to the nucleus seems to be a general feature of apoptosis in mammalian cells (Cande et al.,

2002; Dawson & Dawson, 2004). However, because AIF lacks any intrinsic endonuclease activity, once in the nucleus, it recruits a number of downstream nucleases including cyclophilin A and, at least in the nematode *Caenorhabditis elegans,* endonuclease G (Cande et al., 2004; Wang et al., 2002). Although its physiological role is not clear, it has been suggested to participate in scavenging ROS (Klein et al., 2002). DNA binding by AIF may be required for its apoptogenic function in the nuclear compartment (Dawson & Dawson, 2004).

1.5.1.3. Membrane Permeability Transition (MPT)

This is the phenomenon whereby there is an opening of an unspecific pore, permeability transition pore (PTP), on the mitochondrial inner membrane, allowing the flow of solutes < 1.5 kDa out of the mitochondrial matrix (Connern & Halestrap, 1994). This leads to the collapse of the mitochondrial membrane potential, ψ_m. Cytochrome c leaks through the multiprotein complex (containing hexokinase, porin, and adenine nucleotide translocator (ANT)) (Beutner et al., 1996) into the cytosol where it combines with Apaf-1 to activate procaspase-9 to caspase-9, hence forming the apoptosome, which in turn activates caspase-3, the terminal caspase that executes the apoptotic command. Agents that block the PTP are therefore able to protect against apoptotic death. Such agents include cyclosporin A (CsA), which is also known to block calcineurin (protein phosphatase 2B). The protection by CsA against glutamate excitotoxicity has been reported to occur through calcineurin-dependent and independent mechanisms (Dawson et al., 1993; Ruiz et al., 2000). The opening of the pore may be involved in cellular apoptosis, as AIF is released from the mitochondrial intermembrane space (IMS) as a result of the destruction of the mitochondrial outer membrane after excessive mitochondrial matrix swelling (Susin et al., 1996). PTP formation is enhanced by increased production of ROS (Connern & Halestrap, 1994). The manifestation of PTP is inhibited by substrates for the mitochondrial ANT, e.g., ADP and bonkregic acid, or CsA (Bernardi, 1992).

1.5.2. Necrotic mechanisms

Generally speaking, necrosis results from severe insults such as anoxia and cell trauma and is associated with changes in calcium and sodium ion homeostasis. Major morphological hallmarks of necrotic cell death include the swelling of cells and of organelles (mainly the mitochondria), followed by disruption of the organelles. The plasma membrane ruptures permitting the leakage of cellular contents into the extracellular compartment. Random

DNA degradation also occurs following histone proteolysis. Necrotic cell death is distinct from apoptotic cell death in a number of ways. Large groups of adjacent cells are usually affected -as opposed to individual cells in apoptosis- and there is a promotion of an inflammatory reaction, unlike in apoptosis where no inflammation is evident. Overall, necrosis is usually a pathological event while apoptosis could either be physiological or pathological. In necrosis, the early compromise of the integrity of the cell membrane allowing leakage of intracellular contents is the basis for the use of a number of assays that rely principally on this phenomenon such as the Lactate Dehydrogenase (LDH) Assay for cell death quantification. This perhaps makes the use of these assays for apoptotic cell death measurements unreliable, as the integrity of the cell membrane is not compromised until very late in the apoptotic process.

However, the binary classification of cell death as apoptotic or necrotic now appears severely challenged, as it has been observed that a number of cell death processes such as excitotoxic death often appear as neither entirely apoptotic nor entirely necrotic in that they simultaneously show distinct features of these two death types. This emerging pattern now constitutes what is now referred to as "hybrid cell death," which has recently become a subject of intense investigation (e.g., see Cheung et al., 1998; Martin et al., 1998).

1.5.2.1. Role of poly (ADP-ribose) polymerase (PARP)

Poly (ADP-ribose) polymerase (PARP) (also known as poly (ADP-ribose) synthetase or poly (ADP-ribose) transferase), is a family of abundant chromatin-bound nuclear proteins responsible for the repair of DNA strand nicks and breaks (Cosi et al., 1994). It is therefore important for the maintenance of genomic stability and nuclear homeostasis, although its exact physiological function is still a matter of intense debate. Poly (ADP-ribosyl)ation is now suggested to also regulate gene expression and gene amplification, cellular differentiation and malignant transformation, cellular division, DNA replication, and apoptotic cell death (Chiarugi, 2002). The best known and most important of the 18 members of the PARP family identified to date is PARP-1, a 116-kDa protein that generates more than 95% of the total ADP-ribose polymers in a cell (Dawson & Dawson, 2004; Hong et al., 2004). The family also includes PARP-2, PARP-3, vault PARP and tankyrases (Smith, 2001). PARP-1 generates nicotinamide and long-chained, branched polymers of ADP-ribose (PAR) from oxidized nicotinamide adenine dinucleotide (NAD^+), attaching these polymers (50-200 residues) to nuclear proteins including histones, topoisomerases I and II, DNA polymerases, DNA ligase-2, high-mobility group proteins,

transcription factors, and itself (Smulson et al., 2000; Shall & de Murcia, 2000; Dawson & Dawson, 2004). In response to DNA damage, PARP-1 activity becomes rapidly increased up to 500-fold upon binding to DNA strands and breaks (Dawson & Dawson, 2004). Poly(ADP-ribosyl)ation is a unique biochemical pathway, with PAR synthesis and degradation known to be present in all mitotic and post-mitotic cells with few exceptions in mammalian organisms (de Murcia & Shall, 2000). Despite its important reparatory role, however, overactivation of PARP-1 leads to cell death by a mechanism that involves metabolic derangement resulting from the depletion of NAD^+ and ATP, as the synthesis of every molecule of NAD^+ requires four molecules of ATP. Overactivation therefore leads to a continuous depletion of NAD^+ and ATP, which over time brings about irreversible cellular energy failure and the demise of the cell in a characteristically necrotic manner (Ha & Snyder, 1999). Moroni et al. (2001) reported that PARP inhibitors attenuated necrotic, but not apoptotic, neuronal death in experimental models of cerebral ischaemia. Loss of energy-dependent cellular function also occurs through impairment of the oxidoreduction capacity of NAD^+, which is required in the mitochondrial electron transport chain to maintain its proton gradient and thereby generate ATP (Koh et al., 2005). This is the suicide hypothesis. In fact, it is now recognized that caspase-3 could inactivate PARP in order to turn off an energetically expensive DNA repair pathway and therefore maintain ATP levels required for the execution of apoptosis (Fischer et al., 2003). The extent of oxidant-induced ATP depletion and cell fate could be modified by PARP inhibition (Aito et al., 2004).

Recently, a number of other mechanisms have been mooted to explain the basis for cellular death from PARP-1 overactivation. The most vocal at the moment is the now established link between overactivation of PARP-1 and apoptosis, specifically in relation to the induction of AIF, as it is now known that a variety of environmental and chemical stimuli (e.g., the DNA-alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or NMDA) and free radical/oxidant attacks (e.g., induced by H_2O_2) can trigger the overactivation of PARP-1 in response to DNA damage (Dawson & Dawson, 2004, Hong et al., 2004). This in turn stimulates the translocation of AIF from the mitochondrial intermembrane space to the nucleus triggering chromatin condensation, massive DNA fragmentation and nuclear shrinkage (Yu et al., 2002; Dawson & Dawson, 2004; Hong et al., 2004). Following this, phosphatidylserine becomes exposed, cytochrome c is released at a later time point and caspase-3 is activated. In the context of excitotoxicity, NMDA-induced release of AIF is PARP-dependent and AIF toxicity is caspase-independent (Dawson & Dawson, 2004),

while in general, AIF could mediate both caspase-dependent and caspase-independent cell death, although the cross-talk between AIF and the caspase pathway is complex (Koh et al., 2005). Interestingly, it is also known that cleavage of PARP-1 could be induced by caspases and the presence of cleaved PARP-1 is now a diagnostic tool for the confirmation of apoptosis in many cell types (Lazebnik et al., 1994; Koh et al., 2005). Pharmacological inhibition of PARP-1 or genetic knockout of PARP-1 has been shown to be therapeutically efficacious in experimental models of disorders characterized by DNA damage, such as ischemia, ischemia-reperfusion injury, diabetes, shock, inflammation, ROS-induced injury, cancer, and excitotoxic neuronal cell death (Virag & Szabo, 2002; Skaper, 2003; see Dawson & Dawson, 2004). Excessive PARP-1 activation has also been implicated in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced Parkinsonism and traumatic spinal cord injury (Skaper, 2003). The microglial migration that occurs in secondary neuronal damage is strongly controlled in living brain tissue by the expression of the integrin CD11a, which is under the regulation of PARP-1, thus making PARP-1 downregulation an appealing strategy in protecting neurones against secondary damage (Skaper, 2003). The ability of PARP-1 to modulate neuronal cell death may also arise through regulation of transcriptional activation by modulating the function of NF-κB (Koh et al., 2005). PARP-1 might also modulate the activity of p53 and possibly other effector proteins (Chiarugi, 2002).

The termination of PARP-generated PAR effect occurs through the rapid action of another enzyme, poly (ADP-ribose) glycohydrolase (PARG), which is the only known intracellular enzyme shown to specifically and efficiently catalyze the exo- and endoglycosidic hydrolysis of PAR into free ADP-ribose (Brochu et al., 1994; Ame et al., 2000), although PAR was initially found to be cleaved by phosphodiesterases to generate adenosine 5' monophosphate (AMP) and phosphoribosyladenylate (Miwa et al., 1979). This has a protective role, since the accumulation of PAR leads to cell death by apoptosis. The final, protein-proximal ADP-ribose residue is proposed to be removed from the acceptor protein through a putative ADP-ribosyl protein lyase (Oka et al., 1984). In the CNS, PARP and PARG are present throughout the brain and spinal cord (Koh et al., 2005).

1.6. Experimental systems and models

This section introduces the cell culture technique, the types of cultures commonly employed, and in particular the MC3T3-E1 osteoblast-like cell line and primary cultures of

granule neurones from the cerebellum, highlighting why they were chosen for the experiments.

1.6.1. Cell culture

The cell culture technique is a valuable tool for life science researchers providing a means to maintain and manipulate the cells. Cell culture models permit the study of a single cell group and also allow measurements of cell responses to conditions that are more tightly regulated than the complex environment presented by an entire organism (*in vivo*), e.g., in neurotoxicological studies (Stacey & Viviani, 2001) and thus are a reductionist approach. It allows the *in vitro* investigation of the actual roles of agents in cellular processes. Culture conditions are well optimized with respect to media composition, incubation conditions and cell density, and manipulations are done aseptically. According to Harry et al. (1998), "*in vitro* tests have their greatest potential in providing information on basic mechanistic processes in order to refine specific experimental questions to be addressed in the whole animal".

Primary cultures and cell lines are the two main culture systems used as *in vitro* models of excitotoxicity. By definition, primary cultures are "cells harvested directly from the organism, dissociated into single cells before seeding into the culture vessel, and maintained *in vitro* for periods exceeding 24 hours" (Harry et al., 1998). On the other hand, cell lines are "cultures that have been serially transplanted or subcultured through a number of generations and can be propagated for an extended period of time". Primary cell cultures are the preferred system for use in excitotoxic studies, as it is generally accepted that they more closely represent the *in vivo* situation (Buchhalter & Dichter, 1992). While neuronal cell lines are commercially available, they are generally derived from endogenous tumours or from chemically or virally transformed cells and so often have an atypical nature when established in culture (Lowndes et al., 1994). Some cell lines such as phaeochromocytoma cells are useful for investigating cell death in peripheral neurones, but central neuronal cell lines are expensive and difficult to set up and maintain.

The second culture system comprises the continuous cell lines, which are transformed cells from tumourigenic tissues, with a life span of about 50 divisions. Cell lines initially with limited life span could undergo crisis changing their growth potential and thus making their lifespan unlimited. Clonal cell lines are homogeneous and large quantities of cells could be easily grown from them. Established cell lines have the ability to undergo an unlimited number of cell divisions, altered cell and colony morphology, lack of

locomotion, lack of contact inhibition, lack of density-dependent inhibition of cell multiplication, loss of anchorage dependence and high fibrinolytic activity (Diamond & Baird, 1977). Once the phenotype of the cell line is established, it does not change, although as the number of passages increases, the cells may drift in their physiological responsiveness (Harry et al., 1998).

1.6.2. The MC3T3-E1 osteoblast-like cell line

Lack of reliable *in vitro* models to study the developmental sequence associated with replication of osteoblast precursor and differentiation makes the understanding of the regulation of osteogenesis incomplete. For the investigation of bone formation *in vitro*, a number of cell lines are available. Transformed malignant osteoblast cell lines have uniform phenotype, but also possess unrepressed replicative activity and fail to display the normal coupling of differentiation and growth arrest (Pardee et al., 1978). Thus, they express partially differentiated and replicative functions and so are not ideal as models for studying the process of osteoblast development. On the other hand, studies in primary osteoblast cultures support the concept of sequential expression of the osteoblast phenotype, but various cell populations at various developmental stages as well as de-differentiated cells are present in isolated calvarial and primary cell cultures, making data interpretation difficult. Again, subcultivation of primary cultures results in loss of osteoblast-specific characteristics (Aronow et al., 1990). However, primary cultures of osteoblasts are becoming increasingly important, as it has been demonstrated that primary rat osteoblasts exhibit much closer electrophysiological characteristics to neuronal cells (Gu & Publicover, 2000), although in terms of experimental studies so far, understanding of the osteoblast developmental sequence has been greatly facilitated by an immortalized cell culture system that allows independent investigations of osteoprogenitors, osteoblast differentiation and mature osteoblast function, which is the MC3T3-E1 cell line. It was derived from newborn murine calvariae and is known to display osteoblast-like characteristics after repeated passages (Quarles et al., 1992). It is capable of type 1 collagen synthesis, alkaline phosphatase activity and nodular extracellular matrix mineralization resembling woven bone (Sudo et al., 1983), observations that were supported by Quarles et al. (1992) when they revealed that, in culture, these cells show a temporal sequence of development characterized by distinct proliferative and differentiated stages. They therefore represent immature osteoblasts that undergo a temporal program consistent with osteoblast differentiation in stages analogous to *in vivo* bone formation.

Because the expression patterns of bone-related proteins are temporally regulated during MC3T3-E1 cell differentiation and their regulations are unique compared to other systems, the cell line is also a useful *in vitro* system for the study of developmental regulation of bone-related proteins in relation to the different stages during osteoblast differentiation (Choi et al., 1996).

1.6.3. The cerebellum and cultures of cerebellar granule neurones

The cerebellum is the part of the vertebrate hindbrain that is concerned mainly with somatic motor function, muscle tone control as well as the maintenance of balance (Bear et al., 1996). In a unique way, it represents an important model for cell migration in developing mammalian brain, in view of the well-studied migratory pathway of the granule neurones. Almost all voluntary actions rely on fine control of motor circuits, and the cerebellum is crucial to their optimal adjustment, e.g., with respect to timing (British Neuroscience Association, 2003). Recent evidence suggests that the cerebellum plays a vital role in learning, memory, fear conditioning, and cognitive processing (Wu et al., 2005). In the mammalian brain, cerebellar granule cells are the most abundant neuronal subtype (Burgoyne & Cambray-Deakin, 1988).

In cell culture experiments, cortical, cerebellar granule and hippocampal cultures are the most frequently employed. There is no significant difference between receptor expressions for the "classic" neurotransmitters between these brain areas and the choice of which of these tissues to use in cell culture could be largely dependent on the investigator's personal preference or experience. However, there are certain cases when one of these regions is particularly desirable or more suited to an experimental design or question. For example, the cortex and cerebellum are much larger than the hippocampus, affording the ease of dissection as well as the plating of many more testable cultures when these brain regions are used (Cambray-Deakin, 1995). On the other hand, with regard to electrophysiological studies, some laboratories prefer to use hippocampal cultures to see if a certain electrophysiological property extends to neurotoxicity, as the hippocampus is often used for electrophysiological studies (Buchhalter & Dichter, 1992). It is known that cultured cerebellar granule neurones obtained from young rat or mouse represent an *in vitro* model widely used to study factors that control early neuronal differentiation and cell death such as excitotoxicity (Vaudry et al., 2003). In studying the mechanisms involved in apoptosis, cerebellar granule neurones (CGNs) are frequently more desirable (Kalda and Zharkovsky, 1999), which is why they are most often used in neuropharmacology for studying pathways

of drug-induced apoptosis. For example, they have been used to assess the neurotoxic effects of glutamate (Schramm et al., 1990; Slagsvold et al., 2000) (as did this study), kainic acid (Smith et al., 2003), 1-methyl-4-phenylpyridinium ion (MPP^+), and colchicine (Gorman et al., 1999). In addition, it has been reported that cerebellar interneuron-enriched primary cultures are known to provide an almost pure population of a single type of live neurones (granule cells) (Levi et al., 1989). This therefore overcomes in a unique way one of the main problems encountered in neurobiological or neurochemical studies, which is the great cellular heterogeneity of the CNS preparations available (Levi et al., 1989). Cerebellar granule cells are highly enriched in primary culture (~95% granule cells) and are susceptible to the neurotoxic effect of glutamate (Schramm et al., 1990), although they may be less susceptible to neurodegeneration than the neocortex (cerebrum and cerebral cortex) (Wu et al., 2005). During normal development of the nervous system, neurones are produced in abundance to ensure the target areas are well innervated and neurones that fail to reach their target sites are removed by programmed cell death (apoptosis) (Oppenheim, 1991; Jacobson et al., 1997; Pettmann & Henderson, 1998). This natural arrangement is clearly evident in the cerebellum during the postnatal stage, when neurones migrate from the external to the internal granule layers. Granule cells that receive excitatory inputs from mossy fibres survive and differentiate, while those without this benefit die by apoptosis (Williams & Herrup, 1988; Wood et al., 1993). This requirement for excitatory inputs is simulated *in vitro* by culturing CGNs in medium containing a depolarizing concentration of potassium chloride (e.g., 25mM) (Gallo et al., 1987), or maintaining them in the presence of exogenous trophic agents such as NMDA (Gallo et al., 1987; Balazs et al., 1988) or BDNF (Lindholm et al., 1993). Interestingly, it has been recently reported that foetal chicken CGNs, which develop excitotoxicity rapidly in culture (3 div), may be grown successfully in a physiological concentration of KCl (5mM) (Jacobs et al., 2006). The granule cell migration is prevented by the degradation of the NMDA glycine site neuromodulator, D-serine, or of the enzyme that produces it, serine racemase (Mustafa et al., 2004). Cultured CGNs develop similarly to their *in vivo* counterparts (Gallo et al., 1987), thus making interpretation of data valid for *in vivo* predictions or extrapolations.

1.7. Rationale for the study

The discovery that glutamate is actively secreted in osteoblasts where most of its receptors are also functionally well expressed in a way very similar to their expression patterns in the CNS provided the basis for this comparative study. However, emphasis was on the NMDA

receptors, partly because they are the glutamate receptors that have been most extensively studied so far in osteoblasts and also because they are the receptors that predominantly mediate cell death in the CNS, thus providing a convenient basis for comparison of the responses of the two tissue types- bone and brain- to experimental agents.

1.8. Aims

In the light of the basis for this study as explained, the aims of this work were:

1. To examine the roles of culture media compositions, adenosine and its A_1 and A_{2A} receptors, and glutamate and its NMDA receptors in the modulation of viability of osteoblasts and central neurones.
2. To determine the responses of osteoblasts and central neurones to reactive oxygen species (ROS) generated directly or from a number of other sources, including the kynurenine pathway, since ROS are known to be a major mediator of glutamate-induced damage in the CNS.
3. To investigate the relative involvements of apoptotic and necrotic pathways in the mechanisms of glutamate-induced excitotoxic and ROS-induced cell death, in cultures of osteoblasts and central neurones.
4. To explore the potential roles of adenosine A_1 and A_{2A} receptors in protection against the phenomena of glutamate-induced excitotoxic and ROS-induced damage, in cultures of osteoblasts and central neurones.

2. MATERIALS AND METHODS

2.1. Cell culture reagents and materials

The following reagents and materials were used in setting up the MC3T3-E1 osteoblastic and cerebellar granule neuronal cultures, arranged based on their sources:

(1) **GIBCO (Invitrogen), Paisley, Scotland, UK**: Minimum Essential Medium (MEM), Cat. No. 32360-026; Foetal Calf Serum (FCS) (heat-inactivated), Cat. No. 10500-064; L-glutamine (200 mM stock), penicillin-streptomycin (P/S) (10,000U/10,000µg per ml); gentamicin (50mg/ml); trypsin (1X), liquid 0.25% (1:250), Cat. No. 25050-014; diethylpyrocarbonate (DEPC)-treated water; cell culture freezing medium (with 10% DMSO), phosphate buffered saline (PBS) and Hanks' Balanced Salt Solution (HBSS).

(2) **Sigma, Poole, Dorset, UK**: Foetal Calf Serum (FCS) (heat-inactivated), Cat. No. F9665; L-ascorbic acid (sodium salt), glycerol 2-phosphate (β-glycerol phosphate) (disodium salt hydrate); bovine serum albumin (BSA), trypsin 1:250 (powder, from porcine pancreas); soybean trypsin inhibitor (SBTI), Cat. No. T6414; poly -D- lysine (PDL), hydrobromide; DNase 1, cytosine arabinofuranoside (Ara C) and sodium bicarbonate ($NaHCO_3$) solution 7.5% (sterile-filtered, endotoxin-tested).

(3) **Fisher Scientific UK Ltd., Leicestershire, UK**: D(+)-glucose.

(4) **FSA Laboratory Supplies, Loughborough, England, UK**: Magnesium sulphate ($MgSO_4$).

(5) **BDH Laboratory Supplies, Poole, England**: Potassium chloride (KCl).

All reagents and materials were stored at the appropriate temperature and humidity, as recommended by the respective suppliers. Cell culture materials (dishes, plates, centrifuge tubes, Eppendorf tubes, pipette tips, Pasteur pipettes, millipore filters, hand gloves, etc.) were obtained from a variety of sources based on availability in the University Biomedical Research (IBLS) Stores. However, most plastic (96-well and 24-well) plates were from Corning Incorporated (New York, USA). Glassware and other materials were sterilized by autoclaving.

2.2. Cultures of MC3T3-E1 osteoblastic cells

2.2.1. Stock source and handling

The murine-calvaria derived MC3T3-E1 osteoblast-like cell line (mouse C57BL/6 calvaria, ECACC No. 99072810) used in this study was purchased from the European Community of Animal Cell Culture (ECACC) in partnership with SIGMA. The cells, obtained in a frozen form inside a 1ml plastic cryotube packed in solid carbon dioxide pellets, were of passage two (2) and their expected viability was 99% (viability was monitored during study and average value was 90%). Upon arrival, the ampoule was left for approximately 1 min at room temperature and then transferred to a 37°C water bath until fully thawed (1-2 min). Rapid thawing of cells was ensured so as to minimize any damage to the cell membranes. The ampoule was removed from the water bath and wiped with a tissue soaked in 70% alcohol prior to opening. Its entire content was slowly pipetted into a 25cm^2 tissue culture flask containing 10ml of MEM (supplemented with 10% FCS and 2mM glutamine), as recommended by the supplier. Forty-eight hours later, when the cells were at least 80% confluent, they were harvested with 0.25% trypsin, washed by centrifugation, and resuspended in MEM. The suspension was then split into four parts. Each of the first three parts was transferred into a 25cm^2 flask and incubated at 37°C in a humidified atmosphere of 5%CO_2 / 95%O_2, while the fourth part was frozen down and kept at -80°C for short-term storage, and in liquid nitrogen for long-term storage. All these procedures were carried out under strict aseptic conditions.

2.2.2. Subculturing of cells and treatments

In order to obtain subcultures for experiments, the cells were removed from the flask by trypsinization when the flask had become confluent (>80%). They were then washed and resuspended in fresh medium. Cell density was determined using the haemocytometer and the suspension diluted accordingly with medium in order to obtain the desired seeding density of 1 x 10^5 cells/ml. Cells were then seeded into either the 96-well plates or the 24-well plates. The glass bottle containing the cells was constantly but gently swirled at intervals as the seeding process continued in order to ensure that the cell density was uniform throughout the seeding period.

Cultures in plates were replenished every 2 to 3 days by aspirating the spent medium and adding fresh medium (cells in the culture flasks were fed by aspirating half of the spent medium and replacing it with half of the fresh medium). The medium in which they were maintained was MEM supplemented with 20% (or 10%) FCS and 1% P/S antibiotic combination. The final concentrations of the antibiotics were 100IU/ml for penicillin and 100µg/ml for streptomycin and the medium was stored at 4°C for no longer than 4 days. Subculturing was done on growing cultures that were at least 7 days *in vitro*.
L-ascorbic acid and β-glycerol phosphate are known to accelerate the proliferation and differentiation of the MC3T3-E1 cells (Harada et al., 1991; Quarles et al., 1992). It has also been reported that these supplements are required for the cells to express a maximal glutamatergic phenotype at the time of treatment. Cultures were therefore either continuously maintained in the original culture medium (MEM supplemented with 20% FCS and 1% P/S), referred to as "non-switch" medium, or switched after 3 days to the same MEM but supplemented with 10% FCS, and additionally with 1% of a combination of ascorbate and β-glycerol phosphate (ascorbate and β-glycerol phosphate added to final concentrations of 50µg/ml and 5mM, respectively, from a 100-fold stock), referred to as "switch medium". The cultures were used at 10 div, except otherwise stated, a stage when they are known to have sufficiently differentiated to express functional (including glutamate) receptors. Agents were diluted (from their filter-sterilized stock solutions) to the desired concentrations in culture medium or in MEM only, based on the experiment(s) to be performed.

2.2.3. Freezing down the cells

As it was necessary to keep a stock of cells for future experiments, and as a safeguard against any loss due to contamination, some of the MC3T3-E1 cells were frozen down upon arrival and then routinely afterwards, according to the following protocol:

1. When cells were at least 80% confluent, they were trypsinized by removing the culture medium and adding a volume of 0.25% trypsin solution (preheated to 37°C) sufficient to cover the monolayer of cells. This was 5ml for a 25cm^2 tissue culture flask and 10ml for a 75cm^2 tissue culture flask.
2. The flask was returned to the incubator and checked under the microscope every 5 min for cell detachment.

3. When the cells had detached (evidenced by cells floating in the trypsin solution), the flask was flooded with medium.
4. The entire content of the flask was transferred into a 15ml centrifuge tube (for a $25cm^2$ tissue culture flask), removing as many of the detached cells as was practically possible.
5. The cells were washed in culture medium by initially centrifuging gently (3 min at 500g).
6. The supernatant (medium) was aspirated and the pellet (cells) resuspended in 5ml of medium.
7. Step 5 was repeated and the supernatant was aspirated.
8. The cell culture-freezing medium was added to the pellet of cells.
9. The cells were redispersed by gently and carefully pipetting up and down in the freezing medium and dispensed into 1ml aliquots in sterile plastic cryotubes. The cryotubes were sealed.
10. The aliquots were put in a styrofoam box (or a rack) and cooled at 4°C (fridge) for 30 min.
11. The styrofoam box (or rack) containing the cooled aliquots was then placed in -20°C freezer for 30 min.
12. The frozen aliquots were transferred to -80°C freezer for short-term storage and later to liquid nitrogen for long-term storage.

2.2.4. Resurrecting the frozen cells

The previously frozen cells in each plastic cryotube were gently but rapidly thawed by heating the lower end of the tube in a water bath (37°C). To avoid contamination, complete submersion of the tube was avoided. The entire content of the cryotube was then transferred into a centrifuge tube. 10ml of medium was added and the cells were washed by centrifuging at 500g for 5 min. The medium was aspirated and the cells resuspended in 10ml of medium (or 5ml of medium when the $25cm^2$ flask was used). The whole suspension was then transferred into a flask. In order to ensure all the cells were transferred into the flask, the centrifuge tube was flooded with 10ml of medium and its content again transferred into the flask. This was

repeated so that the cells were finally seeded in 30ml of medium in a 75cm^2 flask. Half of the medium was replaced after 24 h and then every 2-3 days until subculturing.

2.2.5. Assay for alkaline phosphatase (ALP) activity

Alkaline phosphatase is a marker of osteogenic differentiation *in vitro*. As cell lines could show phenotypic drift with multiple passages, it was necessary to characterize the MC3T3-E1 cell line with regard to its development. Cells were maintained for 7, 10 and 17 days, in order to assess and compare the levels of differentiation at these time points for non-switch and switch cultures. At the end of each culture period, ALP activity was assessed. ALP catalyses the conversion of the colourless substrate, p-nitrophenylphosphate (4- nitrophenylphosphate) to free p-nitrophenol (4-nitrophenol), which is yellow and in alkaline solution has strong absorption at a wavelength of 410nm at which the substrate has little or no absorption (Lowry et al., 1954). The equation for this reaction is:

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-nitrophenol} + P_i$$

The method used was adapted from Lowry et al. (1954) and Boyan et al. (1989) as follows:

1. Medium was aspirated from MC3T3-E1 cells in a 24-well plate. The cells were maintained at 4^{0}C by placing and keeping the plate on dry ice.
2. Cells were washed with 0.5ml per well of ice-cold PBS.
3. PBS was aspirated and 0.5ml per well of radioimmunoprecipitation assay (RIPA) buffer was added to lyse the cells. Each 10ml of the RIPA buffer contained 60mg (50mM) TRIS (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl) salt, 87.6mg (150mM) NaCl, 100µl (1%) Igepal, 50µl (0.5%) Triton, and 10mg (0.1%) SDS. These were obtained from suppliers to the University (IBLS) Stores, in most cases Sigma. One tablet of a protease inhibitor (complete mini EDTA-free, ROCHE) was added per 10ml of RIPA buffer a few hours before use to prevent proteolytic degradation.
4. The plate sitting on dry ice was transferred to a shaker and left for 30 min.
5. After gentle scratching using a blue pipette tip, the content of each well was aspirated into an Eppendorf tube and centrifuged at 13 000 rpm for 5 min. The supernatant was collected.

6. 150µl of each of the seven concentrations of the standard was added in triplicate to blank wells in a 96-well plate. The standard consisted of 0.002 - 0.014 µmol /100µl /well of p-nitrophenol (4-nitrophenol) in aminomethylpropanol buffer (0.5M solution of 2-amino-2-methylpropanol, made up in water, pH 10; 8mM p-nitrophenylphosphate, and 2mM $MgCl_2$). 150µl of the aminomethylpropanol buffer was used for blanking.
7. 50µl of the supernatant was used for the detection of ALP activity, added to blank wells in triplicate after vortexing.
8. 100µl of the p-nitrophenylphosphate-containing buffer was then added to sample wells.
9. The plate was left at 37°C for a defined period (usually between 60 and 90 min) in a water bath.
10. 100µl of 0.5M NaOH was added to each well to terminate the reaction.
11. The plate was read at 405nm using the ELISA plate reader (DYNEX Technologies, USA). ALP activity was expressed as µmol/min/mg protein: the value from ALP assay (µmol/ml) was divided by the time (min) taken to incubate in water bath before reading the plate; this new value was then divided by the protein assay value (mg/ml).

2.2.6. Protein assay

This assay was performed using the BIORAD Protein Assay reagent. The stock standard consisted of 20mg BSA in 100ml of distilled water, stored in the refrigerator for a maximum of one month. This stock standard was diluted 1 in 10 with distilled water just prior to use. For the standard curve, BSA concentrations of 0, 0.25, 0.5, 1.0, 1.5 and 2.0 mg/ml (each multiplied by 100, as samples were diluted 1:100) were prepared each in duplicate by making up 0, 50, 100, 200, 300 and 400µl, respectively, of the diluted BSA stock standard to 400µl with distilled water (i.e., adding 400, 350, 300, 200, 100, and 0 µl of water, respectively). The BIORAD reagent (Coomassie Brilliant Blue) was diluted with an equal volume of distilled water prior to use. In order to be able to express the results in mg/ml, 4µl of the supernatant (for each sample) was taken after vortexing and made up to 400µl with distilled water. 200µl of diluted BIORAD reagent was added to every standard and sample and all tubes were vortexed. 200µl of each standard and sample containing diluted BIORAD reagent was pipetted into the wells of a 96-well plate in duplicate and the plate read at 595nm.

2.3. Cultures of cerebellar granule neurones (CGNs)

2.3.1. Stock solutions, preparation buffer, culture medium, well coating and materials

Stock solutions

The following solutions were prepared from their respective original stocks, aliquotted and stored at appropriate conditions:

1 mg/ml trypsin inhibitor (SBTI), 5mg/ml trypsin (from trypsin powder), 1000U/ml DNase I, 1.25mg/ml poly-D-lysine, 1ml of 3.82% w/v $MgSO_4$, and 10 mM Ara C.

A day before passaging, a concentrated solution of SBTI and DNase was prepared by adding 9ml of preparation buffer to 0.5ml SBTI stock and 0.5ml DNase I stock. Then, a weak solution of SBTI and DNase was prepared by adding 3.2ml of this concentrated solution to 16.8ml of preparation buffer. Also, a solution of 0.25mg/ml trypsin was prepared from the trypsin stock by adding 19ml of preparation buffer to 1ml of the stock.

Preparation buffer

This contained 250mg D(+)-glucose, 300mg Bovine Serum Albumin (BSA), and 1ml of 3.82% w/v $MgSO_4$, made up to 100ml with PBS (pH adjusted to 7.4 with 1M NaOH). The preparation buffer was then filter-sterilized using the millipore filter (Minisart®, 0.20μm).

CGN culture medium

The cultures were maintained in Minimum Essential medium (MEM) supplemented with 10% FCS (Sigma), 2mM glutamine, 25mM KCl and 50μg/ml gentamicin, i.e., each 100ml of the CGN culture medium contained 88.1ml of MEM, 10ml of FCS, 1ml of glutamine (200mM), 0.8ml of KCl (2.5M) and 0.1ml of gentamicin (50mg/ml). The KCl concentration here took into account its basal level in the MEM, which was approximately 5mM. The medium was stored at 4°C and used within 3 days of preparation.

Coating of wells

Wells were coated at least 4 days before seeding. Because poly-D-lysine (PDL) represents a good substrate for neurite outgrowth in primary culture (Buchhalter & Dichter, 1992;

Paulsson, 1992), each 96-well plate to be used in neuronal experiments was coated with 50µl/well of 15µg/ml PDL, obtained by diluting a stock solution of PDL (1.25mg/ml) with DEPC-treated water. The stock solution was made by adding 4ml of DEPC-treated water to 5 mg of thawed PDL and stored at -20°C. PDL solution was left in contact with the wells until 24 h before seeding, when it was aspirated and an equal volume of sterile water (50µl/well) added for 1 h to rinse the wells. The water was then aspirated and the plates left to air-dry.

Flame-polished pipettes

The ends of three (3) glass Pasteur pipettes were flame-polished (using the Bunsen burner) to give decreasing diameters, with the largest diameter equivalent to that of a normal Pasteur pipette and the smallest to that of a P200 yellow tip.

2.3.2. Preparation of CGN cultures

2.3.2.1. Animal selection and surgery

8-day old Sprague-Dawley pups of either sex were obtained from pregnant female rats maintained under standard and hygienic conditions (ambient temperature of 21°C ± 2°C, 12-h light: 12 h-dark cycle, food and drinking water available *ad libitum*) in the Central Research Facility (CRF) of Glasgow University. Each rat pup, weighing approximately 10g at postnatal day 8, was anaesthetized by intraperitoneal injection of 0.1ml Euthatal (from Fort Dodge Animal Health, Southampton; 200mg pentobarbital sodium Ph Eur is in 100ml of Euthatal). Once the loss of writhing reflex was confirmed, the pup was quickly decapitated and the head placed in a Petri Dish containing the preparation buffer. With a pair of scissors, an incision was carefully made from the base of the skull through to the frontal region, taking care not to injure the underlying brain. The brain was then neatly exposed with the aid of a pair of forceps, removed and placed in a 20ml glass bottle containing about 5ml of ice-cold preparation buffer. Between five (5) to eight (8) brain samples were typically collected.

2.3.2.2. Microdissection and establishment of CGN cultures

The following procedures were carried out under aseptic conditions:

Brain samples were gently transferred into a 35mm Petri Dish containing a volume of the preparation buffer enough to preserve them during the entire microdissection period. With the aid of a sterile scalpel, needles and forceps, and a dissecting microscope, the cerebella were excised from the whole brain and the meninges and blood vessels attached to them removed as much as possible. All cerebella were then collected in a separate Petri Dish and thoroughly chopped with the scalpel.

Chopped cerebella were transferred to 0.25mg/ml trypsin solution and incubated at 37°C for 20 min, with gentle swirling every 5 min. At the end of the incubation, 20ml of a solution of weak SBTI and DNase was added to the cell suspension before it was centrifuged at 1200 rpm for 2 min at room temperature. The supernatant was then carefully aspirated, after which 2ml of ice-cold solution (kept on dry ice) of concentrated SBTI and DNase was added to the pellet. The pellet was broken up by carefully pipetting up and down with a P1000 blue tip. With the aid of three previously flame-polished glass Pasteur pipettes of decreasing diameters, the cell suspension was triturated, starting with the largest pipette. Approximately ten strokes per pipette were ensured, avoiding the generation of bubbles as much as possible. The 2ml of triturated suspension was made up to 20ml with the preparation buffer, after which it was centrifuged as before. The supernatant was aspirated and 2ml of sterilized CGN media, pre-warmed to 37°C, was added. The cell pellet was again broken up as previously with flame-polished pipettes and the volume of suspension made up with medium to about 15ml.

The percentage of dead cells in the suspension was determined with trypan blue. Cell viability was never less than 99%. Using the haemocytometer (Improved Neubauer), the cell density of the suspension was determined. The period of cell enumeration allowed unwanted debris to fall to the bottom of the suspension. A volume of the cell suspension needed to give the required volume and a seeding density of 1×10^6 cells/ml was then taken and made up with medium. 100µl of this suspension was seeded into each well of the 96-well plates pre-coated with poly-D-lysine. The suspension was gently swirled at intervals to ensure even distribution of cells all throughout the seeding period. The seeded plates were transferred into the incubator where they were maintained at 37°C in a humidified atmosphere of 5% CO_2 / 95% O_2.

2.3.2.3. Elimination of non-neuronal growth

In order to eliminate non-neuronal growth that was predominantly due to the proliferation of the glial population, cytosine arabinoside (Ara C) was added to the cultures 20 h after passaging. The Ara C stock solution (10 mM) was further diluted 100-fold with water on the day of treatment and the diluted solution was then added to each well of the CGN cultures to give a final concentration of 10μM.

2.3.2.4. Cell characterization: Immunocytochemical labelling for neuronal tubulin

The purity of neuronal cultures used in this study was assessed using immunocytochemistry (ICC) to label tubulin present in neuronal cytoskeleton. The procedure was as follows: Culture medium was aspirated from the 96-well plates containing cerebellar granule cultures at 8 div and the cells were washed twice with phosphate-buffered saline (PBS). They were then fixed with 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 30 min at room temperature (RT) inside the fume cupboard (and stored at 4°C, wrapped with tin-foil, if the ICC was to be performed at a later date). They were subjected to three 5-min washes with 0.1M PB and subsequently blocked in 50μl/well of 1.5 % blocking serum (in PBS + 0.3% Triton X-100) for 1 h on shaking table at RT. The blocking serum was from the species in which the secondary antibody had been raised (goat in the present study) (starting from the blocking step, plates were wrapped in tin foil and shielded from light as much as possible). The cells were then reacted at RT with 50μl/well of the primary antibody mouse anti-human β-III tubulin (monoclonal) (Chemicon® International; Cat. No. CBL 412), diluted 1:50 in PBS containing 0.3% Triton X-100. The primary antibody was omitted from the negative control wells (only diluent added). The plates were gently agitated for 1 h and incubation with the primary antibody continued overnight at 4°C. On the next day, cells were washed at RT in three 5-min changes of double strength PBS and incubated for 1 h with 50μl/well of the secondary antibody (goat anti-mouse, conjugated with rhodamine red; from Jackson, Soham, UK, Cat. No. 115-295-146), diluted 1:100 in PBS containing 0.3% Triton X-100. They were then washed in two 2-min changes of PBS and further in PB for 2 min. The cultures were afterwards viewed by fluorescence microcopy on an Olympus DP50 Microscope and images were acquired and stored using DP-Soft Software.

2.3.2.5. Cell treatments and excitotoxicity studies

Neuronal cultures were used for experiments at 8 div except for excitotoxicity studies when they were used at 9 div. Cultures were not fed prior to the day of treatment. On the day of treatment, agents were diluted to the desired concentrations in culture medium and added to cultures after aspirating the spent medium. However, excitotoxicity studies were carried out using Hanks' Balanced Salt Solution (HBSS), adjusted to pH 7.4 with $NaHCO_3$, and treatments lasted for no longer than 1 h. Except otherwise stated, all cultures were restored after treatment to culture medium for at least 18 h before determination of viability. All procedures were carried out aseptically.

2.4. Determination of cell viability: alamar blue assay

The alamar blue assay was used for the determination of cell viability, in which alamar blue reduction varied directly with cell viability. The dye (alamarBlue™; Part No. DAL1100) was obtained from Biosource International, Nivelles, Belgium. The advantages of the assay over other viability assays have been discussed (see section 5.2). In general, the protocol was as follows:

1. The old medium was aspirated and 200µl (100µl for neurones) of fresh medium containing 10% v/v alamar blue was added to all test and control wells in 96-well plates (for 24-well plates, volume was adjusted accordingly). 200µl (or 100µl for neurones) of the medium was used for blanking.
2. The plate was returned to the incubator and allowed to incubate for 4 h (6 h for neurones) at 37°C in an atmosphere of 5% CO_2 / 95% O_2.
3. The absorbance (A) was measured at 540nm (lower wavelength, LW) and then at 595nm (higher wavelength, HW) using a spectrophotometric plate reader (DYNEX Technologies, USA).
4. The absorbance of medium only (average blank value) was automatically subtracted by the plate reader from the absorbance of alamar blue and medium to give the absorbance of the oxidized form of alamar blue in medium. This was carried out at the two wavelengths. The absorbance of the oxidized form at the lower wavelength was designated AO_{LW} while the absorbance of the oxidized form at the higher wavelength was designated AO_{HW}.

5. The correction factor, R_0 was calculated as follows:

$$R_0 = AO_{LW}/AO_{HW}$$

6. Alamar Blue reduction (AR) was then calculated as follows:

$$AR = (A_{LW} - (A_{HW} \times Ro))$$

7. The Alamar Blue reduction was calculated as a percentage of the control as follows:
   AR (% of control) = $(A_{LW} - (A_{HW} \times Ro))tw/ (A_{LW} - (A_{HW} \times Ro))cw \times 100$
   where "tw" and "cw" refer to test wells and control wells, respectively. Cell viability was expressed as a percentage of the control, except otherwise stated.

2.5. Estimation of cell death using trypan blue

An estimation of the number of dead cells (and hence the percentage viability) was carried out using the trypan blue dye. An equal volume of 0.4% trypan blue solution (Sigma) was added to a suspension of cells and the mixture was incubated under standard conditions (as previously) for 5 min. The mixture was then gently mixed with the aid of a pipette and approximately 50µl was taken to fill the two sides of the haemocytometer. Cell counting was done under an inverted binocular microscope (x10 objective), using a hand-held tally counter. The total number of cells and the number of cells that had taken up the dye were determined and the difference between these two values represented the number of cells that had not taken up the dye. Cells that did take up the dye (thus appearing blue) were considered non-viable while those that did not take up the dye were considered viable. In order to determine percentage viability, the number of viable cells was expressed as a percentage of the total number of cells.

2.6. Assessment of morphological changes

The morphology of control cultures and changes to morphology induced by each experimental treatment were monitored on an Olympus DP50 inverted phase-contrast microscope, fitted with a Digital Camera System, which allowed photomicrographs to be taken and saved.

2.7. Results presentation and statistics

The results in this thesis have been presented as mean ± standard error of the mean (SEM) for n cultures or experiments. Treatments were done at least in triplicate (except protein assay

where treatments were done in duplicate). Two sets of means were compared using paired or unpaired t-test as appropriate. For comparison of more than two means, one-way analysis of variance (ANOVA) was used, followed by either the Dunnett's (comparison to control) or the Student-Newman-Keuls' post-hoc test, with a P-value of < 0.05 considered statistically significant.

2.8. Sources and preparation of chemical agents

The chemical compounds used in this study are as follows, grouped according to their sources:

1. **Sigma, Poole, Dorset, UK:**
   Acetylsalicylic acid (ASA, aspirin), adenosine, 2-amino-2-methylpropanol, bovine serum albumin (BSA) protein standard, caffeine, catalase (CAT), 2-chloroadenosine (2-ClA), copper (II) sulphate ($CuSO_4$) pentahydrate, N^6-cyclopentyladenosine (CPA), D-Mannitol, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX); 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ), hydrogen peroxide solution (30%w/w), 3-hydroxyanthranilic acid (3-HAA), 3-hydroxykynurenine (3-HK), kynurenic acid (KYA), L-Glutamic acid sodium salt hydrate (Glutamate or Glu), nicotinamide (NAm), N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), magnesium chloride ($MgCl_2$), *N*-methyl-D-aspartate (NMDA), 3-nitropropionic acid (3-NPA), p-nitrophenol standard, 1,3-Dimethyl-8-phenylxanthine or 8-phenyltheophylline (8-PT); quinolinic acid (QA), S-nitroso-*N*-acetylpenicillamine (SNAP), superoxide dismutase (SOD), xanthine (X), and xanthine oxidase (XO).
2. **Tocris Cookson Ltd., UK:**
   2-p-(2-Carboxyethyl) phenethylamino-5'-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680), cyclosporin A (CsA), 2-amino-5-phosphonopentanoic acid (D-AP5), (+)-MK-801 (dizocilpine), benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone (Z-DEVD-fmk).
3. **Calbiochem (EMD Biosciences Inc.):** p-nitrophenylphosphate (disodium salt).
4. **BDH Biochemicals:** Potassium cyanide (KCN).
5. **Research Biochemicals (International):** 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and allopurinol (AP).

6. **Schering-Plough Centro Ricerche:** 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH58261).
7. **Alexis Biochemicals (Axxora Ltd.), UK:** 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ).
8. **Biorad Laboratories:** Biorad reagent.
9. **Dr. Ongini (AstraZeneca)** gave as a gift 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol (ZM241385).

Most drugs were dissolved in water, with the exception of:

1. Catalase and superoxide dismutase, which were dissolved in normal saline.
2. Acetylsalicylic acid, CNQX, DPQ, kynurenic acid, ODQ, 8-PT, SCH58261, Z-DEVD-fmk and ZM241385, which were dissolved in dimethyl sulfoxide (DMSO).
3. Cyclosporin A, DPCPX and 3-NPA, which were dissolved in ethanol.
4. Allopurinol, quinolinic acid, xanthine and xanthine oxidase, which were dissolved in 1M NaOH.
5. 3-HAA, which was prepared (2mM) in 1M HCl (4% v/v) and diluted with ascorbic acid (100μM) to the desired concentration(s), with the final highest ascorbic acid concentration in cultures of 2.5%v/v. This vehicle had no effect of its own on cultures.

SNAP was dissolved in distilled water containing 0.54mM ethylenediaminetetraacetic acid (EDTA) (pH of water adjusted to 9.0 before adding EDTA). EDTA only helped to stabilize SNAP in storage and had no effect of its own on the cultures. The final concentrations of DMSO, ethanol and NaOH in cultures never exceeded 0.1% (except for Z-DEVD-fmk, which had a final DMSO concentration of 0.2% as recommended by the supplier and for which control solutions therefore included the same concentration of DMSO), which when tested on the cultures had no effect. Normal saline also did not have any effect of its own on the cultures. Stock solutions were filter-sterilized using the millipore filter and stored at appropriate conditions of temperature and humidity as recommended by their suppliers.

3. RESULTS – EFFECTS OF GLUTAMATE AND OXIDATIVE STRESS ON THE VIABILITY OF MC3T3-E1 OSTEOBLASTS

3.1. Alamar Blue (AB) standardization for viability determination

The use of alamar blue (AB) for routine determination of cell viability requires the standardization of each cell type for a number of factors, the most critical being the seeding density and the extent of incubation. Correlations between AB reduction, seeding density and the duration of incubation for the MC3T3-E1 cells were therefore determined. Densities ranging from 2.5 x 10^4 to 4 x 10^5 cells per ml, in consecutive, two-fold increases, were employed for incubation periods of 2, 3, 4, 5 and 6 h. As shown in Fig. 3.1A, AB reduction increased linearly (R^2 = 0.9826) with increasing densities for selected incubation periods (4 h shown). Similarly, AB reduction also correlated linearly (R^2 = 0.9306) with the incubation duration for a given seeding density (1 x 10^5 shown) (Fig. 3.1B). A seeding density of 1 x 10^5 cells per ml and an incubation period of 4 h were eventually chosen for subsequent experiments, except where stated otherwise.

Despite confirmed reports of non-toxicity to cells, it was necessary to examine whether AB had any injurious effect on the MC3T3-E1 cells used in the current study. A set of cultures was pretreated with AB (10%v/v) for 4 h while a parallel set of cultures was similarly treated with vehicle. At the end of pretreatment, at least 16 h was allowed for recovery, after which cell viability was determined using AB itself. There was no difference in viability between cultures pretreated with AB and those pretreated with vehicle (0.61 ± 0.01 vs. 0.60 ± 0.01, for AB- and vehicle-pretreated cultures, respectively; n = 4). A confirmatory trypan blue exclusion experiment also revealed no difference in the viabilities of the two sets of cultures (99.38% ± 0.40 vs. 99.22% ± 0.37; n = 6).

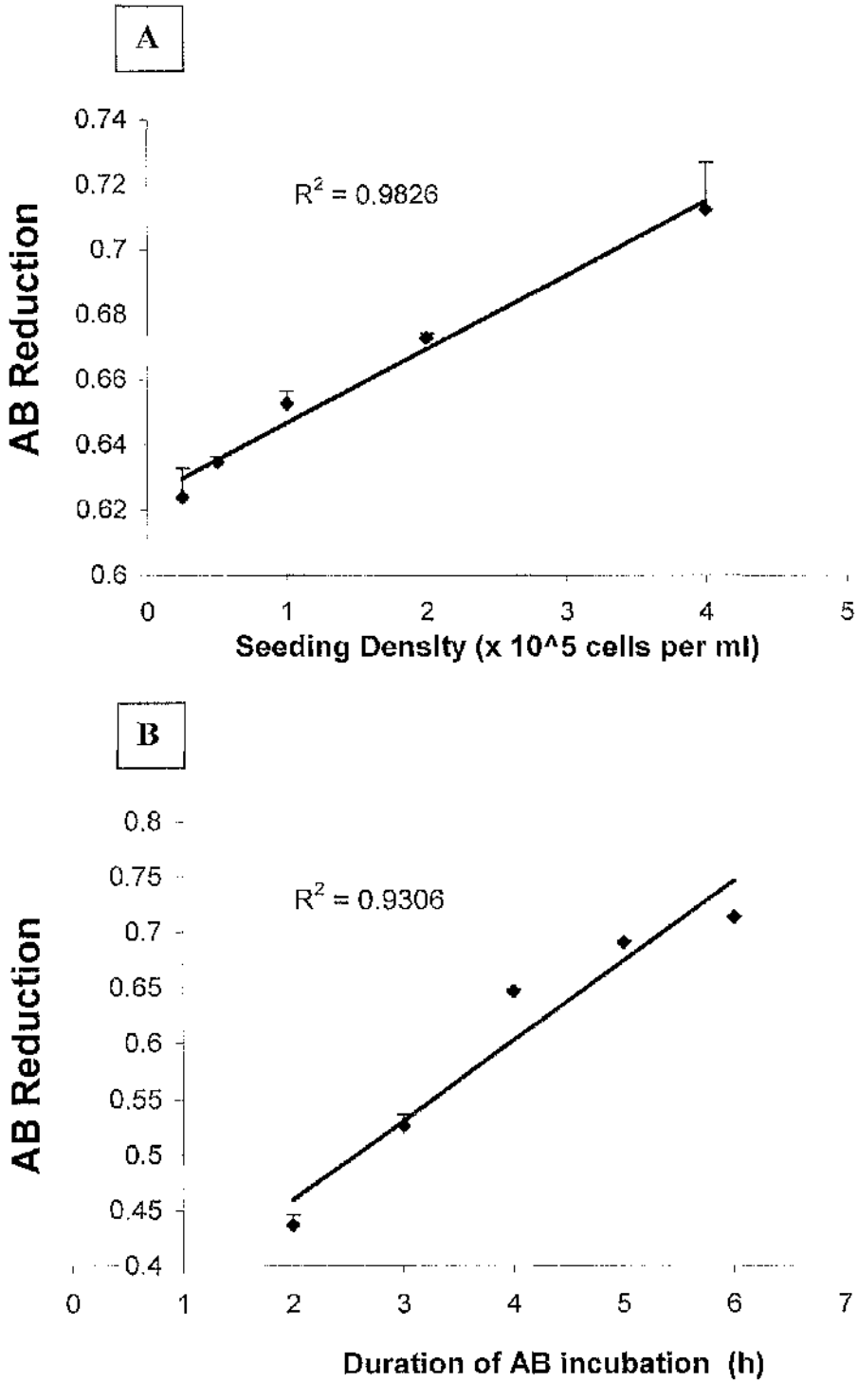


Figure 3.1. Graphs showing the correlation between alamar blue (AB) reduction and **(A)** initial seeding density (4 h of incubation) and **(B)** the duration of AB incubation (seeding density of 1 x 10^5 cells per ml) in the MC3T3-E1 cultures. Each data point represents the mean ± SEM for **(A)** n = 4 or **(B)** n = 3 or 4 cultures.

3.2. Switching the MC3T3-E1 cells

The MC3T3-E1 cells were used at a passage which represented immature osteoblasts. It was necessary therefore to switch these into a differentiated state in order to be representative of mature and functional osteoblasts. A number of recent reports claimed that L-ascorbic acid and β-glycerol phosphate (BGP) are two supplements which, when added together to osteoblast cells in culture, can accelerate both their proliferation and differentiation (Harada et al., 1991; Quarles et al., 1992). These claims were therefore examined to determine if the osteoblast cultures used in this study were competent.

3.2.1. Effect of switching on the proliferation of MC3T3-E1 cells

To assess the effects of these supplements on the proliferation of our MC3T3-E1 cell line, cultures were grown with or without L-ascorbic acid (50μg/ml) and β-glycerol phosphate (5mM) for 5 div. The number of cells was then determined. Significantly higher cell counts were obtained with cultures that received the supplements than with those that were not given the supplements ($2.9 \times 10^5 \pm 0.15$ vs. $2.3 \times 10^5 \pm 0.21$, $P<0.05$, $n = 3$) (Fig. 3.2).

3.2.2. Effect of switching on differentiation

Throughout this thesis, the term "switching" has been used to refer to supplementation of culture medium at 3 div with L-ascorbic acid and BGP, combined with a reduction in serum concentration from 20% to 10%. In order to assess the effect of switching on the differentiation of the MC3T3-E1 osteoblast-like cells, the activity of alkaline phosphatase (ALP), a membrane-bound enzyme known to be an unequivocal marker of differentiation in bone, was determined.

At 1 and 4 days after adding the switch medium, there was no significant difference between switch and control non-switch cultures. At 7 days post-switch, ALP activity was almost three-fold higher in switch than in non-switch cultures ($P<0.001$). The levels of ALP activity in supplemented switch cultures were more than four-fold higher at 11 days post-switch ($P<0.0001$) and were still higher at 14 days post-switch ($P<0.0001$, $n = 5 - 8$) (Fig. 3.3A).

When the plating density was increased three-fold to 3×10^5 cells per ml, a similar result was obtained. However, with this higher density, there were significantly higher levels of ALP activity in non-switch than in switch cultures at one day post-switch ($P<0.001$, $n = 8$), but these non-switch levels of ALP activity failed to rise with increasing days post-switch (Fig. 3.3B).

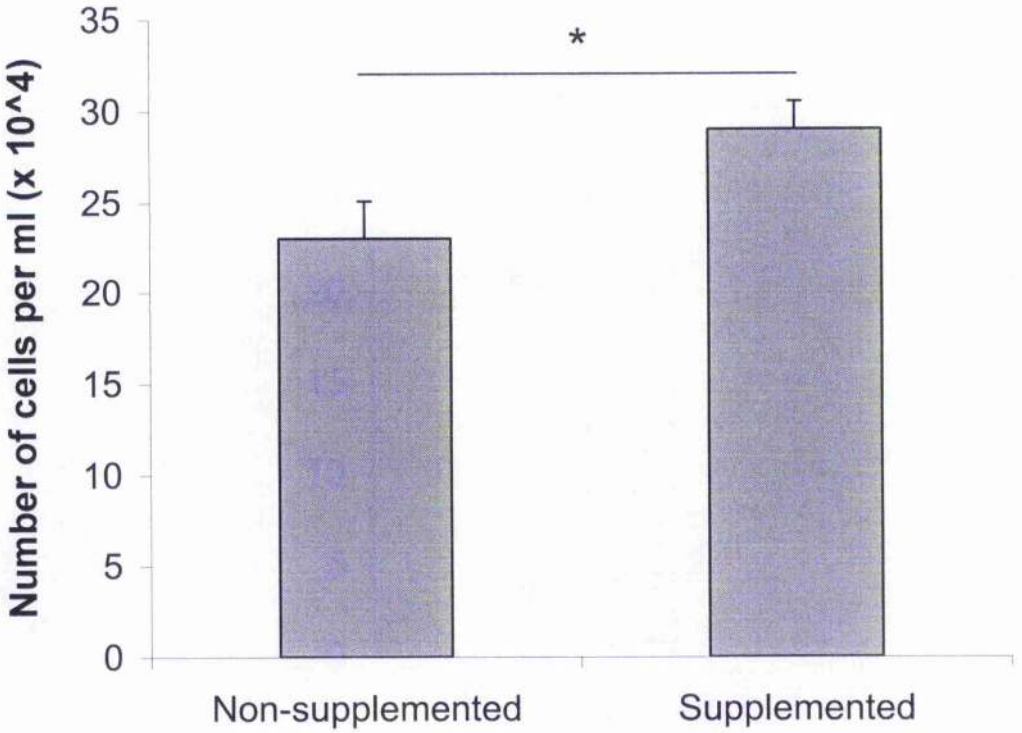


Figure 3.2. Histogram showing the effects of supplementation with L-ascorbic acid and β-glycerol phosphate on the number of MC3T3-E1 cells in cultures at 5 div. The final concentrations of L-ascorbic acid and β-glycerol phosphate were 50μg/ml and 5mM, respectively. Non-supplemented cultures had no L-ascorbic acid and β-glycerol phosphate in the culture medium at any stage, while supplemented cultures had L-ascorbic acid and β-glycerol phosphate in the culture medium from the time of plating until 5 div. Each column represents the mean ± SEM for n = 3 experiments. Statistically significant difference in cell count between supplemented and non-supplemented cultures is indicated as * $P<0.05$.

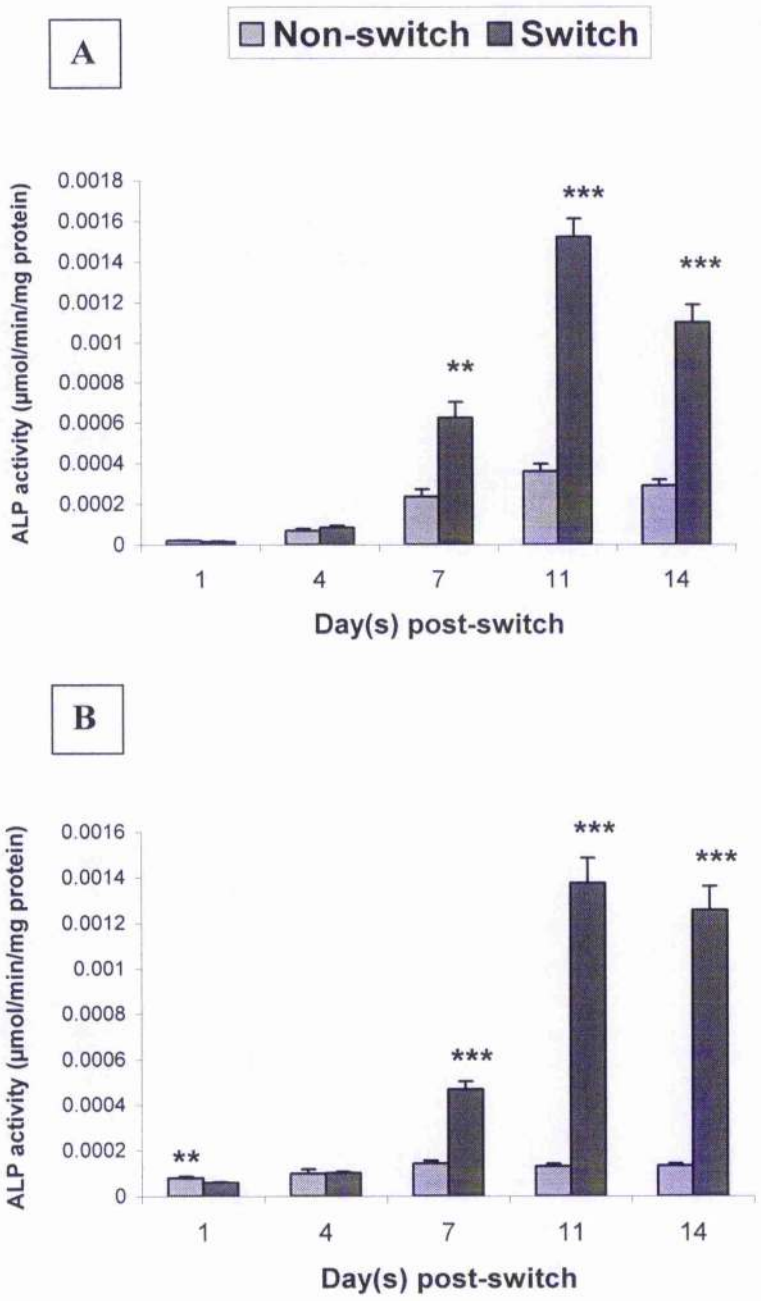


Figure 3.3. Histograms showing alkaline phosphatase (ALP) activity in the osteoblast-like MC3T3-E1 cells with increasing days post-switch when initial plating density was **(A)** 1 x 10^5 cells/ml and **(B)** 3 x 10^5 cells/ml. Switching was carried out at 3 div. N days *post-switch* is therefore equivalent to (N + 3) days *in vitro*. Each column indicates the mean ± SEM for **(A)** n = 5 - 8 or **(B)** n = 8 cultures. ** $P<0.001$, *** $P<0.0001$ for switch vs. non-switch cultures.

3.3. Culture conditions and MC3T3-E1 viability

As it is known that the conditions in which cultures of any cell type are grown could significantly determine their baseline viability or survival, the responses of MC3T3-E1 cells were examined in a range of culture conditions. Whether the process of switching modified the observed responses was also determined.

3.3.1. Effects of increasing serum concentrations and serum withdrawal

Serum is known to be highly essential for the survival of cells (including osteoblasts) in culture (Jilka et al., 1998). The influence of increasing levels of serum on the survival of switch and non-switch MC3T3-E1 cultures and the effect of withdrawing serum from these cultures were therefore investigated. Switch and non-switch cultures were maintained in Minimum Essential Medium (MEM) supplemented with 1, 10 and 20% Foetal Calf Serum (FCS) until 10 div, at the end of which viability was determined. Viabilities of non-switch cultures in the presence of 10 and 20% FCS were each very significantly higher than in the presence of 1% FCS ($P<0.001$, $n = 6$) (Fig. 3.4), but there was little difference in viabilities between non-switch cultures maintained in the presence of 10% FCS and those maintained in 20% FCS. Similarly, for switch cultures, viabilities were very significantly higher in cultures maintained in 10 or 20% FCS compared to those in 1% FCS cultures ($P<0.001$), although viability of switch cultures in the presence of 20% FCS was significantly higher than in 10% FCS ($P<0.01$). With regard to cultures maintained in 1% FCS, survival was significantly higher in non-switch than in switch cultures ($P<0.001$), while there was no difference in survival of non-switch and switch cultures when they were both maintained in 10% FCS. In contrast, maintenance of switch cultures in 20% FCS resulted in significantly higher survival than was the case with non-switch cultures ($P<0.05$). Withdrawal of serum from switch cultures for 0.25, 1, 3 and 6 h at 10 div had no effect on their viabilities ($n = 4$) (Fig. 3.5A). The effect of serum withdrawal for 1, 2 and 3 days before reaching 10 days *in vitro* (div) was also examined ($n = 6$) (Fig. 3.5B). A pattern of steady decline in viability was obvious with increasing durations of withdrawal. Serum withdrawal one day prior to reaching 10 div had no statistically significant effect on switch or non-switch cultures. Serum withdrawal for 2 days before reaching 10 div reduced the viabilities of non-switch and switch cultures significantly to 90.92% ± 1.20 ($P<0.01$) and 89.3% ± 2.20 ($P<0.001$) of the control, respectively, while serum withdrawal for 3 days prior to 10 div more significantly lowered viabilities to 82.34% ± 1.61 and 82.50% ± 2.16

of the control ($P<0.001$). There was, however, no difference between the sensitivities of non-switch and switch cultures to serum withdrawal for any given duration of withdrawal.

3.3.2. Addition of potassium to culture medium

The effect - on survival - of adding potassium at 3 div to the culture medium in which MC3T3-E1 cells are grown was determined. This was carried out in order to verify if these cells compared with neurones, which are known to survive better with K^+ concentrations in the 20-25mM range than in the lower 5-10mM range (Gallo et al., 1987; Xifro et al., 2005). At 10 div, viabilities of non-switch cultures supplemented with 10 and 25mM K^+ were not different from their respective controls whereas 50mM K^+ reduced viability slightly but significantly to 94.06% ± 0.87 of the control ($P<0.01$, $n = 3$) (Table 3.1). On the other hand, the survival of switch cultures in 10mM K^+ was enhanced (104.39% ± 1.46 of the control, $P<0.05$, $n = 3$), while higher concentrations of 25 and 50mM had no effect. Responses of non-switch cultures were not significantly different from those of switch cultures for a given concentration of potassium.

3.3.3. Presence of glutamine in culture medium

Glutamate is considered to support the survival of most cells in culture. The effect of glutamine on MC3T3-E1 viability was therefore examined (Table 3.1). Glutamine added to non-switch and switch cultures at 3 div to a final concentration of 2mM improved their viabilities at 10 div very significantly to comparable levels above the control, with values of 111.46% ± 0.29 and 111.92% ± 0.53, respectively ($P<0.001$, $n = 4$).

3.3.4. Supplementation with ascorbate and β-glycerol phosphate

The addition of each of the switching supplements ascorbate and β-glycerol phosphate alone from 3 to 10 div had no significant effect on survival (Table 3.1). In addition, non-switch and switch cultures had comparable viabilities (expressed in absolute values of AB reduction) at 10 div of 0.68 ± 0.02 and 0.68 ± 0.01, respectively ($n = 4$).

3.3.5. Medium change and feeding protocols

A variety of protocols exist for adequate maintenance of cultures *in vitro*. Osteoblast cultures (in 96-well plates) in this study were fed by complete replenishment of culture medium every 2-3 days. However, the beneficial effect of medium change to overall survival of the MC3T3-E1 cells was assessed. Interestingly, an insignificant (6.8%)

reduction in viability was obtained with cultures maintained without any medium change for 10 div compared to cultures fed every 2-3 days (0.63 ± 0.01 vs. 0.68 ± 0.02, P>0.05, n = 4). Again, cultures fed by replacing only half of the spent medium with fresh medium gave a viability value that was 98.29% ± 1.30 (n = 4) of the viability of cultures fed by total replacement of medium, a difference that was not statistically significant.

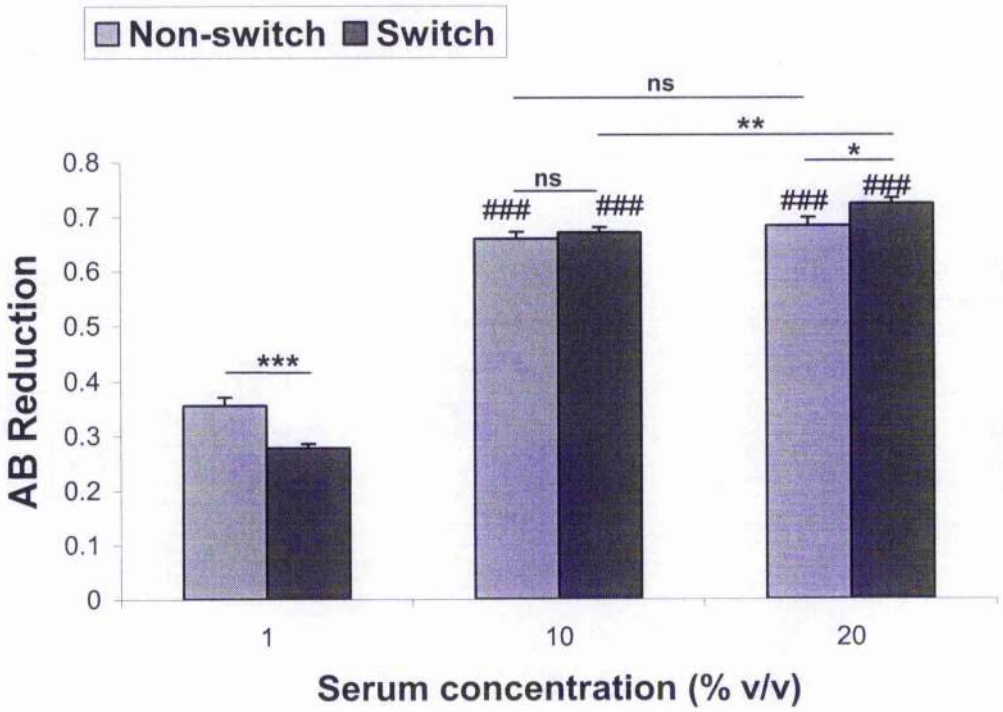


Figure 3.4. Histograms showing the effects of growing non-switch and switch MC3T3-E1 cultures in medium containing increasing concentrations of serum (1, 10, 20%). Viability was determined at 10 div. Each column represents the mean ± SEM for n = 6 cultures. *P<0.05, **P<0.01, ***P<0.001, ns = non-significant; ###P<0.001 compared to corresponding 1% v/v serum concentration.

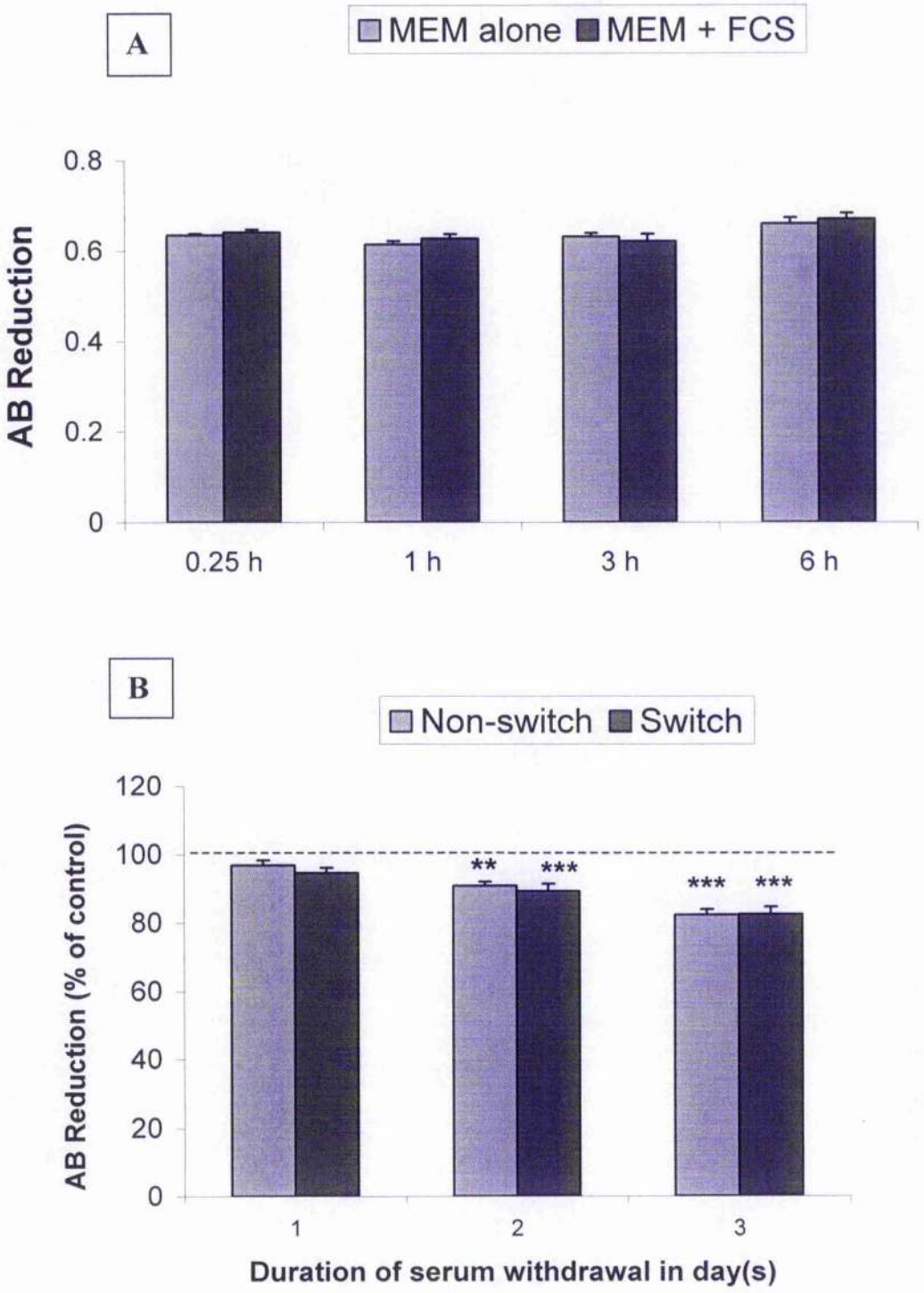


Figure 3.5. Histograms showing **(A)** lack of effects of serum withdrawal for 0.25, 1, 3 and 6 h at 10 div on the viability of switch cultures and **(B)** the effects of serum withdrawal for 1, 2 and 3 days prior to 10 div on the viabilities of non-switch and switch MC3T3-E1 cultures. Each column represents the mean ± SEM for **(A)** n = 4 and **(B)** n = 6 cultures. ** P<0.01, *** P<0.001 compared to control.

CULTURE CONDITION	AB REDUCTION (% OF CONTROL)	
	NON-SWITCH	SWITCH
K^+ concentration (mM)		
10	102.26 ± 0.31 (3)	104.39 ± 1.46 (3) *
25	100.86 ± 0.19 (3)	97.09 ± 1.82 (3)
50	94.06 ± 0.87 (3) **	97.04 ± 0.97 (3)
*Glutamine **2mM***	111.46 ± 0.29 (4) ***	111.92 ± 0.53 (4) ***
*Ascorbate alone **50µg/ml***	-	102.33 ± 1.80 (5)
*β-glycerol phosphate alone **5mM***	-	104.83 ± 1.67 (5)

Table 3.1. Table showing the effects of a range of culture conditions on the viability of non-switch and switch MC3T3-E1 cultures at 10 div. The n values are as indicated in parentheses. Where no value is given (-), the indicated treatment is not applicable. *P<0.05, **P<0.01, ***P<0.001 compared to control.

3.4. Ligands at glutamate and adenosine receptors

3.4.1. Glutamate and NMDA receptor agonists and antagonists

There is increasing evidence for the secretion of glutamate and the presence of glutamate receptors in peripheral tissues including bone (Bhangu et al., 2001; Genever & Skerry, 2001; Gu et al., 2002). Agonists and antagonists at glutamate receptors were therefore tested for their potential effects on the viability of well-differentiated (switch) MC3T3-E1 cultures. All agents were added for 0.25, 1, 3 and 6 h. Fig. 3.6 (A - B) shows the effects, respectively, of the agonist glutamate and the selective agonist NMDA at 10 div. Application of glutamate 10μM for 3 h or less showed a trophic tendency, which was significant at 1 h ($P<0.05$, n = 4 - 5), whereas a 6 h application had no effect. Glutamate 100μM applied for 1 h and 1mM applied for 0.25 h had very significant trophic effects on the cultures ($P<0.01$). No concentration of glutamate at any time point elicited any toxic effect. Similarly, NMDA 100μM applied for 3 h, and 1mM applied for 0.25, 1 and 3 h had significant trophic effects ($P<0.01$ and $P<0.05$, respectively, n = 4 - 5) on osteoblast cultures and none of the treatments caused a significant reduction in viability at any time point investigated.

When applied for 20 h at 4 div, glutamate at 10μM, 100μM, 1mM and 10mM raised viability significantly to 109.75% ± 1.61 ($P<0.05$), 111.63% ± 3.06 ($P<0.01$), 110.95% ± 2.79 ($P<0.01$) and 112.01% ± 1.83 ($P<0.01$), respectively, of the control (n = 6). Consistent with this, NMDA at the same concentration range gave values of 109.01% ± 2.14 ($P<0.05$), 110.06% ± 2.86 ($P<0.01$), 115.52% ± 2.02 ($P<0.01$) and 109.44% ± 0.80 ($P<0.01$) of the control, respectively (n = 4).

Whether the endogenous NMDA receptor agonist, quinolinic acid, could similarly affect osteoblast viability was also tested. A 24 h application of 100μM, 1mM and 10mM quinolinic acid resulted in viability values of 108.18% ± 3.79, 105.91% ± 4.65 and 98.55% ± 3.65 of the control, respectively, but these were not statistically different from the control.

The effects of applications of the non-selective glutamate NMDA receptor antagonist dizocilpine (MK-801) (20μM), and the competitive antagonist D-AP5 (50μM) at 10 div are as shown in Table 3.2. Dizocilpine applied for 0.25 h increased viability significantly beyond control levels ($P<0.05$) but had no significant effect at other time points. D-AP5 showed no significant effect.

3.4.2. Agonists and antagonists at adenosine A_1 and A_{2A} receptors

Application of adenosine for 20 h at 4 div had a significant trophic effect ($P<0.05$) only at 100μM (103.53% ± 1.40, 108.66% ± 2.72 and 103.67% ± 2.51, for 10μM, 100μM and 1mM, respectively; n = 4). The trophic effect by adenosine 100μM was shown by cell count to correspond to a significant increase in cell number ($2.38 \times 10^4 \pm 0.01$ and $1.93 \times 10^4 \pm 0.13$; $P<0.05$ for adenosine 100μM vs. control).

The effects of adenosine receptor ligands on basal viability at 10 div are shown in Table 3.2. Adenosine (10μM - 1mM) had no significant effect on basal viability. While the A_1 receptor agonist CPA (50nM) showed a tendency to improve basal viability, the selective antagonist DPCPX (50nM) significantly raised viability above control values on application for 1 h. However, modulations of viability by DPCPX and another selective A_1 receptor antagonist, 8-PT (20μM), were not clear from these data, appearing to be biphasic, depending on the time point used. The A_{2A} receptor agonist CGS21680 (20nM) showed a trophic tendency on the osteoblast cultures, whereas application of the antagonists ZM241385 and SCH58261 at 50nM caused a reduction in viability at most time points. This reduction was significant when ZM241385 was applied for 0.25 h.

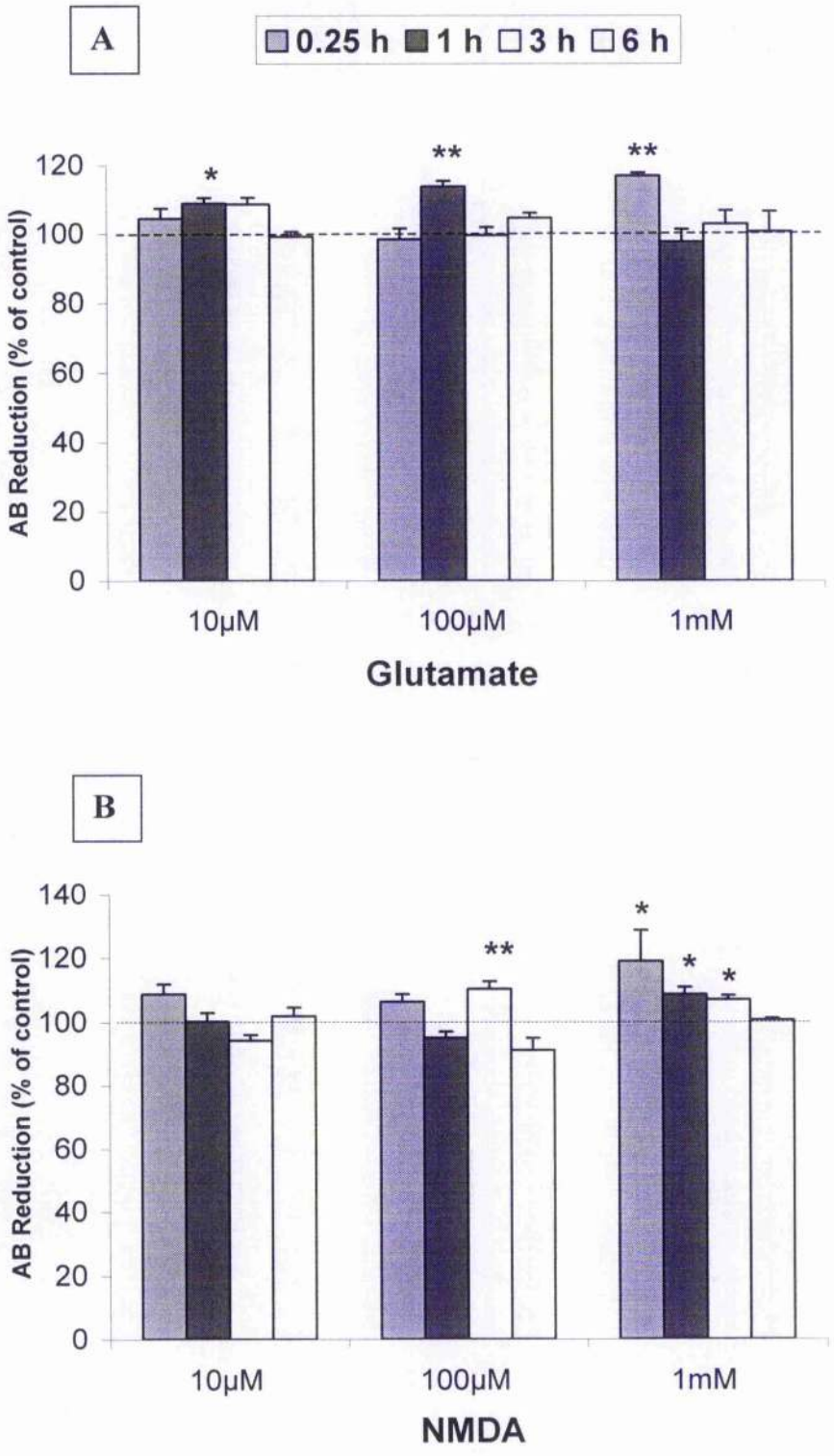


Figure 3.6. Histograms showing the effects of 0.25, 1, 3 and 6 h applications of **(A)** glutamate and **(B)** NMDA on the viability of MC3T3-E1 cultures at 10 div. Each column represents the mean ± SEM for n = 4 or 5 cultures. * $P<0.05$, ** $P<0.01$ compared to control.

LIGAND	ALAMAR BLUE REDUCTION (% OF CONTROL)			
	0.25 h	**1 h**	**3 h**	**6 h**
NMDA receptor				
MK-801 20μM	107.05 ± 2.45*	102.12 ± 2.89	103.27 ± 8.42	95.85 ± 3.68
D-AP5 50μM	105.92 ± 7.08	108.48 ± 6.42	97.91 ± 8.42	98.81 ± 8.02
Adenosine receptors				
Adenosine 10μM	111.18 ± 6.42	94.70 ± 2.66	99.75 ± 8.44	106.34 ± 7.00
100μM	102.55 ± 5.70	98.36 ± 8.06	105.83 ± 8.57	96.53 ± 4.79
1mM	104.30 ± 5.04	107.47 ± 6.85	99.98 ± 8.96	99.00 ± 8.19
Caffeine 1mM	104.35 ± 3.85	102.67 ± 3.97	103.75 ± 11.68	103.92 ± 5.09
A_1 receptors				
CPA 50nM	139.36 ± 19.38	114.58 ± 9.95	99.80 ± 9.87	127.32 ± 14.43
DPCPX 50nM	134.71 ± 20.34	118.78 ± 3.60*	69.71 ± 15.78	100.19 ± 5.4
8-PT 20μM	146.82 ± 16.39	119.81 ± 12.90	93.40 ± 6.66	136.12 ± 13.47
A_{2A} receptors				
CGS21680 20nM	147.23 ± 20.10	127.54 ± 15.75	109.23 ± 8.83	144.00 ± 18.92
ZM241385 50nM	90.21 ± 2.68*	91.91 ± 3.84	94.50 ± 3.58	101.64 ± 5.12
SCH58261 50nM	96.61 ± 2.60	90.81 ± 4.47	91.20 ± 3.00	98.17 ± 2.89

Table 3.2. Table showing the effects of ligands at glutamate NMDA receptors and at adenosine receptors on the viability of MC3T3-E1 cells at 10 div. Each value as presented represents the mean ± SEM for n = 4 or 5 cultures. *P<0.05 compared to control.

3.5. Modulation of osteoblastic viability by reactive oxygen species (ROS)

3.5.1. Hydrogen peroxide and osteoblastic viability

Fig. 3.7 illustrates the concentration - response graph obtained when switch MC3T3-E1 cultures were exposed to hydrogen peroxide (100μM - 5mM) at 10 div for 0.25, 1, 3 and 6 h. Hydrogen peroxide reduced osteoblast viability in a concentration- and time-dependent manner, compared to untreated controls ($P<0.05$ or $P<0.01$, $n = 4$). H_2O_2 100μM had no significant effect. Overall, a concentration of at least 200μM applied for 1 h was required to produce a statistically significant effect. The extent of damage at 6 h was not significantly different from that obtained at 3 h. Effects of hydrogen peroxide on the morphology of osteoblast cultures were consistent with the results from AB reduction (Fig. 3.8), as there was massive loss of cells when cultures were treated with concentrations of hydrogen peroxide beyond 100μM, while the remaining cells appeared isolated and rounded, in contrast to control culture cells which adhered to one another at full confluence. A working concentration of 500μM was chosen for most of the subsequent experiments- except otherwise stated- in order to allow increases or decreases in viability to be clearly shown.

Because H_2O_2 100μM did not significantly damage osteoblast cultures at 10 div, the possible effects of concentrations from 100μM and below on MC3T3-E1 cultures at an earlier stage of development (4 div) were investigated. With 20 h exposure at 4 div, statistically significant increases in viability ($P<0.01$) were obtained for H_2O_2 at 6.25, 12.5, 25 and 50μM (112.33% ± 0.33, 112.08% ± 2.54, 113.71% ± 1.74 and 111.53% ± 2.36, of the control, respectively; $n = 4$) while 100μM resulted in a decrease in viability to 93.06% ± 3.13, which was not significant.

3.5.1.1. Effect of recovery duration

The influence of the length of recovery period after hydrogen peroxide insult on the eventual survival of osteoblast cultures was examined. The recovery period was taken as the period between the end of treatment with hydrogen peroxide and the reading of viability values with the plate reader. This period was, therefore, inclusive of the 4 h gap for which the cultures were incubated with alamar blue before readings were taken. As shown in the left panel of Fig. 3.9, cultures treated for 1 h with H_2O_2 200μM had a significantly better survival with a recovery period of 24 h than with 4 h ($P<0.05$). In contrast, treatment for a longer period of 6 h resulted in reduced survival with 24 h

compared to 4 h of recovery ($P<0.05$, $n = 4$). With a higher concentration of peroxide (500μM), there were no differences in survival outcomes for cultures treated for 1 or 6 h when recovery period was increased from 4 to 24 h ($n = 4$) (Fig. 3.9, right panel).

3.5.1.2. Cellular differentiation and sensitivity to oxidative damage by H_2O_2

At this stage, the influence of differentiation induced by ascorbate and β-glycerol phosphate on the sensitivity of MC3T3-E1 cells to hydrogen peroxide-mediated oxidative damage was assessed. Non-switch cultures were exposed to the same concentrations of hydrogen peroxide (100μM-5mM) at 10 div as were switch cultures, and the viability values obtained by alamar blue reduction compared with previous values for switch cultures as shown in Table 3.3. It appeared that sensitivity to H_2O_2-induced oxidative damage was enhanced by switching-induced differentiation, although the differences in sensitivities of switch and non-switch cultures to the damage failed to reach statistical significance for most concentrations.

3.5.1.3. Catalase prevents the damaging effect of hydrogen peroxide

Catalase (CAT) is an antioxidant enzyme known to destroy hydrogen peroxide by reducing it to water and molecular oxygen according to the equation:

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Catalase was therefore tested for its ability to prevent the reduction in osteoblastic viability caused by hydrogen peroxide at 10 div. Catalase at 1250U/ml had a mitogenic effect on its own which was statistically significant at 6 h and also completely abolished the damaging effects of hydrogen peroxide 500μM, raising viability beyond control level at each time point ($n = 4$) (Fig. 3.10). A series of consecutive half dilutions of catalase, starting from 1250U/ml, was also tested against H_2O_2. Table 3.4 shows the results obtained when each of these dilutions was added alone and when combined with H_2O_2. All of the catalase concentrations showed some evidence of mitogenicity and were able to prevent damage by peroxide.

In order to confirm that the observed effect of catalase on hydrogen peroxide cytotoxicity was entirely due to its catalytic (enzymatic) activity and not attributable to some other protein that could be present in the "cocktail", catalase was inactivated in a water bath at 60°C for 5-7 min and then the ability of this inactivated enzyme to prevent hydrogen peroxide cytotoxicity at the longest time point (6 h) was tested. The heat-inactivated

catalase (250U/ml) at 6 h had no mitogenic effect when applied alone and also completely failed to prevent hydrogen peroxide cytotoxicity (n = 3) (Fig. 3.11).

3.5.1.4. Aspirin (acetylsalicylic acid, ASA) and hydrogen peroxide cytotoxicity

The ability of catalase to induce cyclooxygenase (COX), especially COX-2, and the relevance of this induction to mitogenicity elicited by catalase has been reported in a number of tissues (Chen et al., 1998; Choi et al., 2001; Litvinov &Turpaev, 2004; Jang et al., 2004; Jang et al., 2005). Therefore, the possible effect of aspirin (acetylsalicylic acid), a COX inhibitor, on hydrogen peroxide cytotoxicity was examined (n = 3) (Fig. 3.12A). The results showed that aspirin increased basal viability to 112.96% ± 1.15 of the control when applied alone for 1 h ($P<0.01$) but had no effect at 6 h. However, when tested for its potential to modulate hydrogen peroxide toxicity, it showed no ability to either prevent or exacerbate the deleterious effect, whether at 1 or 6 h.

3.5.1.5. Superoxide dismutase (SOD) and hydrogen peroxide

Hydrogen peroxide is a dismutation product of the superoxide anion radical, the antioxidant enzyme superoxide dismutase (SOD) being responsible for catalyzing this reaction according to the equation:

$$2O_2^{\cdot -} + 2H^{-} \xrightarrow{SOD} H_2O_2 + O_2$$

The effect of SOD on hydrogen peroxide-induced reduction in the viability of osteoblasts was therefore assessed. Hydrogen peroxide at 500μM reduced viability in a time-dependent manner, with significance at 1, 3 and 6 h (n = 4) (Fig. 3.12B). When SOD 250U/ml was added alone, there were increases in cell viability, reaching significance at 1 h, although these data were largely fraught with significant variability between replicates. Co-administration of hydrogen peroxide and SOD resulted in marginal improvements in viability compared to hydrogen peroxide alone. After 3 h of treatment, SOD raised viability significantly from 15.19% ± 2.06 for peroxide alone to 33.80% ± 8.96 ($P<0.05$).

3.5.1.6. Hydrogen peroxide in the presence of copper (II) ion

Through the Fenton reaction, hydrogen peroxide is able to combine with transition metal ions such as iron (Fe^{2+}) or copper (Cu^{2+}) to generate the hydroxyl radical (Mazzio & Soliman, 2003). The possibility of interactions between hydrogen peroxide and copper (II)

ion (generated from $CuSO_4$) in MC3T3-E1 cultures was therefore investigated with an H_2O_2 concentration of 100μM. As shown in Fig. 3.13A, this concentration had no effect on osteoblast viability when applied alone for 1 h. The addition of $CuSO_4$ 1mM alone for 1 h reduced osteoblast viability significantly to 77.85% ± 3.59 of the control ($P<0.01$, $n = 4$). A co-administration of H_2O_2 100μM and $CuSO_4$ 1mM for 1 h reduced viability to 48.72% ± 6.08 of the control ($P<0.001$), a reduction significantly more than was obtained with $CuSO_4$ alone ($P<0.001$). In order to determine whether any production of hydroxyl radical was involved in this phenomenon, the effect of mannitol, a known scavenger of the hydroxyl radical, was assessed on the damage caused by a co-administration of H_2O_2 and $CuSO_4$ (Fig. 3.13B). At 1 h, mannitol 50mM had no effect on its own, but surprisingly, it significantly exacerbated the reduction in viability caused by a combination of H_2O_2 and $CuSO_4$. Viability was significantly decreased 30.58% to 69.42% ± 6.37 of the control ($P<0.001$, $n = 4$) by a combination of H_2O_2 and $CuSO_4$. In the presence of mannitol, a greater (91.28%) reduction in viability was obtained, bringing this value down to 8.72% ± 1.48 of the control ($P<0.001$). This reduction in the presence of mannitol was more significant than the reduction caused when only H_2O_2 and $CuSO_4$ were combined ($P<0.001$). The morphological effects of these treatments also confirmed that a combination of H_2O_2 and copper (II) ion was more damaging in the presence than in the absence of mannitol (Fig. 3.14).

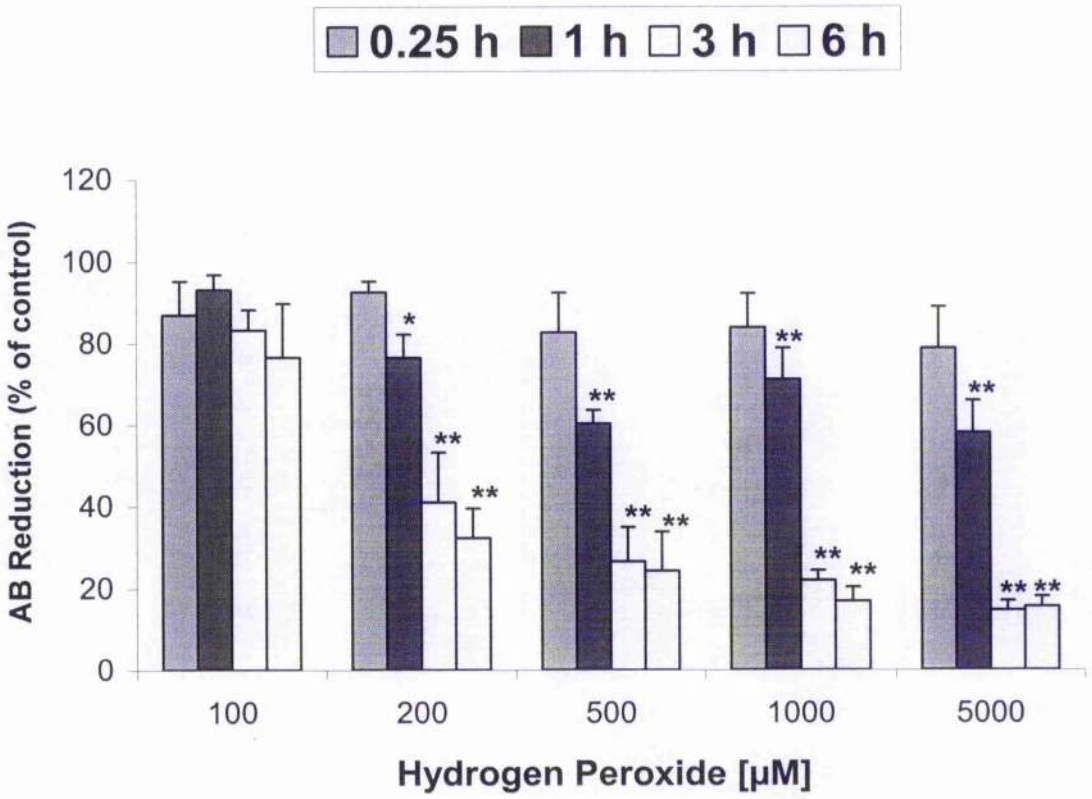


Figure 3.7. Histograms showing the effects of hydrogen peroxide (100µM – 5mM) applied for 0.25, 1, 3 and 6 h on the viability of MC3T3-E1 osteoblast-like cells at 10 div. Each column represents the mean ± S.E.M for n = 4 cultures. *P < 0.05, ** P< 0.01 compared to control.

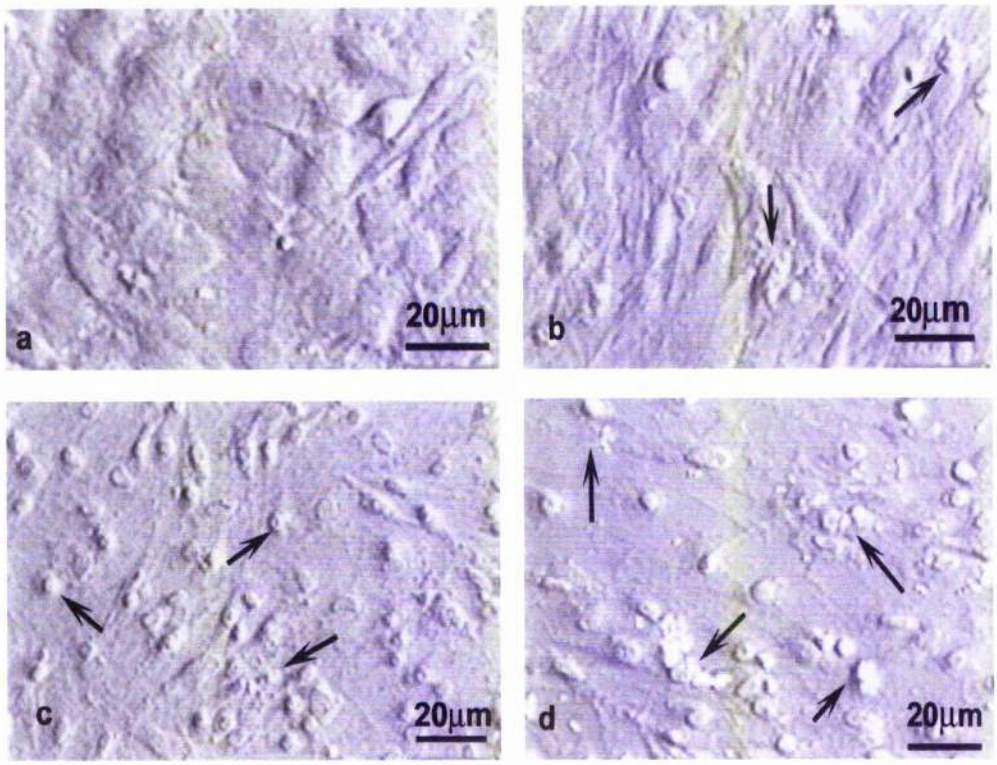


Figure 3.8. Photomicrographs showing the effects of H_2O_2 on the phenotype of MC3T3-E1 cells as shown by phase contrast microscopy. (**a**) Control cells maintained in switch medium for 10 div are confluent and closely adhered when compared to cultures treated for 6 h with (**b**) 200μM, (**c**) 500μM, and (**d**) 1000μM H_2O_2, where cells rounded up (arrows) and a progressive loss of cells was evident. Bar = 20 μm.

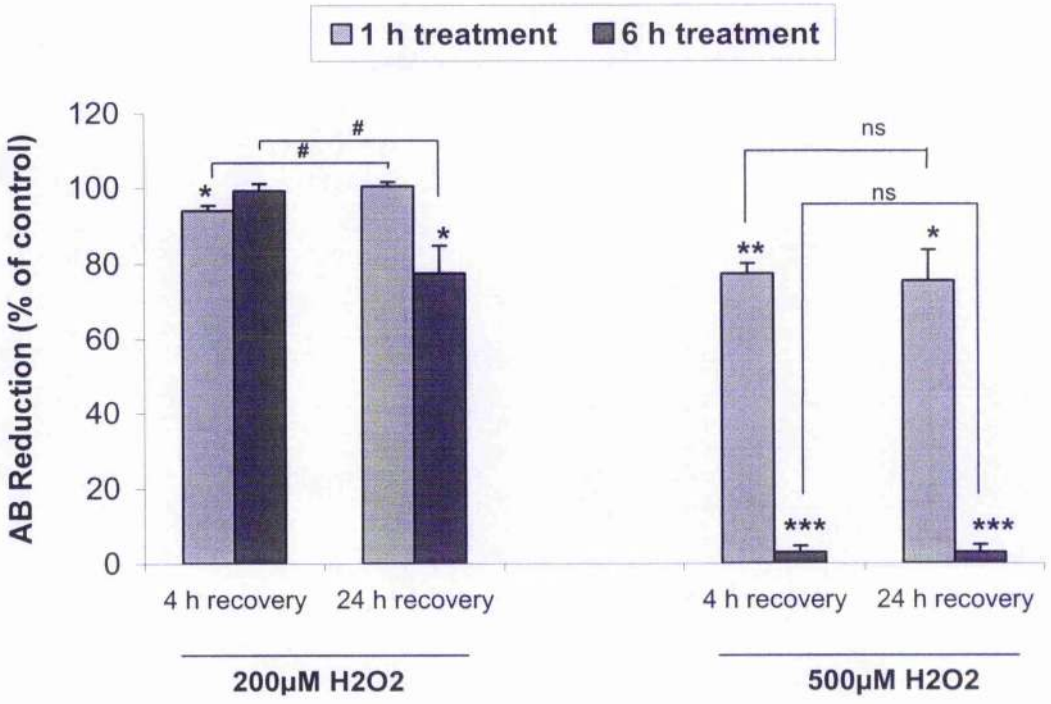


Figure 3.9. Histograms showing the effects of duration of recovery period on the outcome of damage by 200µM (left panel) and 500µM (right panel) hydrogen peroxide. The recovery period is the period between the end of treatment with hydrogen peroxide and the reading of viability values with the plate reader, inclusive of the 4 h gap for which the cultures were incubated with alamar blue before readings were taken. Each column shows the mean ± SEM for n = 4 cultures. *P<0.05, **P<0.01, ***P<0.001 compared to control; #P<0.05, ns = non-significant for 4 h vs. 24 h recovery.

H_2O_2 (μM)	AB REDUCTION (% OF CONTROL)	
	NON-SWITCH	SWITCH
0.25 h		
100	97.09 ± 3.10	87.02 ± 8.25
200	97.50 ± 4.87	92.59 ± 2.60
500	92.50 ± 9.55	82.64 ± 9.75
1000	94.18 ± 4.48	83.85 ± 8.36
5000	80.76 ± 3.06	78.69 ± 10.16
1 h		
100	94.30 ± 2.15	93.18 ± 3.67
200	91.11 ± 4.34	76.44 ± 5.77
500	88.01 ± 3.54	60.30 ± 3.30**
1000	75.96 ± 4.93	71.14 ± 7.68
5000	54.57 ± 4.49	58.08 ± 7.88
3 h		
100	99.84 ± 3.96	83.20 ± 5.08*
200	82.48 ± 7.38	40.97 ± 12.29*
500	41.91 ± 7.37	26.57 ± 8.28
1000	37.36 ± 3.23	21.93 ± 2.40**
5000	24.13 ± 10.68	14.69 ± 2.29
6 h		
100	106.40 ± 3.78	76.52 ± 13.25
200	78.61 ± 12.93	32.23 ± 7.27*
500	38.20 ± 8.17	24.29 ± 9.48
1000	12.05 ± 5.76	16.83 ± 3.49
5000	12.31 ± 2.81	15.54 ± 2.53

Table 3.3. Table showing the differential sensitivities of non-switch and switch cultures to hydrogen peroxide (100μM – 5mM) at 10 div. Each value indicates the mean ± SEM for n = 4 cultures. *P<0.05, **P<0.01 compared to non-switch cultures.

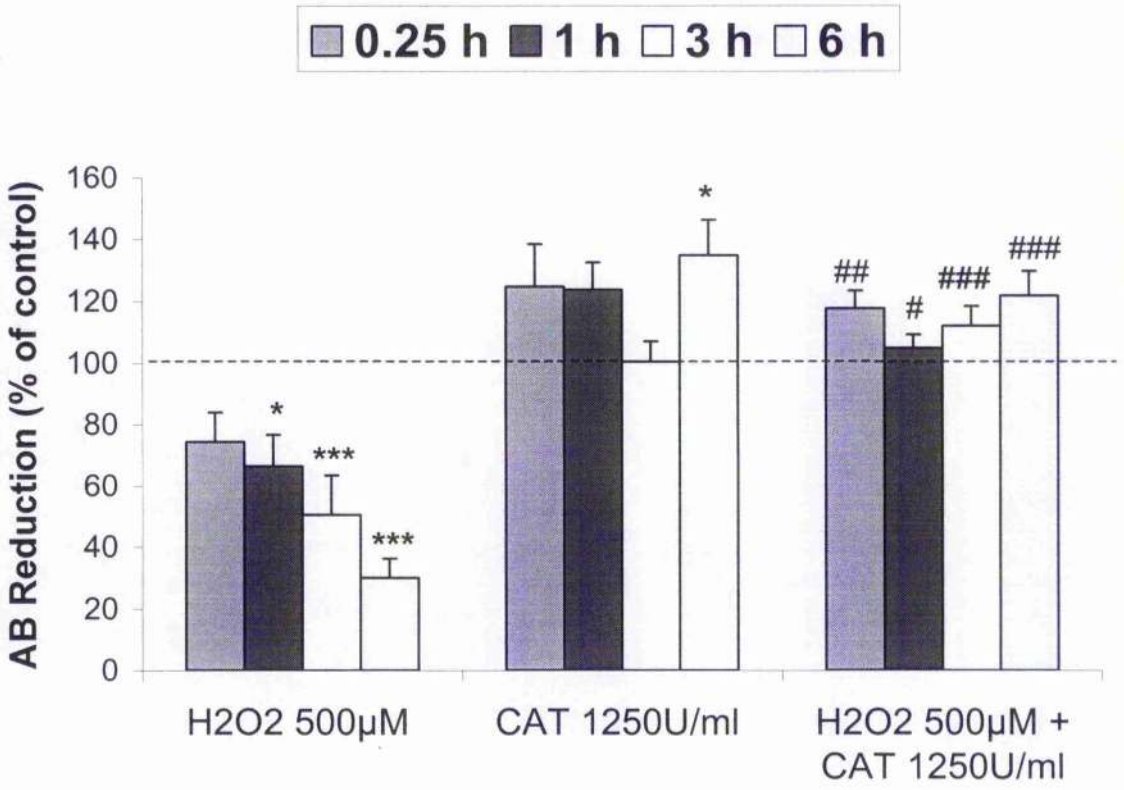


Figure 3.10. Histograms showing the effects of catalase (CAT) 1250U/ml on the viability of MC3T3-E1 cultures and the hydrogen peroxide-mediated cytotoxicity in these cultures. Each column shows the mean ± SEM for n = 4 cultures. *P<0.05, ***P<0.001 compared to control; #P<0.05, ###P<0.001 compared to corresponding H_2O_2 500µM.

	AB Reduction (% of control)			
	0.25 h	**1 h**	**3 h**	**6 h**
***CAT** (U/ml)*				
39	120.13 ± 14.62	114.92 ± 14.29	93.35 ± 11.14	109.82 ± 8.44
78	106.98 ± 11.60	108.50 ± 11.67	109.24 ± 10.12	87.19 ± 10.90
156	125.28 ± 8.48	114.43 ± 13.21	105.43 ± 13.30	113.36 ± 6.41
313	84.35 ± 22.77	127.89 ± 13.90	107.09 ± 10.16	118.69 ± 3.97
625	115.45 ± 13.31	112.88 ± 6.61	130.77 ± 8.93	114.42 ± 5.76
H_2O_2				
500µM	74.43 ± 9.53	66.43 ± 10.30*	50.43 ± 13.00***	30.01 ± 6.19***
H_2O_2 500µM + ***CAT*** (U/ml)				
39	106.57 ± 2.66[a]	104.14 ± 4.20[a]	93.72 ± 10.87[c]	111.39 ± 10.31[c]
78	102.57 ± 7.24	107.65 ± 6.81[a]	102.73 ± 2.31[c]	101.97 ± 2.19[c]
156	124.43 ± 14.49[b]	110.30 ± 6.60[a]	108.10 ± 3.06[c]	110.65 ± 6.48[c]
313	99.97 ± 6.19	121.47 ± 16.98[b]	107.25 ± 3.20[c]	118.30 ± 10.47[c]
625	110.10 ± 4.87[a]	115.16 ± 10.74[a]	115.82 ± 7.00[c]	110.90 ± 4.92[c]

Table 3.4. Table showing the effects of increasing catalase concentrations (39U/ml – 625U/ml) on the viability of MC3T3-E1 cultures and the cytotoxicity mediated by hydrogen peroxide in these cultures at 10 div. Each value represents the mean ± SEM for n = 4 cultures. *P<0.05, ***P<0.001 compared to control; [a]P<0.05, [b]P<0.01, [c]P<0.001 compared to corresponding H_2O_2 500µM.

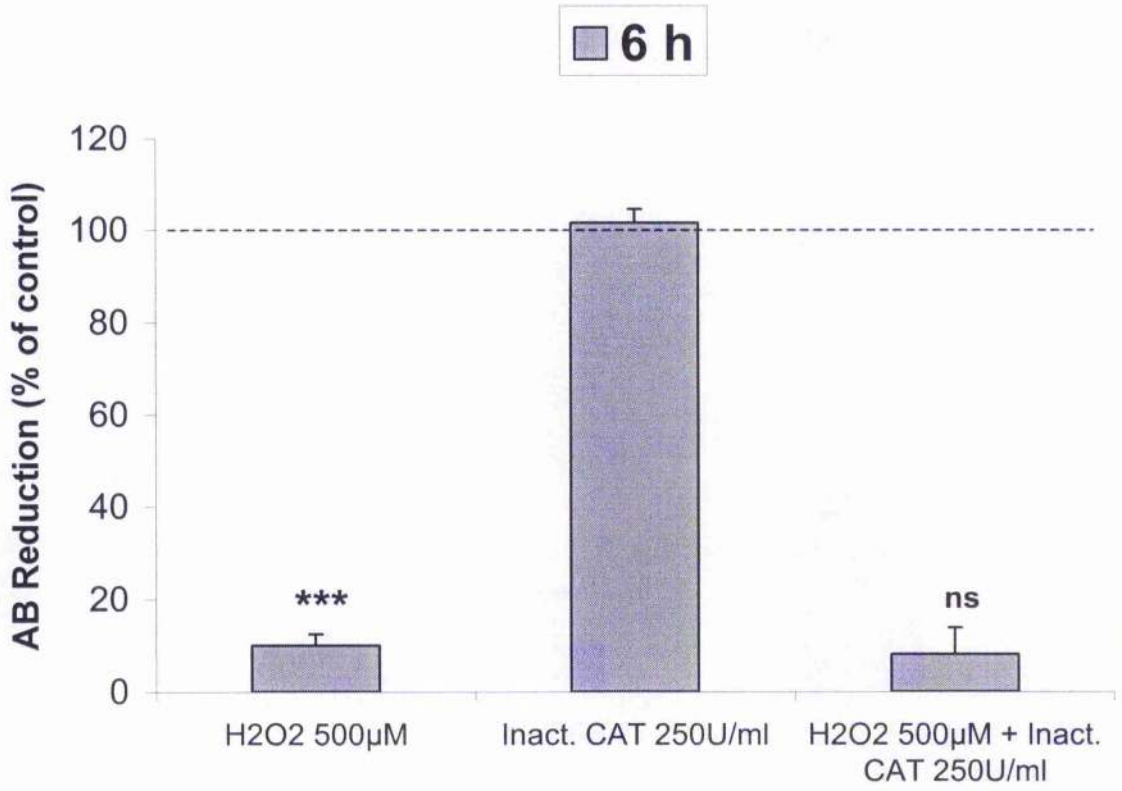


Figure 3.11. Histogram showing lack of effect of heat-inactivated catalase (250U/ml) on the viability of MC3T3-E1 cultures and the cytotoxicity mediated by H_2O_2 (500µM) in these cultures. The inactivation of 250U/ml catalase by heat treatment caused a complete loss of its mitogenic activity and its ability to prevent H_2O_2-mediated cytotoxicity after 6 h of exposure. Each column represents the mean ± SEM for n = 3 cultures. *** $P<0.001$ compared to control; ns = non-significant compared to H_2O_2 - treated cultures.

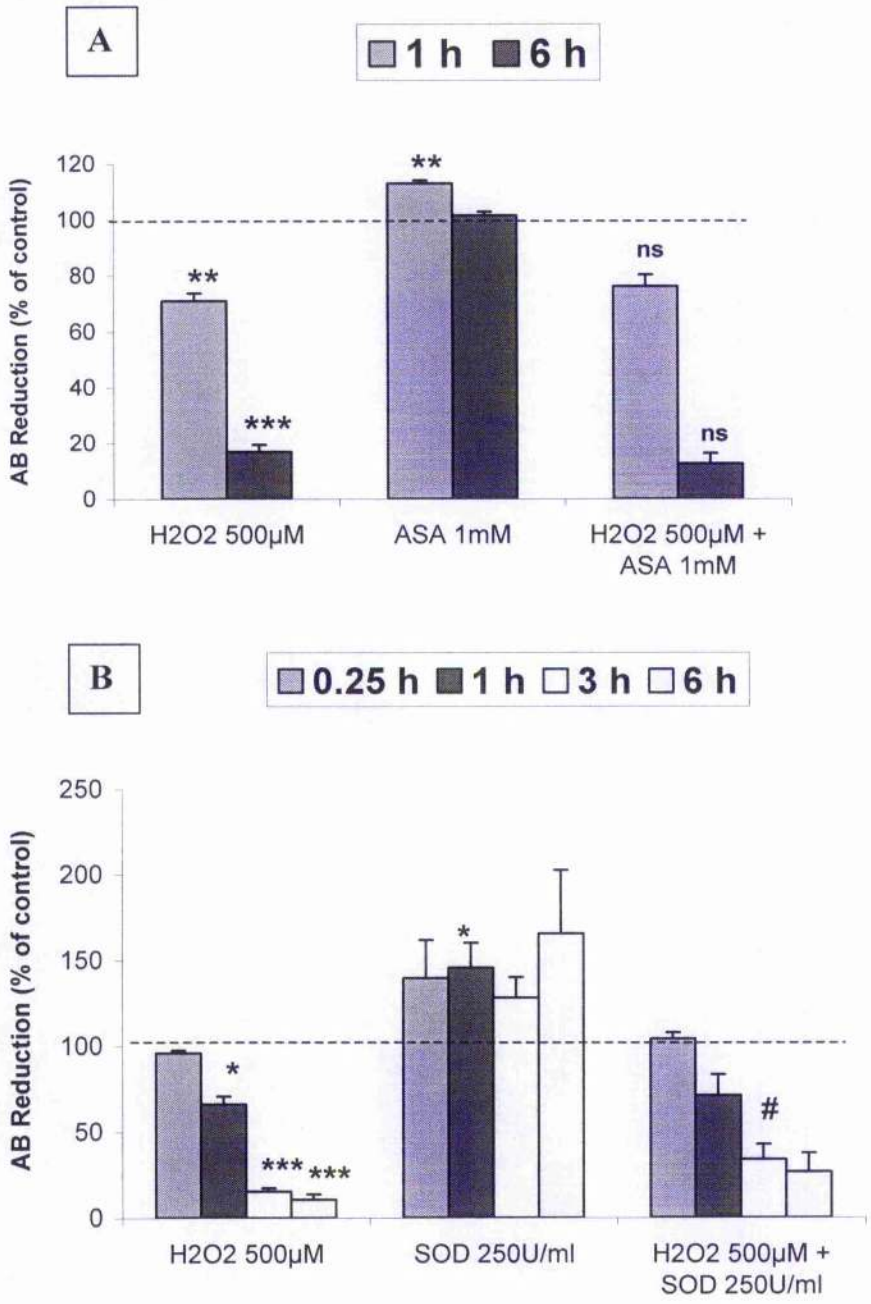


Figure 3.12. Histograms showing the effects of **(A)** aspirin (ASA, 1mM) and **(B)** the antioxidant enzyme superoxide dismutase (SOD) 250U/ml on the viability of MC3T3-E1 cultures when each was applied alone and in combination with hydrogen peroxide (500µM) for up to 6 h. Each column represents the mean ± SEM for **(A)** n = 3 or **(B)** n = 4 cultures. *P<0.05, **P<0.01, *** P<0.001 compared to control; #P<0.05, ns = non-significant compared to corresponding H_2O_2 500µM.

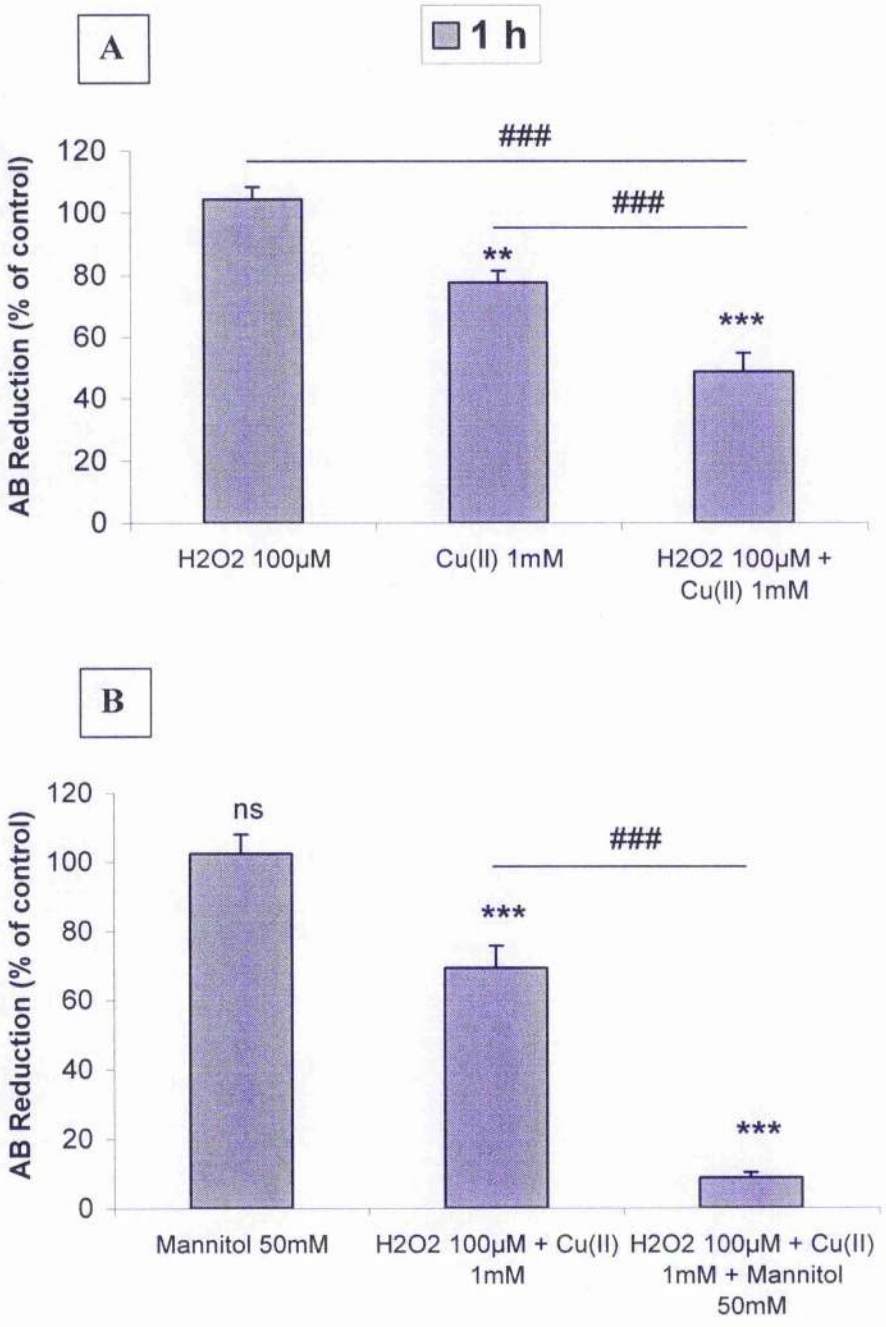


Figure 3.13. Histograms showing **(A)** the effects of $CuSO_4$ 1mM alone and in combination with hydrogen peroxide 100µM for 1 h on the viability of MC3T3-E1 cultures and **(B)** the effect of the hydroxyl radical scavenger mannitol 50mM on the reduction in viability of MC3T3-E1 cells induced by a combination of hydrogen peroxide (100µM) and copper (II) ion (1mM) for 1 h. Each column represents the mean ± SEM for n = 4 cultures. **P<0.01, ***P<0.001, ns = non-significant compared to control; ###P<0.001.

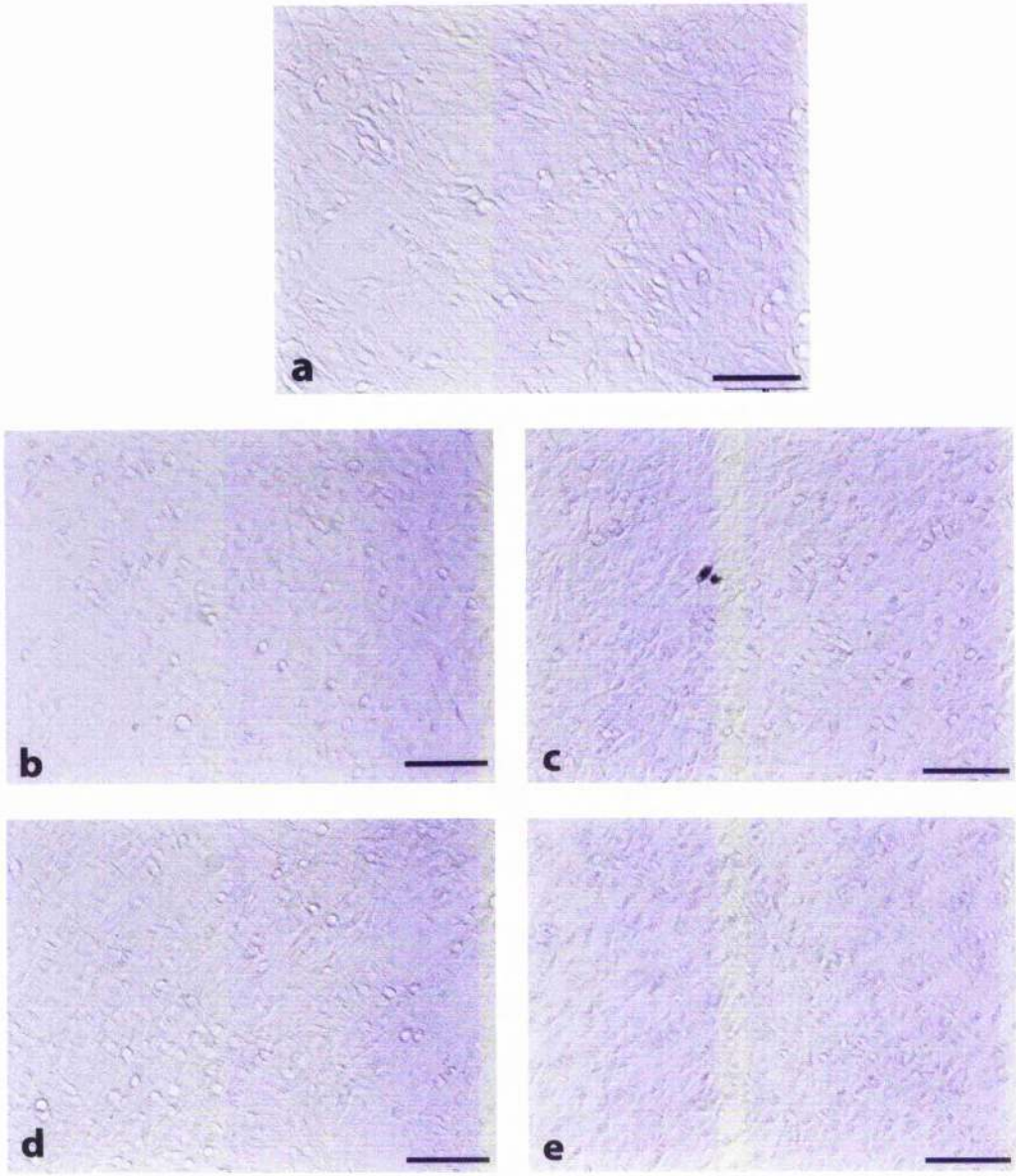


Figure 3.14. Photomicrographs showing the morphological effect of 1 h combination of H_2O_2 100μM and Cu^{2+} 1mM on the viability of MC3T3-E1 cells and the exacerbation of this effect by the hydroxyl radical scavenger, mannitol 50mM. **(a)** Control **(b)** H_2O_2 100μM **(c)** Cu^{2+}1mM **(d)** H_2O_2 100μM + Cu^{2+} 1mM, and **(e)** H_2O_2 100μM + Cu^{2+} 1mM + Mannitol 50mM. Cultures treated with hydrogen peroxide and copper (II) ion showed damaged phenotype **(d)**, which was worse in the presence of mannitol **(e)**. Bar = 50μm.

3.5.2. Mechanisms of H_2O_2-induced osteoblastic cell damage and death

With a view to having a clearer understanding of the nature of cell death induced by hydrogen peroxide in MC3T3-E1 cells, the relative contributions of the processes of apoptosis and necrosis to the eventual demise of the cells after treatment with the oxidant were investigated.

3.5.2.1. Amelioration of cell death by caspase-3 inhibition

Caspases are known to be the predominant mediators of apoptotic cell death (Cohen, 1997). Caspase-3 is believed to be the major and most widely studied executive caspase (Du et al., 1997). The role of caspase-3 in cell death mediated by hydrogen peroxide in MC3T3-E1 cells was therefore examined using the compound Z-DEVD-fmk, a potent caspase-3 inhibitor, at a concentration that had no effect on basal viability when applied alone. Hydrogen peroxide at a concentration of 200μM reduced viability significantly at 6 h ($P<0.001$, $n = 3$) (Fig. 3.15A). Z-DEVD-fmk (40μM) was unable to protect significantly against this insult, only raising viability 6% from 77.60% ± 3.85 for H_2O_2 alone to 82.55% ± 0.66. At a higher concentration of 500μM, hydrogen peroxide reduced viabilities very significantly to 63.98% ± 2.85 and 8.11% ± 2.38 of the control, for 1 and 6 h treatments, respectively ($P<0.001$, $n = 3$) (Fig. 3.15B). In the presence of Z-DEVD-fmk, the damage by H_2O_2 500μM was unaltered at 1 h, but was very significantly protected against at 6 h ($P< 0.001$).

3.5.2.2. Membrane permeability transition (MPT) in H_2O_2-induced cell death

A critical requirement for caspase-mediated apoptosis is the release of cytochrome c from the mitochondria through the process of membrane permeability transition, leading to the opening of the inner membrane permeability transition pore. The involvement of this phenomenon in H_2O_2-induced osteoblastic cell death was therefore assessed by the use of cyclosporin A, an agent that blocks the opening of the transition pore. Cyclosporin A (CsA) at 0.5, 1 and 10μM had no effect on its own when applied for 1 or 6 h ($n = 4$) (Fig. 3.16A). At 1 h, hydrogen peroxide (500μM) reduced osteoblast viability very significantly to 61.18% ± 3.32 ($P<0.001$, $n = 4$) (Fig. 3.16B). CsA had no effect on this reduction. In contrast, however, while 6 h application of H_2O_2 500μM reduced viability very significantly to 18.82% ± 2.34 ($P<0.001$, $n = 3$), CsA significantly ameliorated this reduction in a concentration-dependent manner.

3.5.2.3. *Poly (ADP-ribose) polymerase (PARP) inhibition in H_2O_2-mediated cell death*

Evidence now shows that overactivation of the nuclear protein PARP, especially PARP-1, is inextricably involved in the induction of necrotic cell death (Ha & Snyder, 1999; Moroni et al., 2001). In this study, the level of necrotic death involved in hydrogen peroxide-mediated osteoblastic cell death was examined using PARP inhibitors. Nicotinamide (NAm) is known to have some potential to inhibit PARP while DPQ is a very potent inhibitor. When tested at 6 h, nicotinamide at a concentration of 30μM had no effect on its own and also failed to modify the deleterious effect of H_2O_2 which reduced viability significantly to 21.60% ± 7.01 of the control ($P<0.001$, $n = 4$) (Fig. 3.17A). At a higher concentration of 1mM, nicotinamide still had no effect when applied alone, but significantly protected against H_2O_2 damage, raising viability from 32.42% ± 2.39 for peroxide alone to 66.46% ± 3.14 ($P<0.001$, $n = 3$) (Fig. 3.17B). DPQ (10μM) had no effect on the cultures when applied alone. It also failed to elicit any protection when tested against the cytotoxicity of H_2O_2 200μM that reduced viabilities significantly to 85.08% ± 4.21 at 1 h ($P<0.01$) and to 81.98% ± 3.43 at 6 h ($P<0.001$, $n = 5$) (Fig. 3.18A). When tested against H_2O_2 500μM ($n = 3$) (Fig. 3.18B), DPQ (10μM) did not improve viability significantly at 1 h (a 6.9% rise from 66.27% ± 4.77 to 73.17% ± 3.11), but at 6 h, there was a very significant (33.22%) rise in viability from 7.98% ± 2.39 to 41.20% ± 3.65 ($P<0.001$).

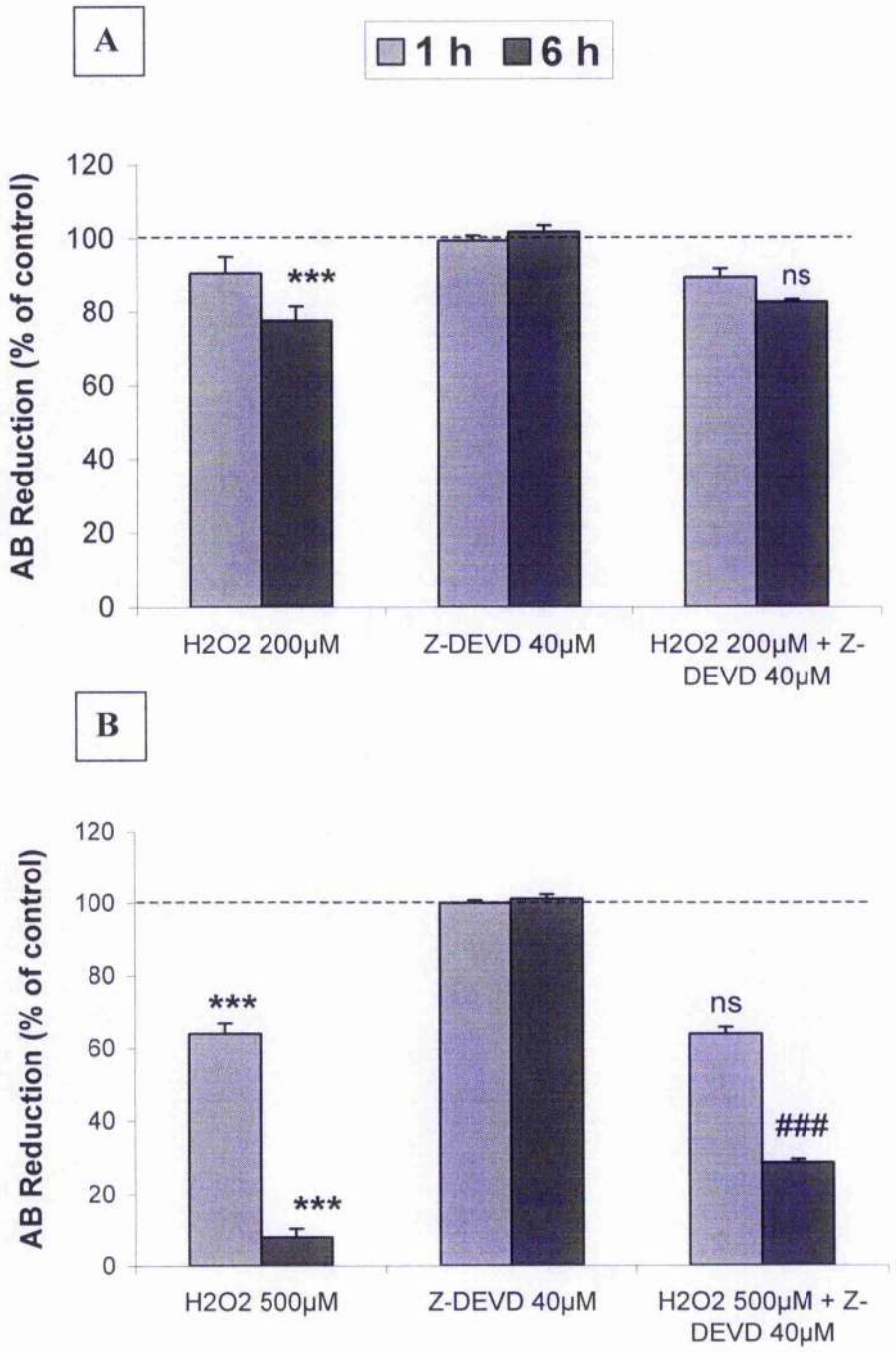


Figure 3.15. Histograms showing **(A)** the lack of effect of a caspase-3 inhibitor, Z-DEVD-fmk 40µM, on the cytotoxicity mediated by H_2O_2 200µM in MC3T3-E1 cells and **(B)** the protective effect of the inhibitor on the cytotoxicity mediated by H_2O_2 500µM in MC3T3-E1 cells. Each column represents the mean ± SEM for n = 3 cultures. ***P<0.001 compared to control; ns = non-significant, ### P<0.001 for the protective effect of the inhibitor vs. cultures treated with **(A)** 200µM H_2O_2 or **(B)** 500µM H_2O_2 in the absence of the inhibitor.

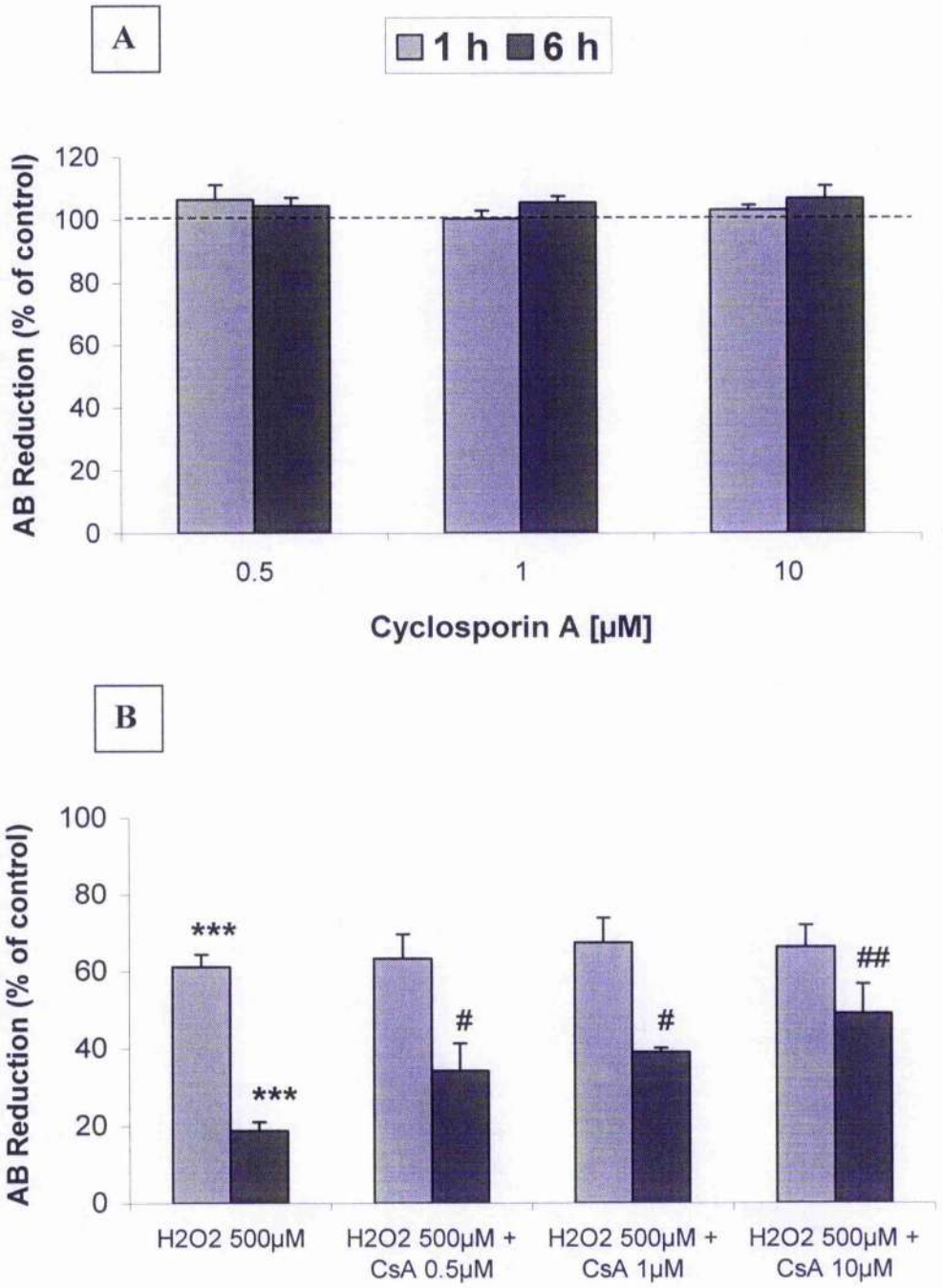


Figure 3.16. Histograms showing **(A)** the lack of effect of the inner membrane permeability pore blocker, cyclosporin A (CsA), on the viability of MC3T3-E1 cultures and **(B)** the effects of the blocker on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cultures, for 1 or 6 h of exposure. Each column shows the mean ± SEM for **(A)** n = 4 cultures and **(B)** n = 4 cultures (1 h treatments) or n = 3 cultures (6 h treatments). ***P<0.001 compared to control; #P<0.05, ##P<0.01 compared to corresponding H_2O_2 500µM treatment.

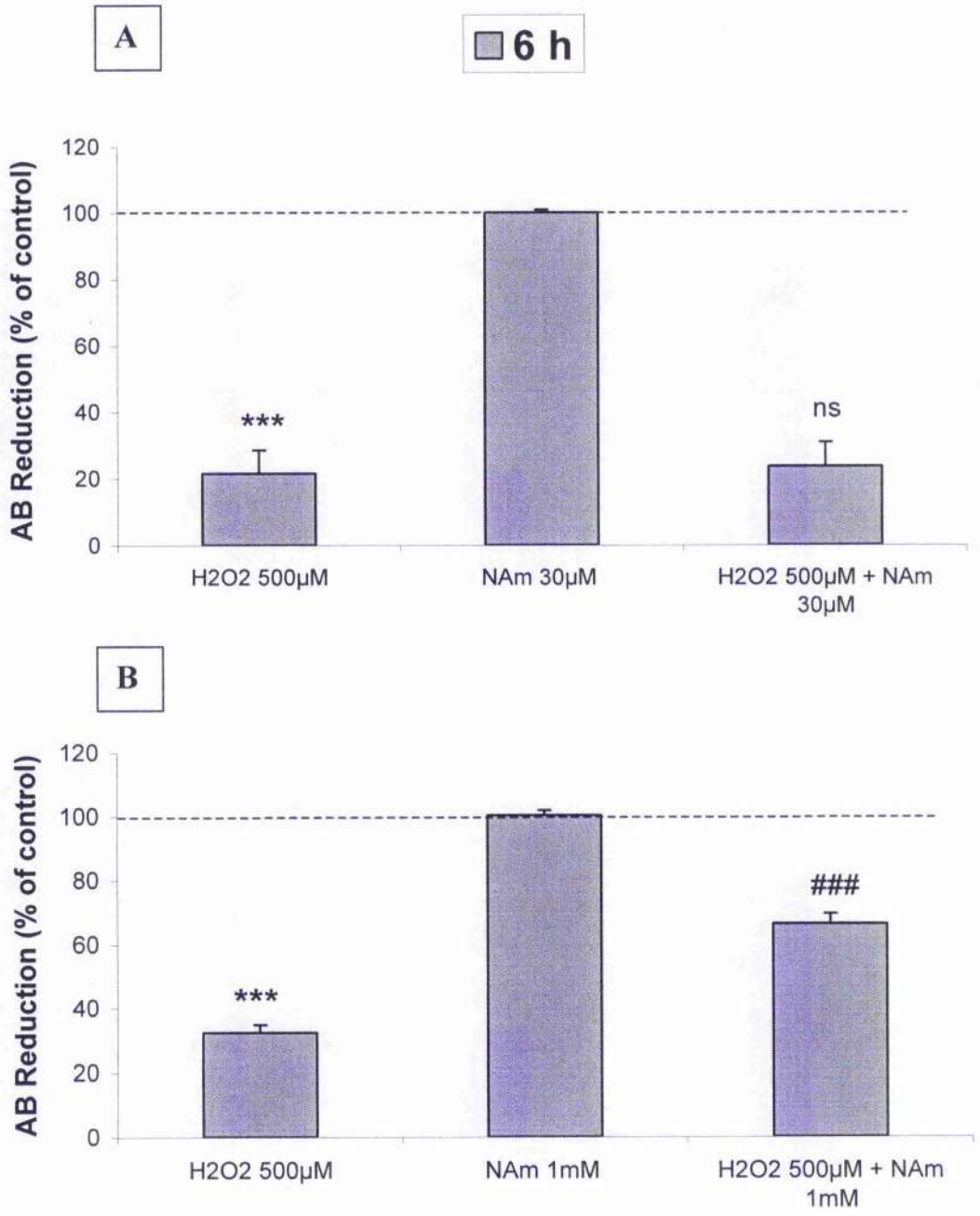


Figure 3.17. Histograms showing **(A)** the lack of effect of a low concentration of the poly (ADP-ribose) polymerase (PARP) inhibitor, nicotinamide (NAm 30µM) and **(B)** the effect of a high concentration of the PARP inhibitor (NAm 1mM) on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cultures. Each column shows the mean ± SEM for **(A)** n = 4 or **(B)** n = 3 cultures. ***P<0.001 compared to control; ###P<0.001, ns = non-significant compared to H_2O_2 500µM.

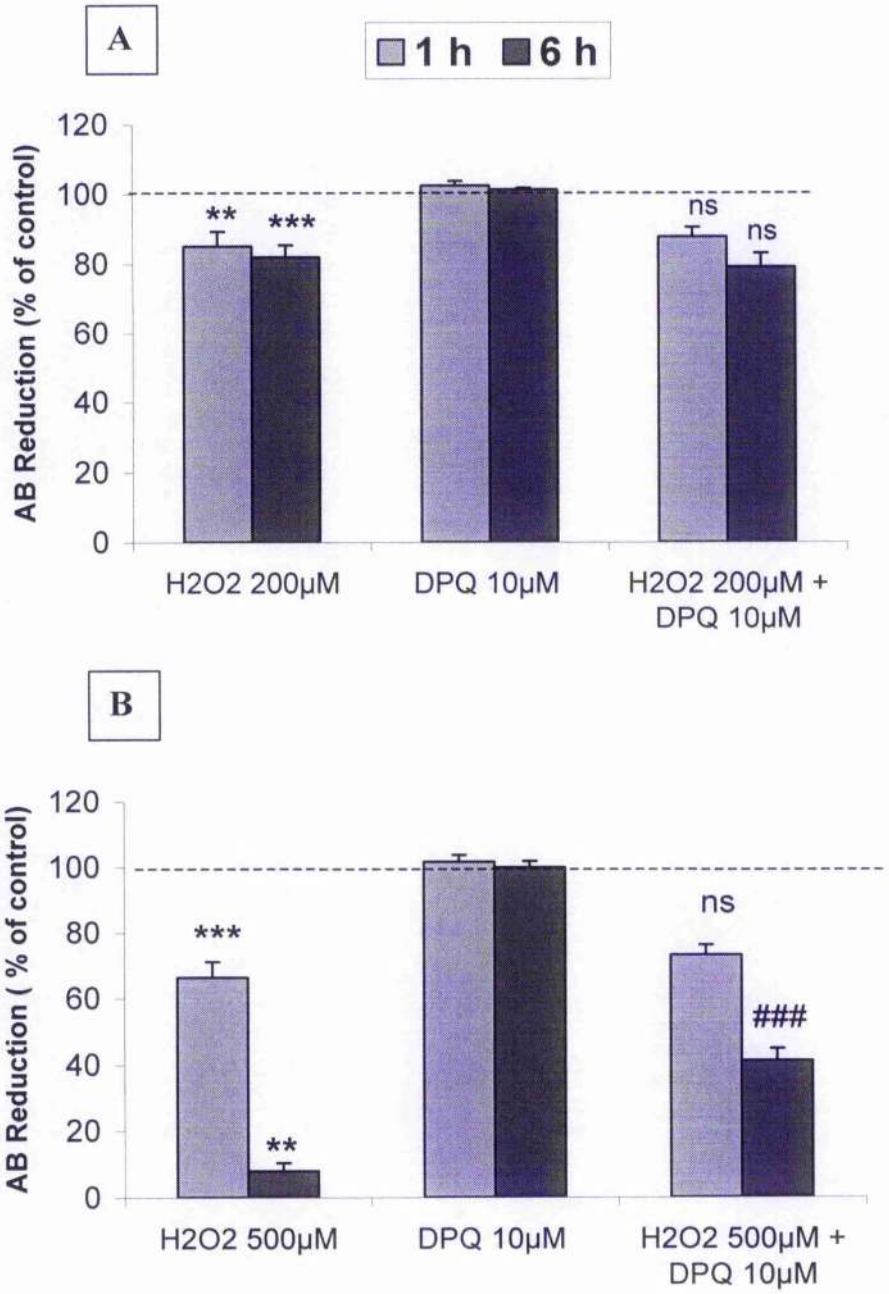


Figure 3.18. Histograms showing **(A)** the lack of effect of the potent poly (ADP-ribose) polymerase (PARP) inhibitor, DPQ (10µM), on the cytotoxicity mediated by H_2O_2 200µM and **(B)** the effect of the PARP inhibitor on the cytotoxicity mediated by H_2O_2 500µM in MC3T3-E1 cultures. Each column shows the mean ± SEM for **(A)** n = 5 or **(B)** n = 3 cultures. **P<0.01, ***P<0.001 compared to control; ns = non-significant compared to H_2O_2 alone; ###P<0.001 compared to corresponding H_2O_2 500µM.

3.5.3. *Roles of ligands at glutamate and adenosine receptors in hydrogen peroxide-mediated cytotoxicity*

3.5.3.1. Glutamate and NMDA receptor ligands and H_2O_2 cytotoxicity

In view of the trophic effects of a number of glutamate receptor ligands tested earlier, their possible roles were investigated in modulating damage caused by H_2O_2 on switched osteoblasts at 10 div, a stage at which they are believed to express functional glutamate receptors (Quarles et al., 1992). Hydrogen peroxide at 500μM reduced osteoblast viability in a time-dependent manner compared to untreated controls, with values down to 95.26% ± 2.29, 84.13% ± 1.94, 72.20% ± 7.01, and 57.84% ± 13.04, for 0.25, 1, 3 and 6 h of treatment, respectively (n = 4 - 5) (Table 3.5). The observed reduction in viability was significant at 1, 3 and 6 h. As shown in the table, both agonists and antagonists failed to protect against the damaging effect of H_2O_2. In fact, rather than elicit any protective effect, they all showed a substantial tendency to potentiate the effects of peroxide. At 0.25 h, hydrogen peroxide had no significant effect (95.26% ± 2.29 of the control), but in the presence of either the non-competitive NMDA receptor antagonist MK-801 (20μM) or the selective antagonist D-AP5 (50μM), H_2O_2 was able to reduce significantly viability to 86.81% ± 4.54 ($P<0.05$) and 77.36% ± 2.29 ($P<0.001$) of the control, respectively.

3.5.3.2. Interactions between adenosine receptor ligands and hydrogen peroxide

Adenosine receptor ligands were also tested for their potential ability to modify damage to osteoblasts caused by the reactive oxygen species hydrogen peroxide. The results are as shown in Table 3.6 (n = 4 - 5). In each case, hydrogen peroxide (500μM) reduced viability in a time-dependent manner. Adenosine had no significant effect on the damage caused by hydrogen peroxide. The selective A_1 receptor agonist CPA applied at 50nM for 0.25 h increased viability significantly from 95.97% ± 1.58 for H_2O_2 alone to 105.82% ± 3.45 when combined with H_2O_2. However, it did not significantly improve H_2O_2 damage caused by longer treatments. The selective A_{2A} agonist CGS21680 (20nM) did not improve damage at any time point. Caffeine, an adenosine receptor antagonist, at 1mM ameliorated the H_2O_2 damage at 3 h, improving viability significantly to 62.19% ± 6.66 from 44.62% ± 4.02 for H_2O_2 alone ($P<0.05$). There were no significant effects on H_2O_2 damage when the selective A_1 antagonists, DPCPX (50nM) and 8-PT (20μM), and the selective A_{2A} antagonists, ZM241385 (50nM) and SCH58261 (50nM), were used.

Experiments were also performed to examine if a more stable adenosine analogue, 2-chloroadenosine, and higher concentrations (100nM for each) of the A_1 and A_{2A} receptor ligands could modify damage caused by hydrogen peroxide. In this regard, the selective agonists and antagonists were tested for 1 and 3 h. Interestingly, 2-chloroadenosine, even when applied at a concentration of 1μM, protected against H_2O_2 damage, with viability significantly improved from 72.87% ± 2.83 to 92.27% ± 4.57 ($P<0.01$) after 3 h and enhanced from 50.00% ± 4.68 to 71.35% ± 5.72 ($P<0.01$) after 6 h of treatment (n = 3) (Fig. 3.19). CPA 100nM when applied alone for 1 h caused a significant increase in viability, which was 108.58% ± 0.75 of the control ($P<0.001$), and also afforded protection against oxidant injury when present for 3 h ($P<0.05$, n = 4) (Fig. 3.20). DPCPX applied for 1 h at 100nM increased the viability of MC3T3-E1 cells significantly to 107.85% ± 1.25 ($P<0.001$), but failed to modify H_2O_2 damage (Fig. 3.20). CGS21680 increased baseline viability significantly when applied alone for 1 h ($P<0.05$), and also improved viability in H_2O_2-treated cultures ($P<0.01$ for 1 h, n = 4; and $P<0.001$ for 3 h, n = 3) (Fig. 3.21). ZM241385 100nM increased viability to 106.77% ± 0.77 of the control level ($P<0.01$) after 1 h application, but did not modify the effect of H_2O_2 either at 1 or 3 h (Fig. 3.21). The effects of these treatments on the morphology of the MC3T3-E1 cells are as shown in Fig. 3.22, where arrows point to damaged cells and the agonists only partially improved damage by peroxide.

LIGAND (μM)		ALAMAR BLUE REDUCTION (% OF CONTROL)			
		0.25 h	**1 h**	**3 h**	**6 h**
H_2O_2 500μM		***95.26 ± 2.29***	***84.13 ± 1.94****	***72.20 ± 7.01****	***57.84 ± 13.04****
NMDA	**10**	82.37 ± 8.19	66.61 ± 9.59*	47.82 ± 15.00*	45.34 ± 14.56*
	100	82.27 ± 4.83	70.94 ± 8.30*	49.82 ± 16.35*	46.67 ± 14.52*
Glu	**100**	82.46 ± 8.28	72.88 ± 5.60**	52.69 ± 15.53*	47.73 ± 14.63*
	1000	88.72 ± 0.10	70.98 ± 7.00**	52.89 ± 15.81*	47.70 ± 14.23*
MK-801	**20**	86.81 ± 4.54*	71.13 ± 9.60*	56.18 ± 14.43*	43.02 ± 13.53**
D-AP5	**50**	77.36 ± 2.29[a] ***	74.03 ± 7.14**	46.27 ± 14.13**	46.84 ± 12.36**

Table 3.5. Table showing the effects of some NMDA receptor ligands on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cultures. Each value as shown, representing the mean ± SEM for n = 4 or 5 cultures, is for the indicated ligand in combination with H_2O_2 500μM. *P<0.05, **P<0.01, *** P<0.001 compared to control; [a]P<0.001 compared to H_2O_2 500μM.

LIGAND (μM, except stated)		AB REDUCTION (% OF CONTROL)			
		0.25 h	1 h	3 h	6 h
H_2O_2	*500μM*	*104.27 ± 7.34*	*88.26 ± 7.14*	*44.62 ± 4.02****	*30.88 ± 8.67***
Adenosine	**10**	101.49 ± 7.00	83.28 ± 5.08	55.84 ±5.59	23.61 ± 4.78
	100	107.92 ± 8.71	91.00 ± 8.49	54.14 ± 6.26	19.71 ± 4.67
	1000	101.41 ± 8.33	88.47 ± 5.52	45.61 ± 10.41	27.03 ± 7.56
Caffeine	**1000**	112.66 ± 6.87	87.32 ± 3.39	62.19 ± 6.66[a]	29.27 ± 6.03
H_2O_2	*500μM*	95.97 ± 1.58	66.30 ± 4.70**	15.19 ± 2.06***	10.65 ± 2.96***
CPA	**50nM**	105.82 ± 3.45[a]	71.37 ± 5.48	21.98 ± 5.75	12.24 ± 1.62
CGS21680	**20nM**	102.34 ± 3.36	75.30 ± 4.35	21.02 ± 7.26	15.50 ± 2.65
DPCPX	**50nM**	97.58 ± 3.10	74.75 ± 5.55	21.68 ± 4.07	15.93 ± 5.70
8-PT	**20**	101.76 ± 3.88	74.53 ± 2.98	22.33 ± 8.51	15.69 ± 0.84
H_2O_2	*500μM*	81.34 ± 8.05	67.34 ± 7.27*	56.36 ± 16.39	46.64 ± 20.08
ZM241385	**50nM**	80.69 ± 8.24	73.50 ± 9.57	54.30 ± 17.60	46.24 ± 19.28
SCH58261	**50nM**	83.18 ± 6.25	68.06 ± 9.41	57.89 ± 16.95	43.31 ± 18.64

Table 3.6. Table showing the effects of some adenosine A_1 and A_{2A} receptor ligands on hydrogen peroxide-mediated reduction in viability of the MC3T3-E1 cultures. Each value as shown, representing the mean ± SEM for n = 4 or 5 cultures, is for the indicated adenosine receptor ligand in combination with H_2O_2 500μM. *P<0.05, **P<0.01, *** P<0.001 compared to control, [a]P<0.05 compared to H_2O_2 500μM.

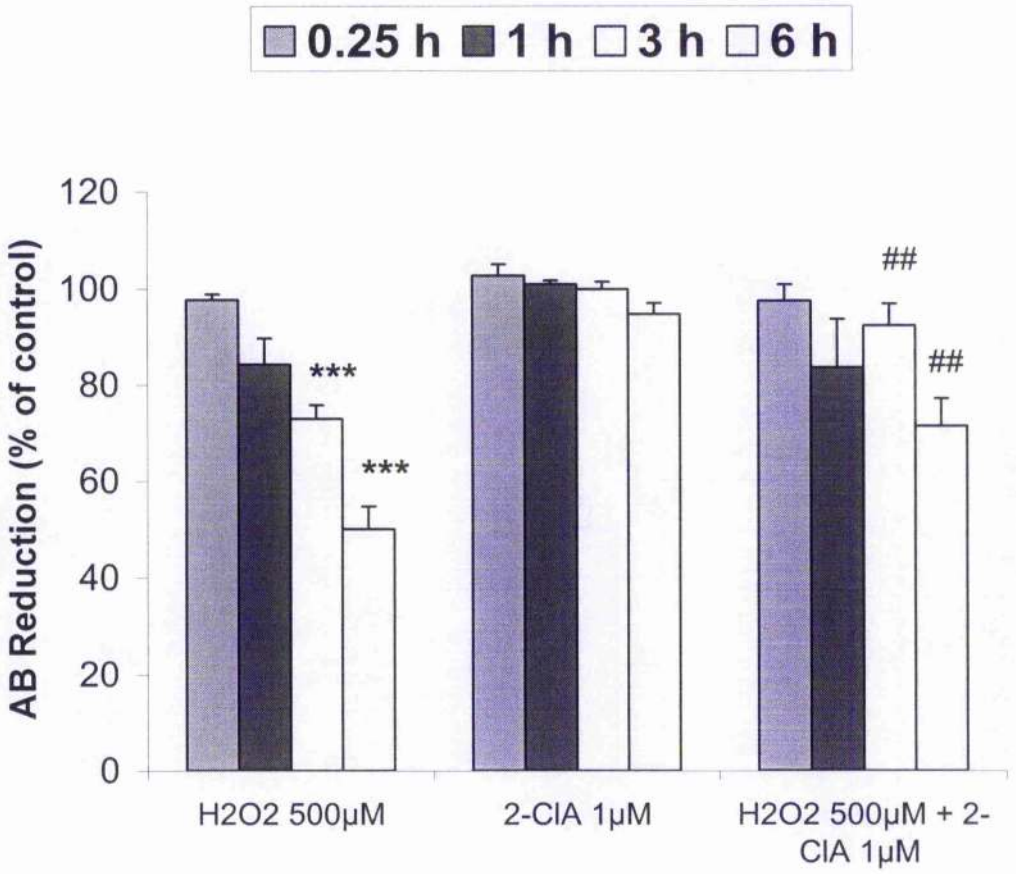


Figure 3.19. Histograms showing the effects of a stable adenosine analogue, 2-chloroadenosine (2-ClA 1µM), on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cells. Each column represents the mean ± SEM for n = 3 cultures. ***P<0.001 compared to untreated controls; ## P<0.01 compared to H_2O_2 500µM.

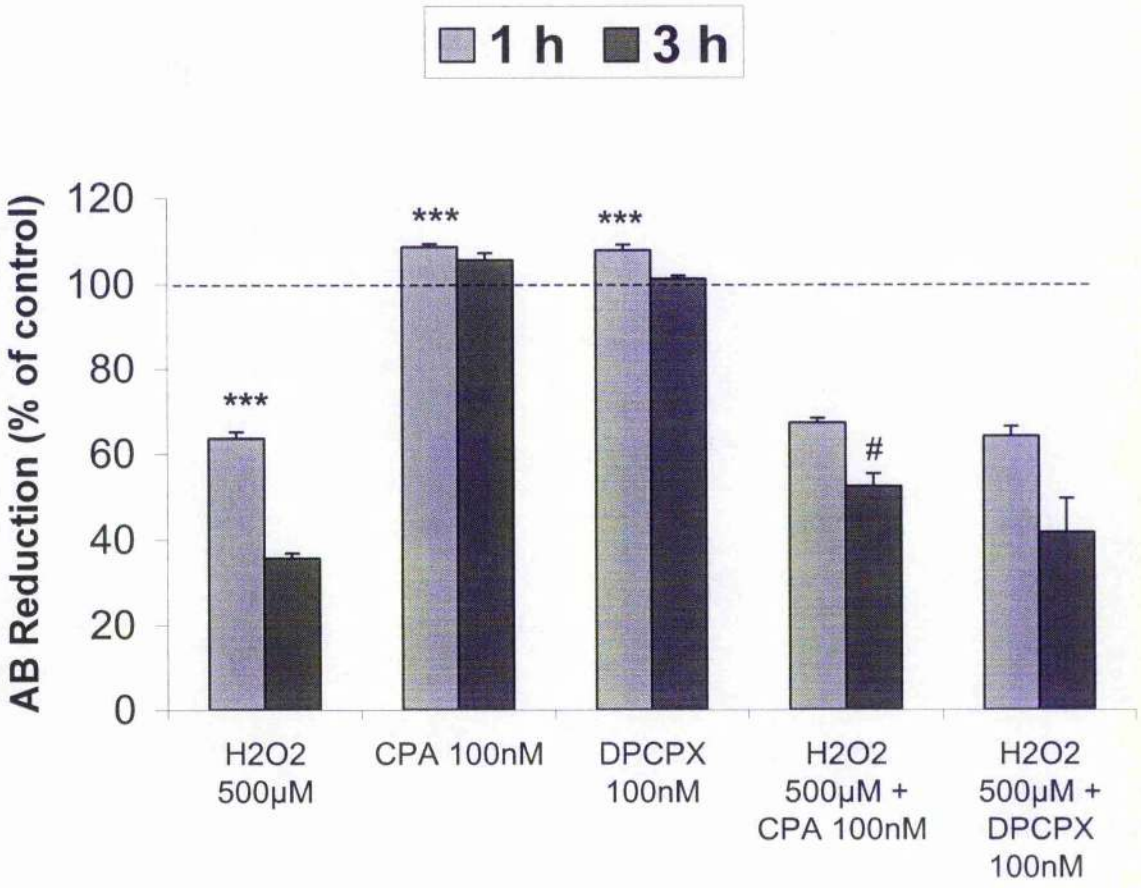


Figure 3.20. Histograms showing the effects of a selective adenosine A_1 receptor agonist, CPA (100nM), and a selective A_1 receptor antagonist, DPCPX (100nM), on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cells. Each column represents the mean ± SEM for n = 4 cultures. *** $P<0.001$ compared to control; # $P<0.05$ compared to H_2O_2 500µM.

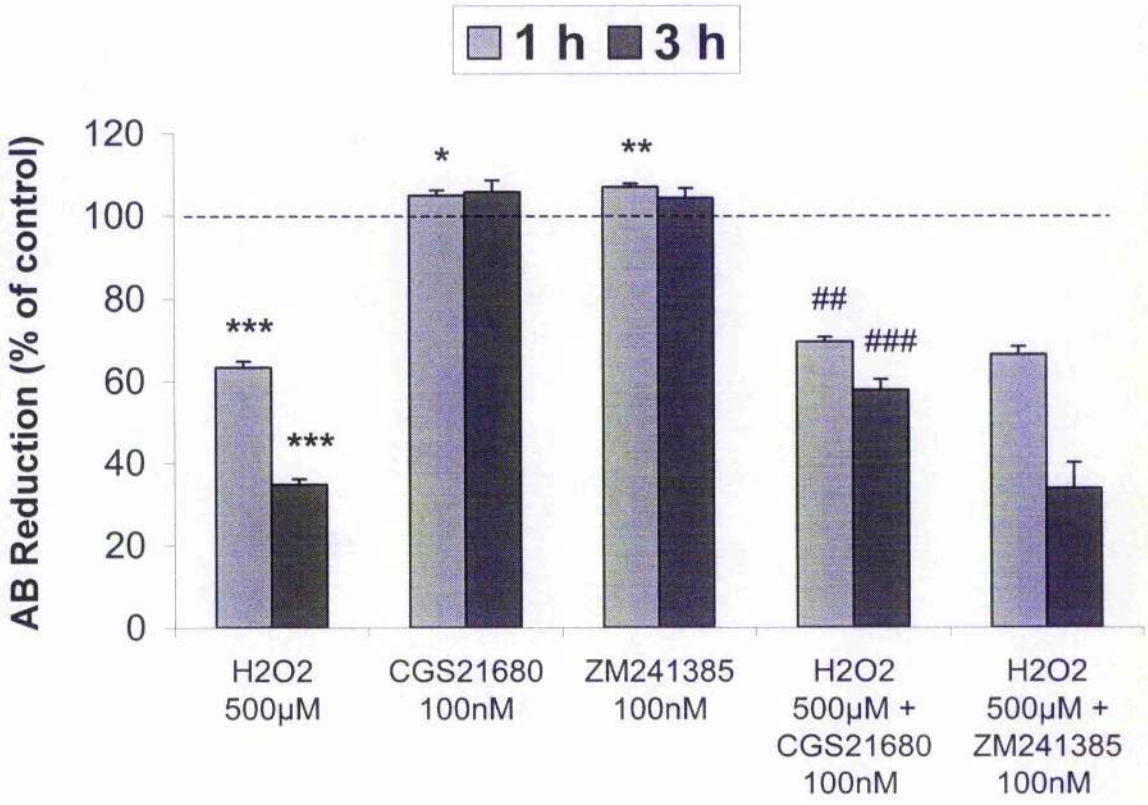


Figure 3.21. Histograms showing the effects of a selective adenosine A_{2A} receptor agonist, CGS21680 (100nM), and a selective A_{2A} receptor antagonist, ZM241385 (100nM), on hydrogen peroxide-mediated cytotoxicity in MC3T3-E1 cells. Each column shows the mean ± SEM for n = 4 cultures for 1 h treatments or n = 3 cultures for 3 h treatments. *P<0.05, **P<0.01, *** P<0.001 compared to control; ## P<0.01, ### P<0.001 compared to corresponding H_2O_2 500µM.

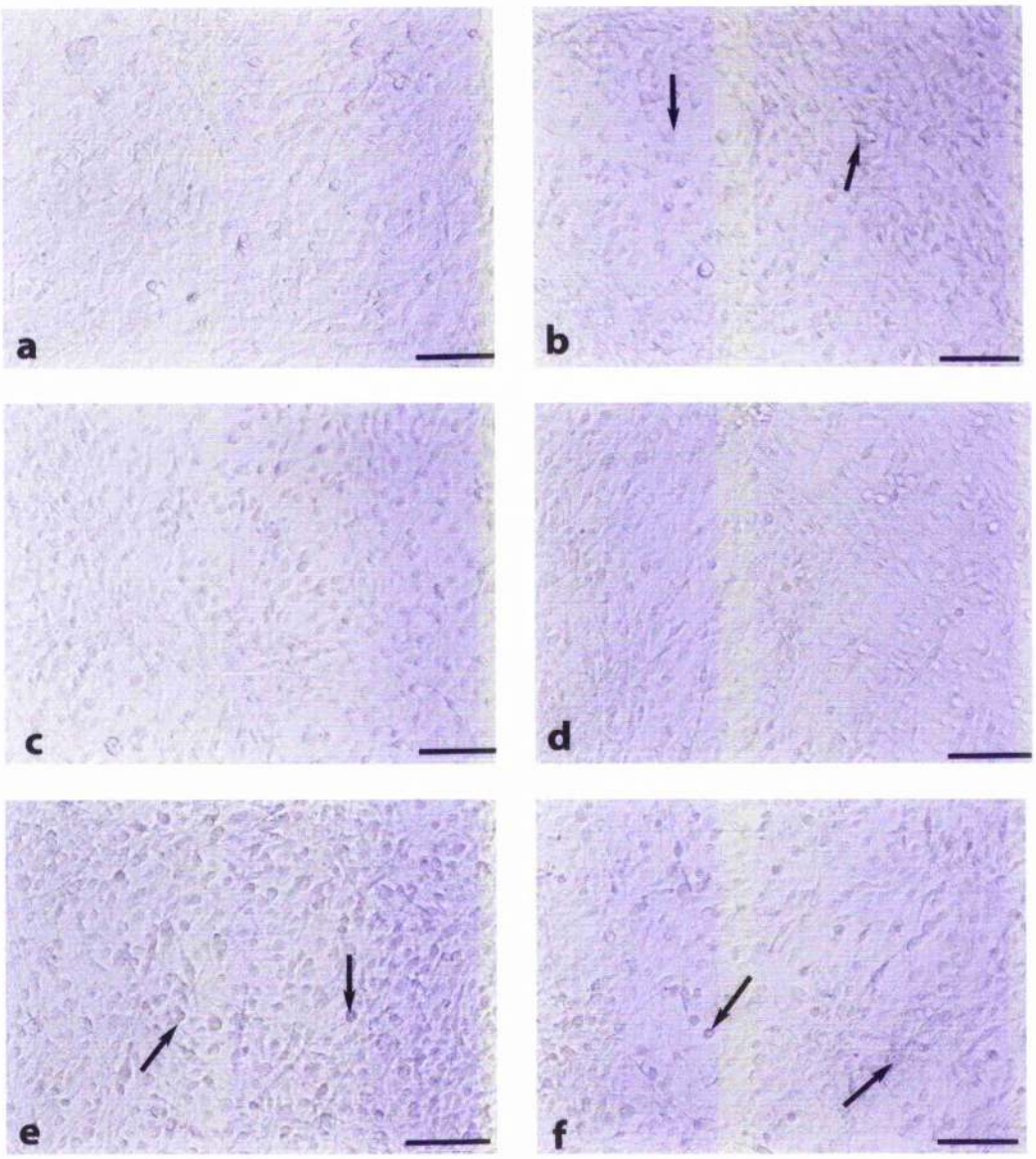


Figure 3.22. Morphological effects of 3 h application of H_2O_2 500μM alone and in combination with specific A_1 and A_{2A} adenosine receptor agonists and antagonists (each at 100nM). **(a)** Control **(b)** H_2O_2 **(c)** H_2O_2 + CPA **(d)** H_2O_2 + CGS21680 **(e)** H_2O_2 + DPCPX, and **(f)** H_2O_2 + ZM241385. Arrows indicate damaged cells that have rounded up to a shrunken phenotype. Bar = 50μm.

3.5.4. *Other generators of reactive oxygen species*

3.5.4.1. Xanthine (X) and xanthine oxidase (XO)

The effect of the superoxide-generating system of xanthine (X) and xanthine oxidase (XO) on osteoblast viability was also examined for 1, 6 and 24 h. Xanthine (100μM) alone applied for 1 or 6 h had no effect on viability but at 24 h significantly increased baseline viability beyond control level to 108.01% ± 2.82 ($P<0.01$, n = 4 - 5) (Fig. 3.23). The application of xanthine oxidase at an activity of 0.02U/ml had no effect on osteoblast viability. Xanthine and xanthine oxidase when combined together produced no effect on osteoblast viability at 1 or 6 h, but increased viability significantly to 108.38% ± 0.83 of the control at 24 h ($P<0.05$). There was no difference between this effect and that produced when xanthine was applied alone for 24 h.

3.5.4.2. Metabolites of the kynurenine metabolic pathway

The kynurenine pathway is involved in the metabolism of the amino acid tryptophan. A number of metabolites produced along this pathway are known to be potent generators of reactive oxygen species. They include 3-hydroxyanthranilic acid (3-HAA) and 3-hydroxykynurenine (3-HK). Osteoblast cells were therefore cultured with these metabolites to observe any potential effects on baseline viability. 3-HAA at 1 and 10nM had no effect when applied for up to 24 h. At 100nM, 3-HAA induced a slight but significant increase in viability after 1 h exposure ($P<0.05$), but had no effect when treatment lasted for 6 or 24 h (n = 3) (Fig. 3.24A). Osteoblast cultures were exposed to 3-HK for a period between 1 and 24 h. The application of 10 and 100μM 3-HK for up to 6 h had no effect (n = 5) (Fig. 3.24B). Even when cultures were treated for a longer period of 24 h, 3-hydroxykynurenine at 1, 10 and 100μM still had no effect, giving viability values of 99.80% ± 2.43, 105.73% ± 1.66 and 98.03% ± 0.97 of the control, respectively. However, profound time-dependent reductions in viability were obtained with 3-HK at 1mM. This concentration reduced osteoblastic viability significantly to 85.44% ± 1.34 of the control at 1 h, representing a 14.56% reduction ($P<0.01$) (Fig. 3.24B). With a longer exposure period of 6 h, viability in the presence of 3-HK 1mM was decreased to 39.13% ± 4.77 of the control ($P<0.01$), representing a significantly greater reduction of 60.87% ($P<0.001$). Morphologically, cultures treated with 3-HK 1mM for 1 h showed some loss of intact cells while treatment with the same

concentration of the kynurenine for a longer period of 6 h revealed greater loss of cells and the rounding up of remaining cells (Fig 3.25).

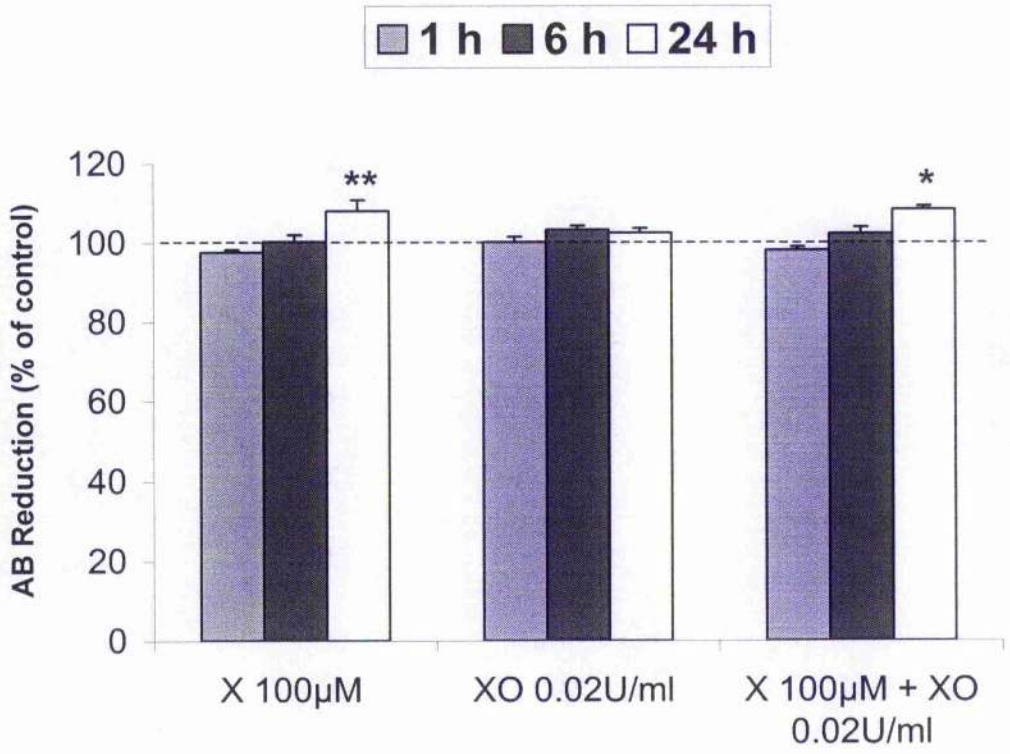


Figure 3.23. Histograms showing the effects of xanthine 100µM and xanthine oxidase 0.02U/ml on the viability of MC3T3-E1 cultures when each of them was applied alone and when they were combined for 1, 6 or 24 h. Each column represents the mean ± SEM for n = 4 or 5 cultures. *P<0.05, **P<0.01 compared to control.

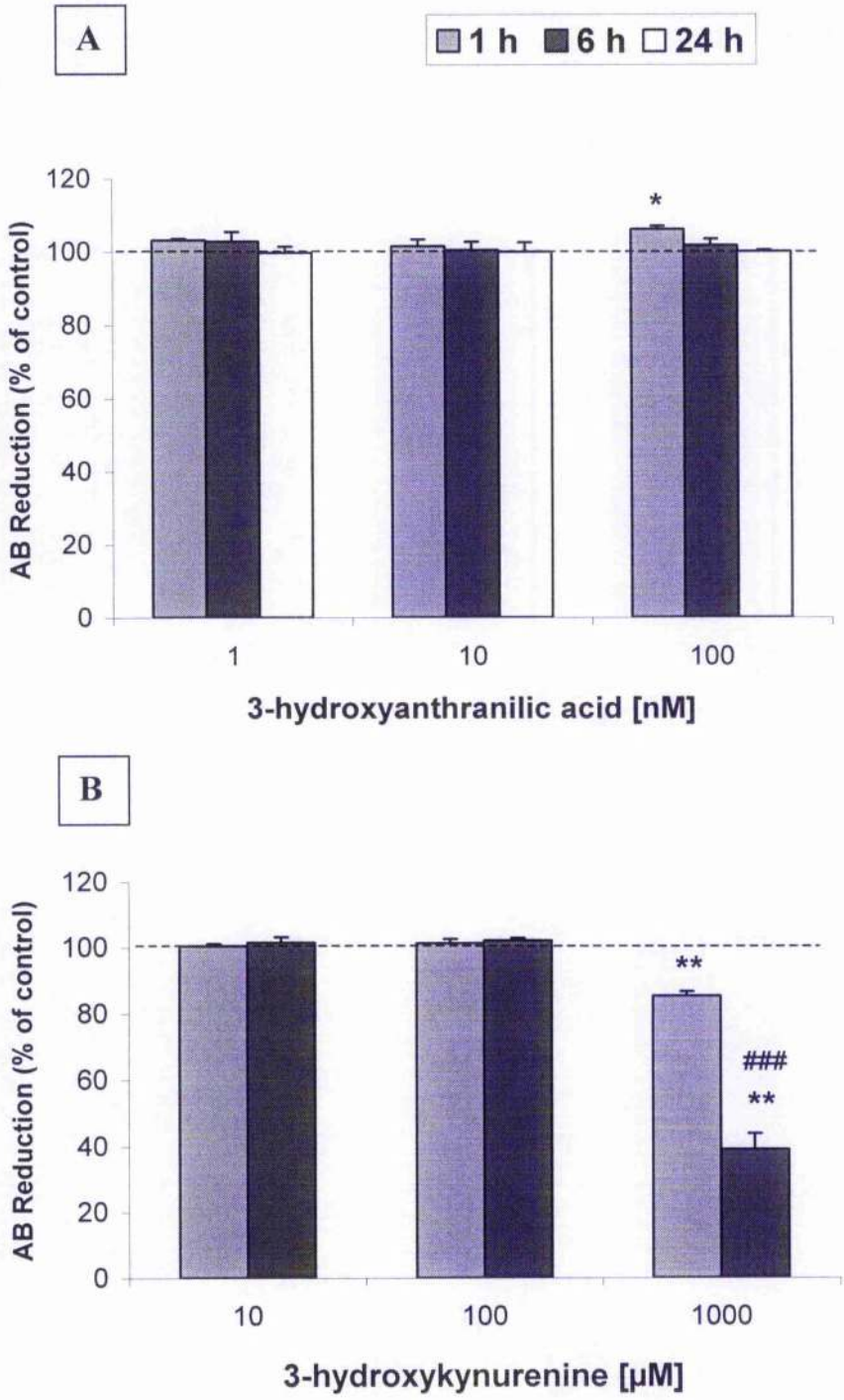


Figure 3.24. Histograms showing the effects on the viability of MC3T3-E1 osteoblast cultures of **(A)** a kynurenine pathway metabolite, 3-hydroxyanthranilic acid (3-HAA) applied for 1, 6 or 24 h and **(B)** another kynurenine pathway metabolite, 3-hydroxykynurenine (3-HK), applied for 1 or 6 h. Each column shows the mean ± SEM for **(A)** n = 3 or **(B)** n = 5 cultures. *P<0.05, **P<0.01 compared to control; ###P<0.001 for comparison of effects of 3-HK 1mM at 1 and 6 h (SNK).

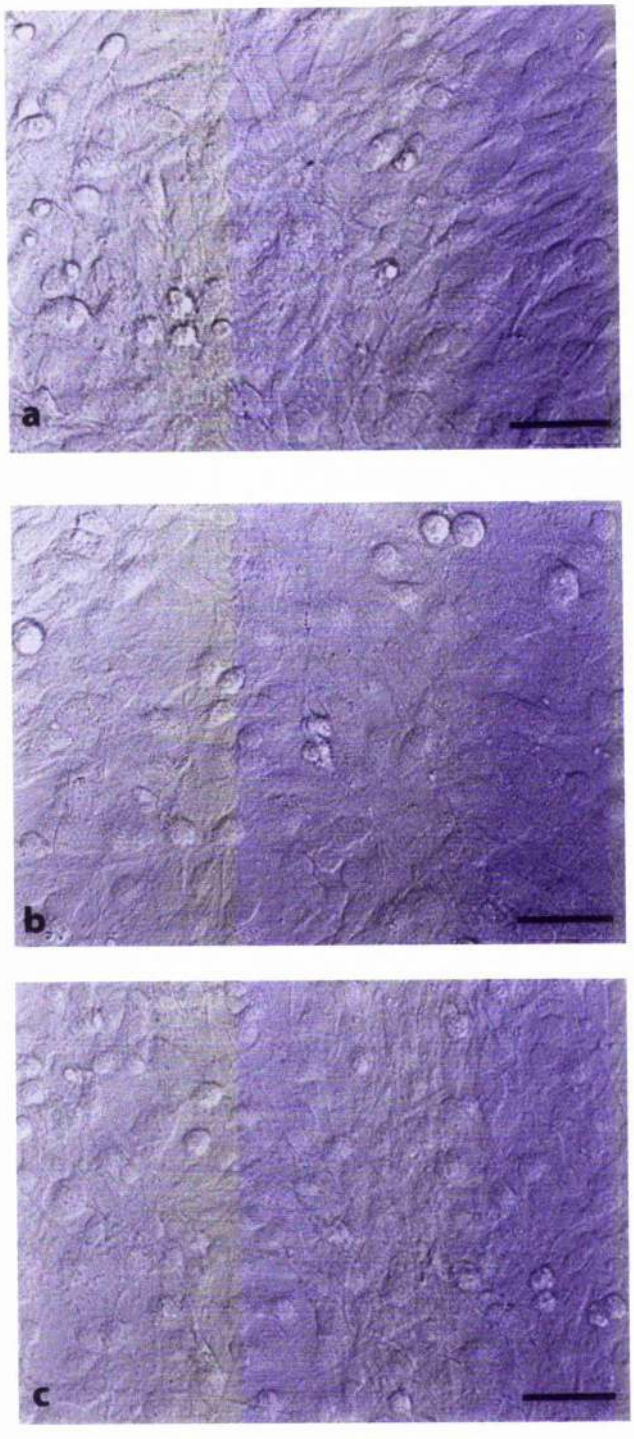


Fig. 3.25. Effects on the morphology of MC3T3-E1 cells of 3-hydroxykynurenine (3-HK). **(a)** Control **(b)** 1mM (1 h) **(c)** 1mM (6 h). Treatment with 3-HK 1mM for 1 h showed initiation of damage to previously intact cells **(b)**, which was more pronounced with the longer treatment (6 h) **(c)**. Bar = 50μm.

3.5.5. Nitrosative stress and osteoblastic viability

We investigated whether the effects elicited by reactive oxygen species in MC3T3-E1 cells are shared by reactive nitrogen species like nitric oxide by using the nitric oxide donor S-nitroso-*N*-acetyl penicillamine (SNAP). Baseline viability was unchanged with 1 or 6 h exposure to SNAP 10μM, but a 24 h exposure resulted in a significantly increased viability that was 109.13% ± 3.50 of the control ($P<0.05$, $n = 4$) (Fig. 3.26A). SNAP 100μM elicited no effect at any time point examined. Cultures were subsequently exposed to higher SNAP concentrations for 24 h. SNAP 1mM failed to produce a significant effect, reducing viability less than 7% to 93.08% ± 6.57 of the control ($n = 4$) (Fig. 3.26B). However, SNAP at 5mM was profoundly toxic to the osteoblasts, with almost 94% reduction of viability to 6.10% ± 0.72 of the control ($P<0.01$), an effect that suggested that complete cell death was induced with this SNAP concentration.

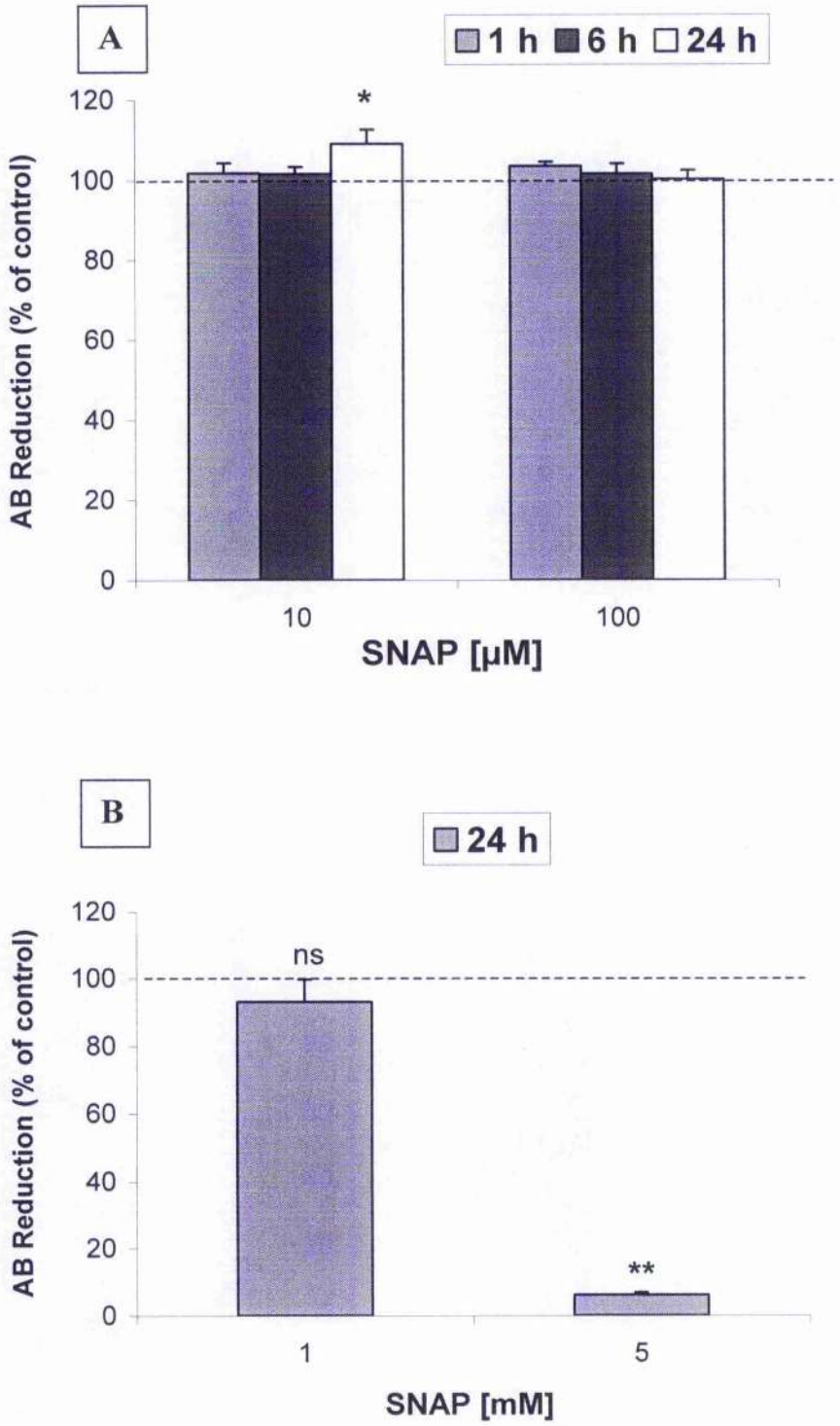


Figure 3.26. Histograms showing **(A)** the lack of effect of low concentrations (10, 100µM) of a nitric oxide donor, S-nitroso-*N*-acetyl penicillamine (SNAP) applied for 1, 6, or 24 h and **(B)** the effects of high concentrations (1, 5mM) of the donor, applied for 24 h, on the viability of MC3T3-E1 cultures. Each column represents the mean ± SEM for n = 4 cultures. *P<0.05, **P<0.01, ns = non-significant compared to control.

3.5.6. Mitochondrial poisoning and osteoblastic viability

Considering the observed relative resistance of MC3T3-E1 cultures to both oxidative and nitrosative stress - evidenced by a requirement for prolonged exposure to high concentrations of inducers of oxidative and nitrosative stress to elicit significant reduction of viability - the degree of their sensitivity to mitochondrial damage was established. Two known potent mitochondrial poisons were used: potassium cyanide (KCN) and 3-nitropropionic acid (3-NPA). Interestingly, exposures of MC3T3-E1 cultures to both low and high concentrations of KCN (10μM – 10mM) were unable to produce any statistically significant damage with treatments lasting for up to 24 h, although 100μM and 1mM applications were potentially toxic at 24 h, reducing viabilities to 85.06% ± 4.74 and 85.33% ± 7.02 of the control (n = 4 - 5) (Fig. 3.27A). Similarly, exposure to 3-NPA at 10, 100 and 1000μM for up to 6 h produced no effect on osteoblast viability. However, with 24 h of exposure, 3-NPA 100μM significantly reduced osteoblast viability 33.36% to 66.64% ± 15.62 of the control ($P<0.05$, n = 4) (Fig. 3.27B), while 3-NPA 1mM elicited a greater reduction (57%) to 42.86% ± 7.04 of the control ($P<0.01$).

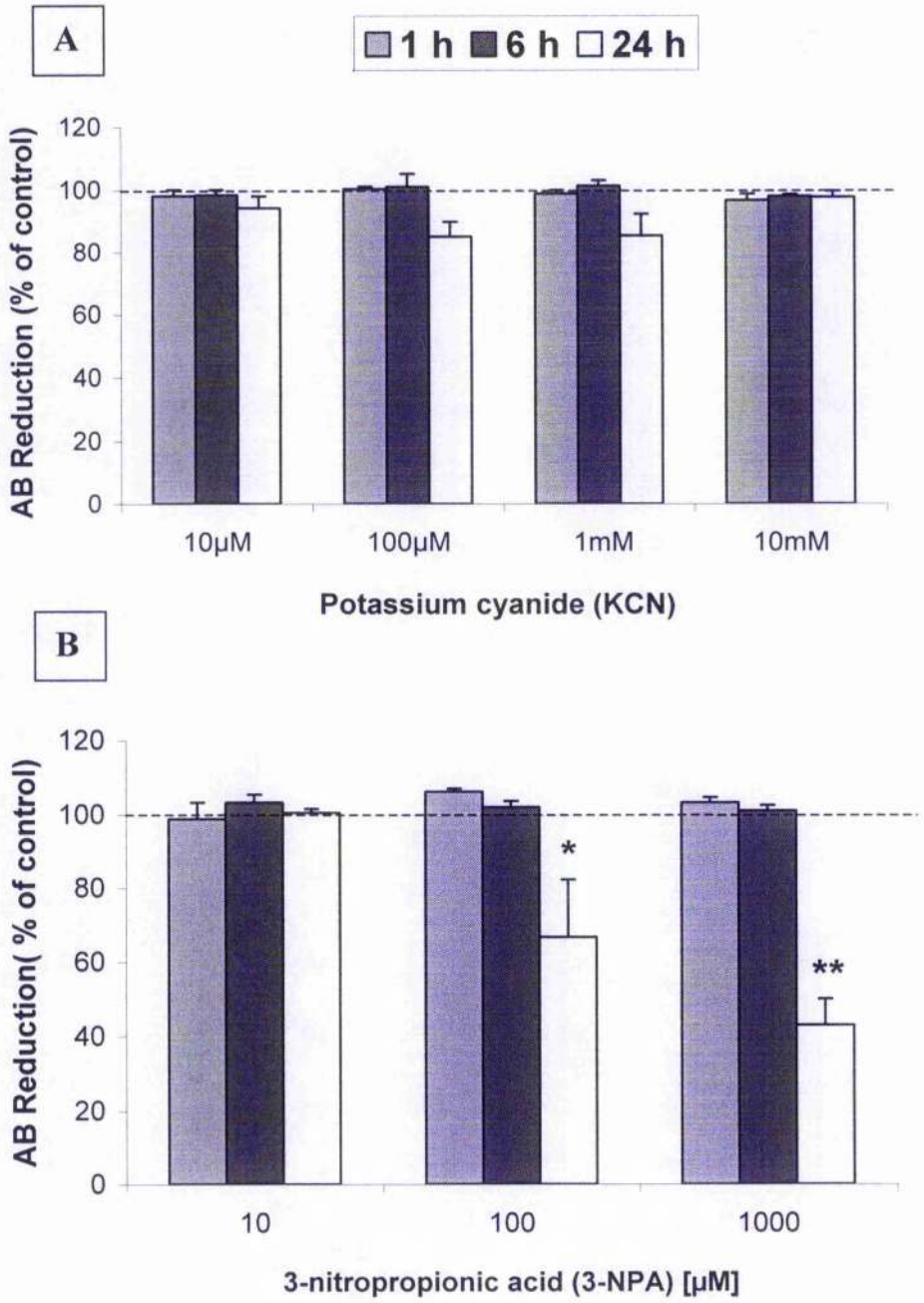


Figure 3.27. Histograms showing **(A)** lack of effect of the mitochondrial poison, potassium cyanide (KCN) and **(B)** the effects of another mitochondrial poison, 3-nitropropionic acid (3-NPA), each applied for up to 24 h, on the viability of MC3T3-E1 cultures. Each column represents the mean ± SEM for **(A)** n = 4 or 5 or **(B)** n = 4 cultures. *P<0.05, **P<0.01 compared to control. No statistically significant effect was produced by KCN.

4. RESULTS – EFFECTS OF GLUTAMATE AND OXIDATIVE STRESS ON THE VIABILITY OF CEREBELLAR GRANULE NEURONES (CGNs)

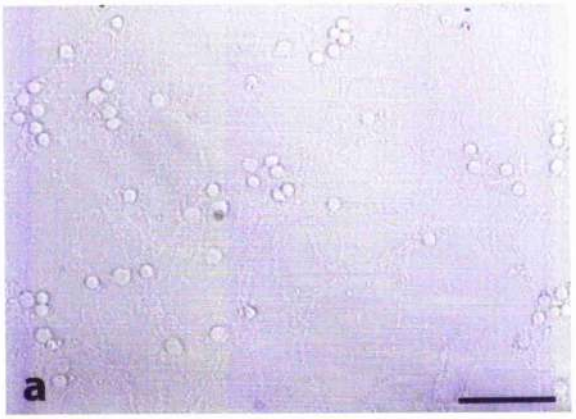
4.1. Immunocytochemical labelling for neuronal tubulin

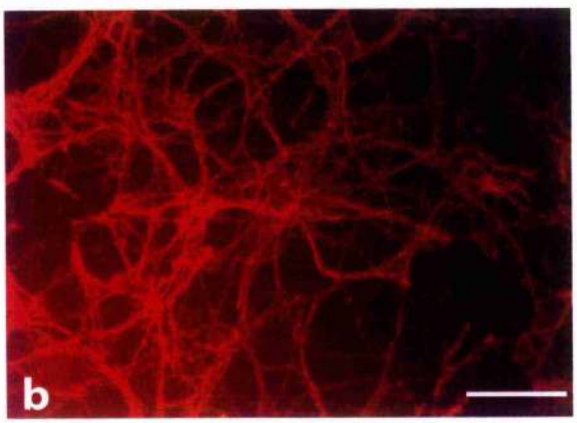
In order to determine their purity, the cultured cerebellar granule neurones were immunolabelled with an antibody specific for the β-III isoform of neuronal tubulin. This isoform of the cytoskeletal protein tubulin is found throughout the cytoplasm, but not nucleus, of the perikarya and extends into the neurite processes; it is specific for neurones (Geisert & Frankfurter, 1989; Farina et al., 1999). The results are as shown in Fig. 4.1. At 8 div, the cultures showed strong immunoreactivity against the mouse anti-human β-III isoform of tubulin monoclonal antibody (Fig. 4.1b), while control cultures that were not treated with the (primary) antibody lacked such staining (Fig. 4.1d). The corresponding bright field appearances have been included (Fig. 4.1a and Fig. 4.1c).

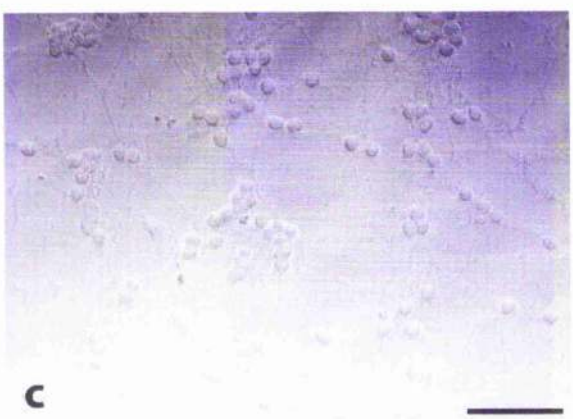
4.2. Alamar Blue (AB) standardization

The routine alamar blue determination of viability in cerebellar granule neurones (CGNs) was standardized with regard to seeding density and duration of incubation with the dye, as was the case with the MC3T3-E1 cells. Seeding densities were in the range 2.5×10^5 to 2×10^6 cells per ml, in consecutive, two-fold increases, incubated for 2, 4, 6, and 8 h. For a given incubation period (6 h), AB reduction increased linearly ($R^2 = 0.9002$) with increasing densities (Fig. 4.2A). Similarly, for a given seeding density (1×10^6 cells per ml), AB reduction increased linearly ($R^2 = 0.9739$) with time (Fig. 4.2B). In all experiments therefore, a seeding density of 1×10^6 cells per ml and an incubation period of 6 h were chosen as standard.

The non-toxicity of AB to CGNs was also confirmed. Two sets of cultures were pretreated for 6 h with either AB (10%v/v) or an equal amount (10% v/v) of vehicle. No disruption or lysis of neuronal cells was observed. Cultures were then restored to the culture medium for at least 16 h before viability was determined. When quantified with AB itself, there was no difference in the viability of cultures pretreated with AB and that of cultures pretreated with vehicle dilution (0.120 ± 0.003 vs. 0.124 ± 0.004, for AB- and vehicle-treated (control) cultures, respectively; $n = 6$). The use of the trypan blue exclusion test also confirmed no difference in the viabilities of the two sets of cultures ($92.93\% \pm 3.17$ vs. $90.55\% \pm 0.40$, $n = 4$).







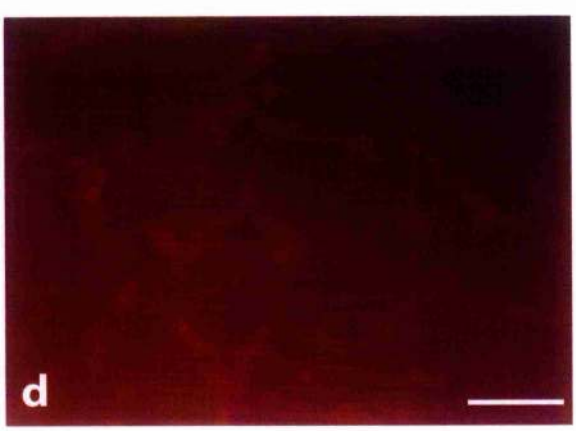


Figure 4.1. CGN cells cultured for 8 div to identify positively the neuronal phenotype by immunocytochemical labelling with antibody against β-III isoform of tubulin, which is a specific marker for the neuronal cytoskeleton.

(a) bright field and **(b)** fluorescent micrographs of neurones with extensive neurite outgrowth evident.

(c) bright field and **(d)** fluorescent micrographs of neurones when primary antibody was omitted as a staining control (note only faint background outline of neuronal cell bodies and no staining into the neurite processes).
Bar = 50μm.

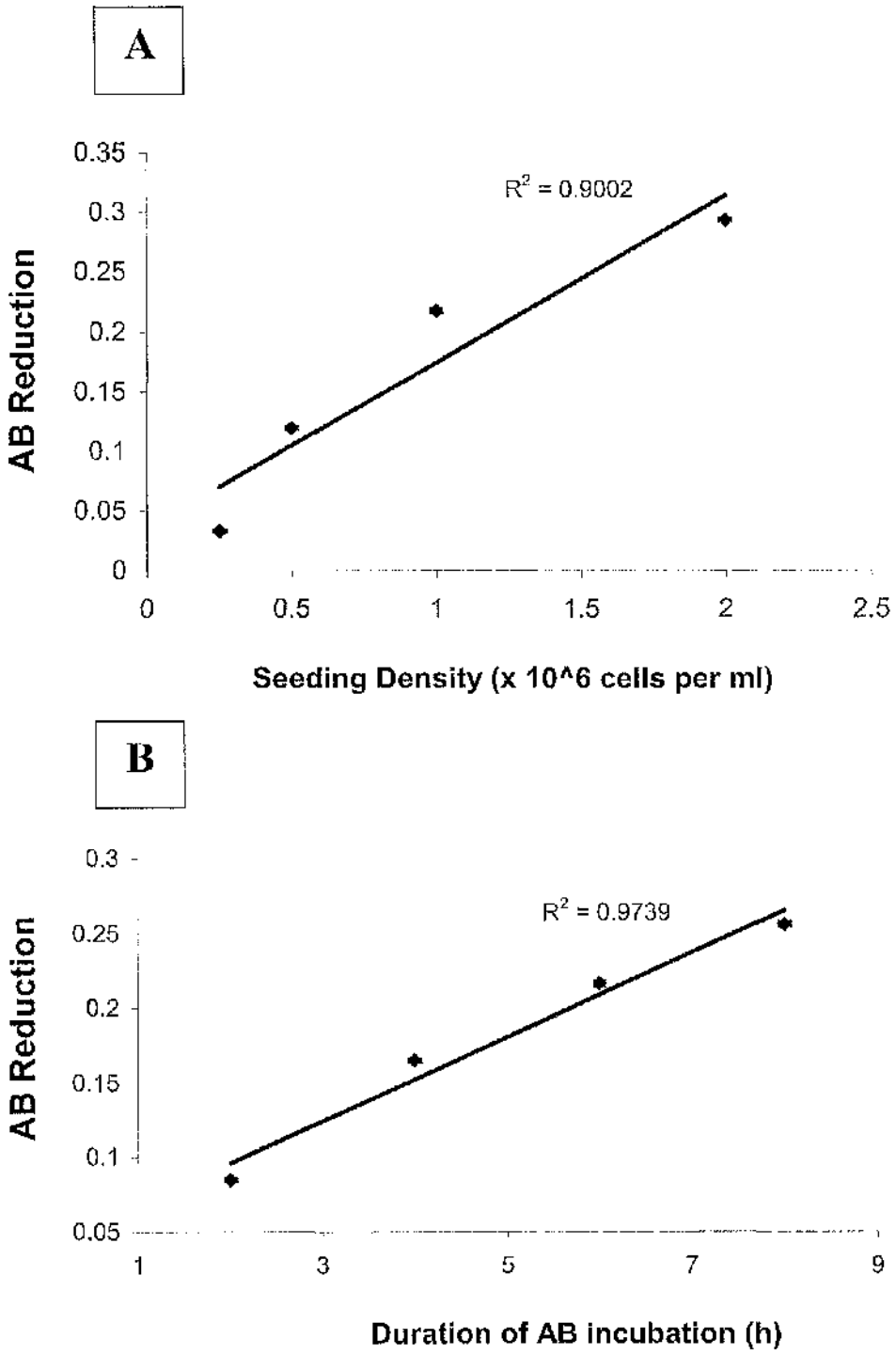


Figure 4.2. Graphs showing the linear correlation between alamar blue (AB) reduction and **(A)** the initial seeding density when duration of incubation was 6 h and **(B)** the duration of AB incubation for a given seeding density of 1 x 10^6 cells per ml in cerebellar granule neuronal (CGN) cultures. Each data point represents the mean ± SEM for n = 4 cultures.

4.3. Serum and high potassium concentration withdrawals (deprivation)

Serum and raised potassium ion concentrations are two culture supplements whose inclusions in the culture medium are known to significantly promote the survival of CGN cultures (Hopp et al., 1987; Hetman et al., 1999; Gallo et al., 1987; Balazs et al., 1988; Lindholm et al., 1993). The degree of sensitivity of the CGN cultures to the absence of these supplements was therefore assessed. Cerebellar granule cultures grown in 10% FCS were serum-deprived for 1 and 6 h at 8 div. This was followed by a recovery period of 20 h before viability determination. While 1 h of serum deprivation had no effect on neuronal survival, cultures without serum for 6 h showed a very significant (32.42%) reduction ($P<0.01$, $n = 6$) in survival when compared with non serum-deprived control (67.58% ± 2.21 of the control) (Fig. 4.3). Cultures that underwent serum withdrawal especially at 6 h showed a grossly shrunken morphology with extensive damage to their neurite processes (Fig. 4.4a-c) compared to control cultures with robust appearance and intact neurite processes. Withdrawal of high potassium concentration was achieved by reducing the concentration of potassium in the culture medium from 25mM to 5mM for 2 days from 6 to 8 div. This reduction took into account the level of potassium in the basal medium (MEM) used, which was approximately 5mM. The potassium withdrawal protocol was therefore essentially the withdrawal of the potassium added at plating. The full medium was restored for at least 20 h at the end of the 2 days of high potassium withdrawal, after which viability was determined. As shown in Fig. 4.3, a very significant (31.44%) reduction ($P<0.01$) in viability compared to control cultures that were maintained in high potassium concentration was observed (68.56% ± 5.74 of the control) and this was well correlated with morphological effects, as low-potassium cultures contained shrivelled neurones with damaged processes as opposed to high-potassium (control) cultures that retained intact cell bodies and neurite interconnections (Fig. 4.4d-e). The results from serum withdrawal experiments determined the subsequent protocol throughout, which subjected CGNs for no longer than 1 h without serum in Hanks' Balanced Salt Solution (HBSS) when undertaking excitotoxicity studies.

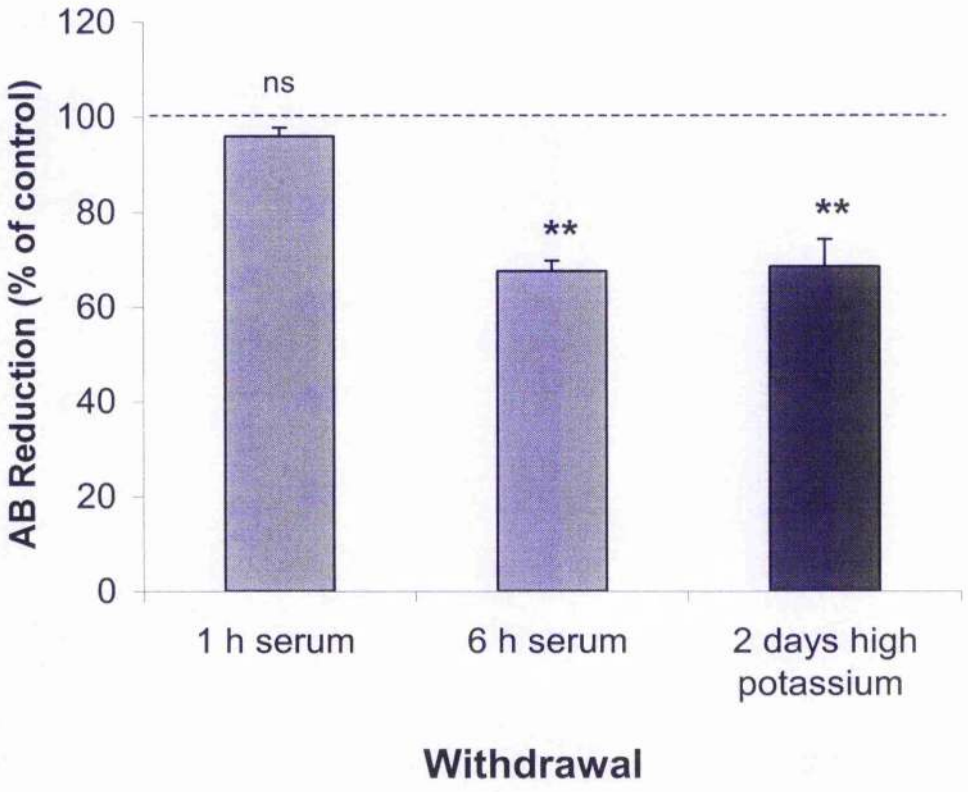


Figure 4.3. The effects of withdrawing FCS and high potassium concentration on the viability of CGNs. Serum was withdrawn at 8 div for the indicated duration, while high potassium concentration (25mM) was withdrawn for 2 days from 6 to 8 div. High potassium concentration withdrawal implies removal of the extra potassium added to the culture medium when cultures were set up. This was achieved by restoring the cultures to MEM supplemented with FCS, without adding extra potassium. Basal potassium level in the MEM used was approximately 5mM. Each column represents the mean ± SEM for n = 6 cultures. **P<0.01, ns = non-significant compared to control.

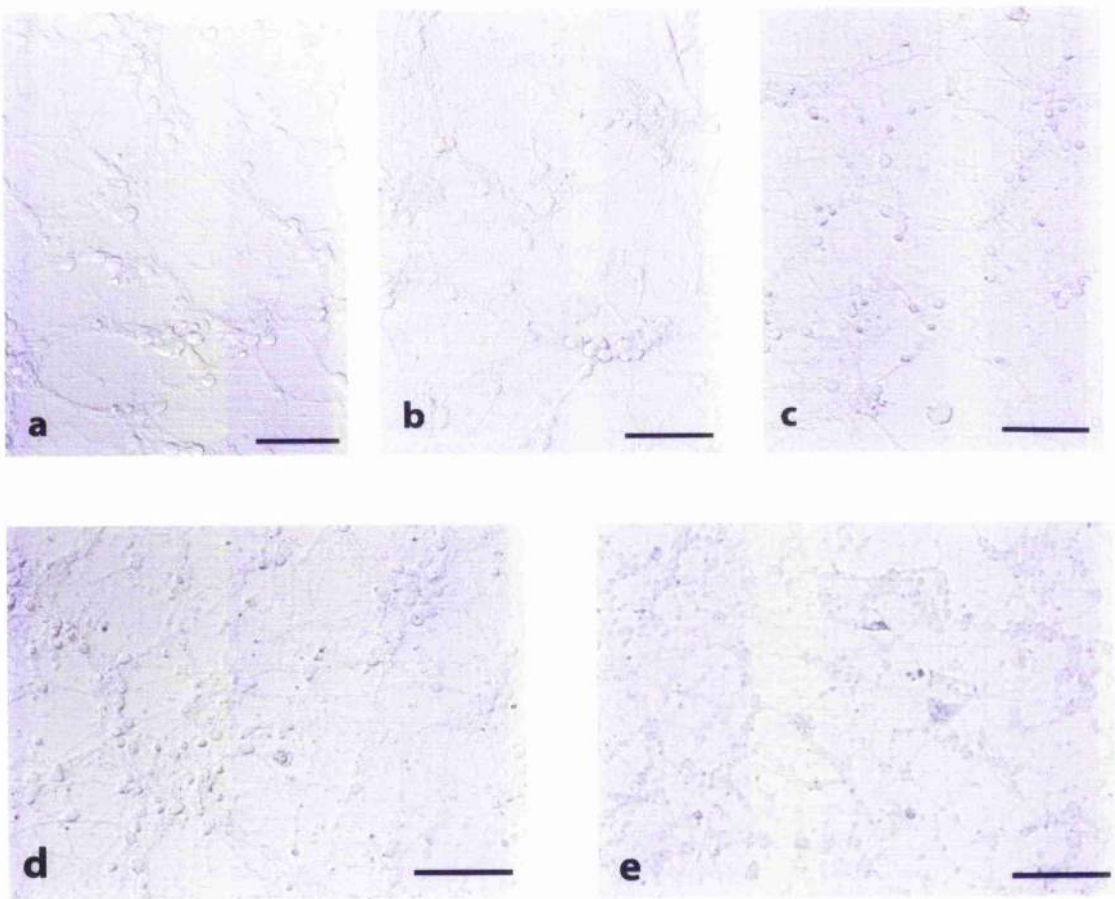


Figure 4.4. Effects on cerebellar granule neurones of **(a-c)** withdrawing serum for 1 or 6 h and **(d-e)** reducing potassium concentration for 2 days from 25mM (high K^+) to 5mM (low K^+). Cultures were supplemented with or without 10% FCS.

(a) Control (no serum withdrawal) **(b)** 1 h serum withdrawal **(c)** 6 h serum withdrawal, and **(d)** Control (25mM K^+) **(e)** 2 days of low K^+ (6 - 8 div). Note the gross shrunken appearance of cultures that were serum-deprived for 6 h **(c)** and those that were maintained in low potassium concentration **(e)**, in comparison to their respective control cultures **(a** and **d)**, which showed intact neurite processes. Bar = 50μm.

4.4. Excitotoxicity in the cerebellar granule neurones

Excitotoxicity studies were carried out at 9 div in Hanks' Balanced Salt Solution without serum, due to the earlier observations that serum may significantly compensate for and thus occlude the neurotoxic effect of glutamate.

4.4.1. Glutamate and NMDA-induced excitotoxicity

The effects of glutamate and the selective agonist NMDA on the viability of cerebellar granule neurones were examined using two different post-treatment protocols. One restored the cultures, after 1 h treatment, to serum-free MEM enriched with D-glucose 21mM (MEM + glucose) and the other restored cultures to MEM supplemented with 10% FCS (MEM + serum). Cultures were allowed to recover for at least 18 h. With both protocols, glutamate (10μM - 1mM) reduced the viability of CGNs significantly in a concentration-dependent manner, compared to the control ($P<0.01$, $n = 4 - 6$) (Fig. 4.5). However, there were greater reductions in viability when cultures were restored to MEM + glucose than when restored to MEM + serum, which was significant for 10μM and 100μM of glutamate. NMDA (10μM - 1mM) also induced a concentration-dependent reduction in CGN viability with both conditions ($n = 4 - 6$) (Fig. 4.6). NMDA was less potent in reducing CGN viability than glutamate at comparable concentrations, and no difference was observed between the effects of the two restoration protocols for any given concentration of NMDA. Morphologically, there was severe damage to the cell bodies and neurites of cultures treated with glutamate or NMDA, and at high concentrations, cell loss was evident (Fig. 4.7). The loss of cells following treatment with 100μM glutamate was significantly greater when cultures were restored to MEM + glucose than when restored to MEM + serum. For all subsequent excitotoxicity experiments, cultures were restored after 1 h of treatment to serum-containing MEM, as this favoured baseline survival significantly more than restoration to serum-free, glucose-enriched MEM.

4.4.1.1. Blockade by antagonists of glutamate-induced excitotoxicity

By employing both non-competitive and selective antagonists at the NMDA receptor, the involvement of the NMDA receptor in the observed reduction in CGN viability induced by either glutamate or NMDA was clarified. The non-competitive NMDA antagonist dizocilpine (MK-801) (10μM) and the selective antagonist D-AP5 (50μM) had no significant effects on the CGN cultures when applied alone, giving viability values of 107.30% ± 2.04 and 105.83% ± 2.96 of the control, respectively ($n = 3$). For glutamate or

NMDA, a working concentration of 300μM was chosen, as this concentration produced approximately 50% reduction in viability, thus allowing increases or decreases induced by other agents to be clearly observed. Glutamate 300μM reduced the viability of CGN cultures to 62.44% ± 1.22 of the control ($P<0.01$, n = 3) (Fig. 4.8A). MK-801 (10μM) significantly attenuated this effect of glutamate ($P<0.05$), raising viability to 79.94% ± 6.78, a level that was still significantly lower than the control ($P<0.05$ compared to control), while the application of D-AP5 at 50μM restored viability to 91.03% ± 6.58 ($P<0.01$), a level comparable with the control. This restoration of viability by D-AP5 correlated with morphological improvement in which damage to cell bodies was improved as shown in Fig. 4.11a-c. The involvement of the AMPA/kainate receptors in the reduction of viability by glutamate was examined using the AMPA/kainate receptor blocker, CNQX, at 10μM. CNQX had no significant effect on its own and also failed to attenuate the effect of glutamate that reduced CGN viability to 58.73% ± 8.31 of the control ($P<0.001$, n = 4) (Fig. 4.8B). Kynurenic acid (KYA), a product of the kynurenine pathway of tryptophan metabolism (Stone & Darlington, 2002), blocks NMDA receptor activation by acting as an antagonist at the glycine-sensitive co-agonist site (Perkins & Stone, 1982). Its effect on glutamate-induced reduction in viability of CGNs was therefore investigated. At 1mM, KYA completely blocked the significant (23.92%) reduction ($P<0.01$) in viability induced by glutamate 300μM (n = 4) (Fig. 4.9A). Complete blockade of glutamate effect was also obtained with KYA 100μM (n = 4) (Fig. 4.9B). These concentrations of KYA elicited no significant changes in viability when applied alone (107.92% ± 5.36 and 112.45% ± 3.28, for 100μM and 1mM KYA, respectively; $P>0.05$, n = 3). However, with regards to morphological outcomes, KYA when applied with glutamate preserved neuronal cell number but not neurite outgrowth (Fig. 4.11a, b, d).

4.4.1.2. Antagonists and NMDA-induced excitotoxicity

Application of NMDA 300μM resulted in a significant decrease in the viability of CGNs to 61.89% ± 2.87 ($P<0.001$) (Fig. 4.10A). This effect was completely abolished by both MK-801 (10μM) and D-AP5 (50μM) ($P<0.001$, n = 3). Interestingly, in the presence of D-AP5, NMDA-treated cultures retained a morphology that was almost identical to that of control cultures (Fig. 4.11a, e, f). In Fig. 4.10B, NMDA reduced viability to 67.63% ± of the control ($P<0.001$, n = 3). Kynurenic acid at 100μM totally blocked this NMDA effect, while at 1mM, it raised viability in the presence of NMDA significantly beyond the control level ($P<0.01$).

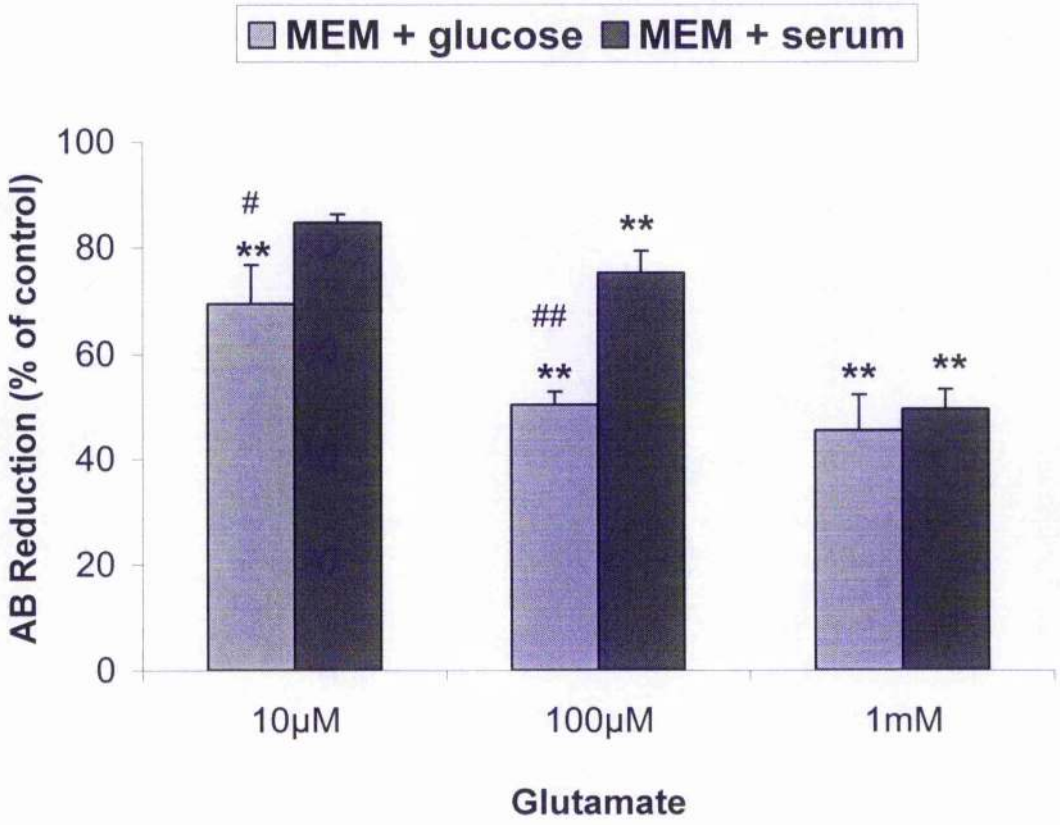


Figure 4.5. The effects of 1 h application of glutamate on the viability of cerebellar granule neuronal cultures at 9 div. Treatments were carried out in Hanks' Balanced Salt Solution (HBSS) without serum. At the end of the treatment period, cultures were either restored to serum-free MEM containing D-glucose 21mM (MEM + glucose), or to medium containing 10% FCS without glucose (MEM + serum). Each column shows the mean ± SEM for n = 4 or 6 cultures. **P<0.01 compared to control; #P<0.05, ##P<0.01 for comparison between MEM + glucose and MEM + serum cultures.

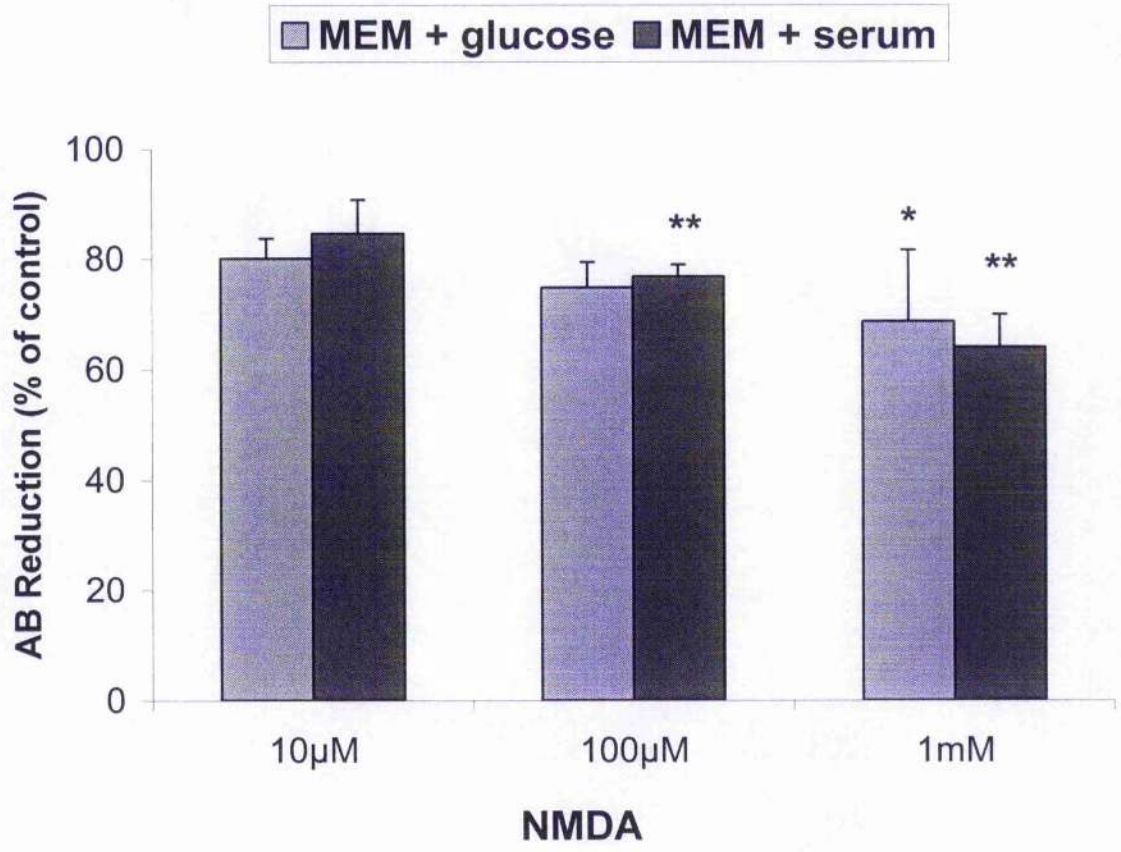


Figure 4.6. Histograms showing the effects of 1 h application of NMDA on the viability of cerebellar granule neuronal cultures at 9 div. Treatments were done in Hanks' Balanced Salt Solution without serum. At the end of the treatment period, cultures were either restored to serum-free MEM containing D-glucose 21mM (MEM + glucose), or to medium containing 10% FCS without glucose (MEM + serum). Each column shows the mean ± SEM for n = 4 or 6 cultures. *P<0.05, **P<0.01 compared to control.

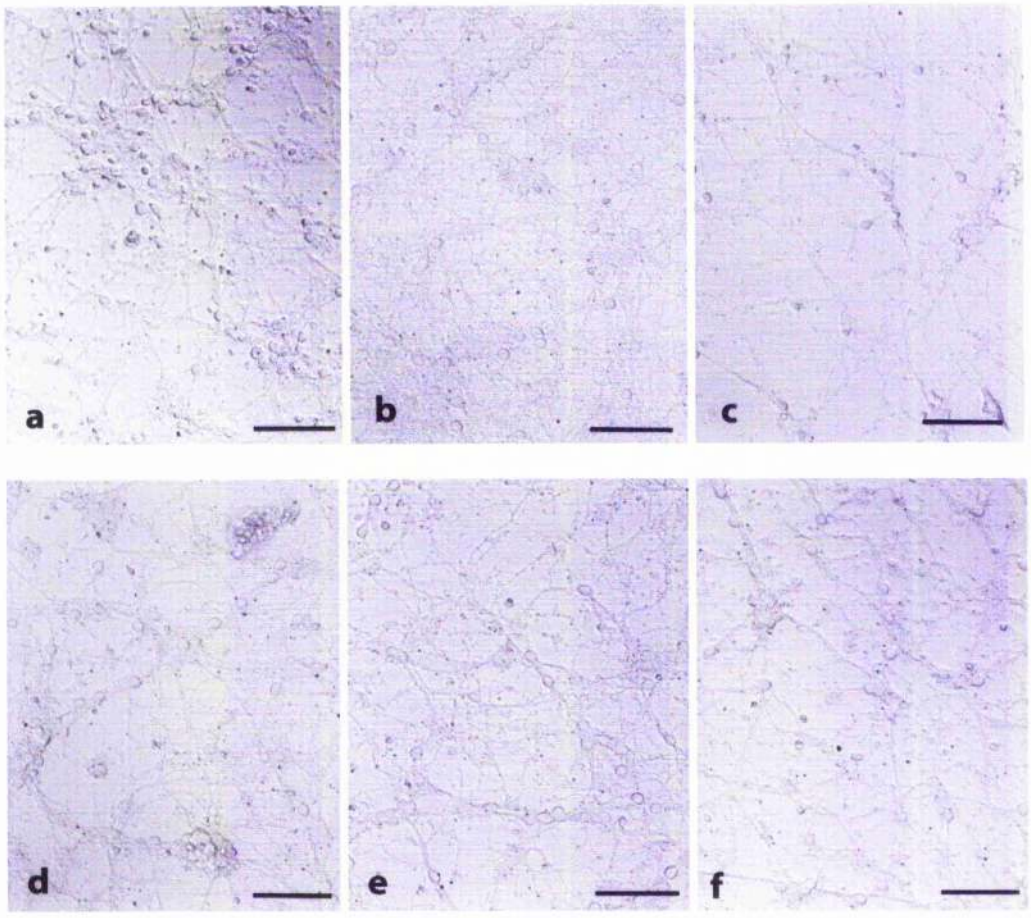


Fig. 4.7. Morphological effects of increasing concentrations of glutamate and NMDA on cerebellar granule neuronal cultures at 9 div that were restored to MEM + serum (except otherwise stated). **(a)** Control **(b)** Glutamate 100μM **(c)** Glutamate 100μM (MEM + glucose) **(d)** Glutamate 1mM **(e)** NMDA 100μM **(d)** NMDA 1mM. Glutamate-treated **(b, c, d)** or NMDA-treated **(e, f)** cultures suffered damage to their cell bodies and neurites. There was greater cell loss with restoration to MEM + glucose **(c)** than with restoration to MEM + serum **(b)** for glutamate 100μM. Bar = 50μm.

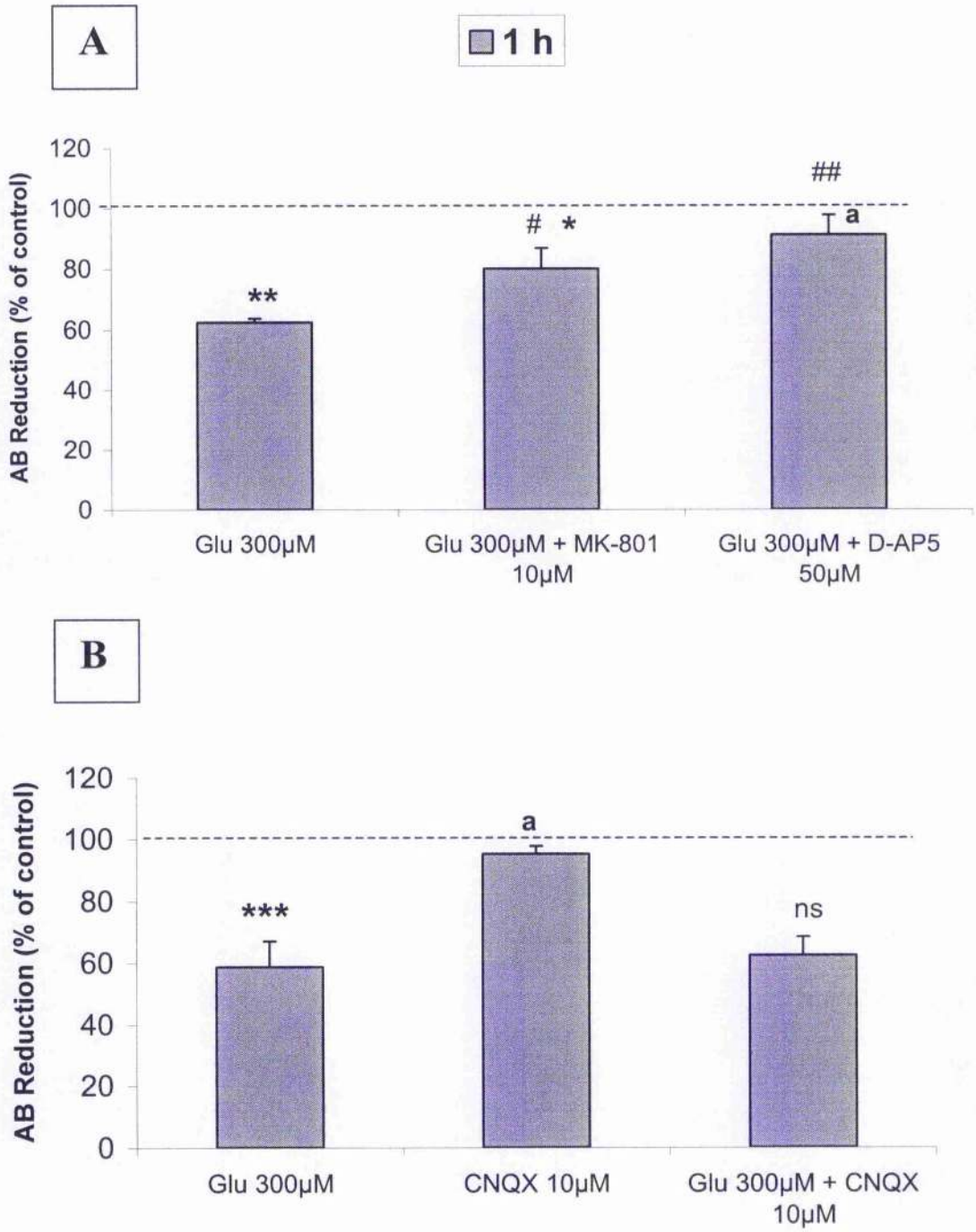


Figure 4.8. Histograms showing the blockade of the excitotoxic effects on CGNs of 1 h application of glutamate (Glu 300µM) by **(A)** the non-competitive NMDA receptor antagonist, MK-801 (10µM), and the selective NMDA receptor antagonist, D-AP5 (50µM), but not by **(B)** the AMPA/kainate receptor antagonist, CNQX (10µM). Each column shows the mean ± SEM for **(A)** n = 3 or **(B)** n = 4 cultures. *P<0.05, **P<0.01, ***P<0.001, **a** = non-significant compared to control; #P<0.05, ##P<0.01, ns = non-significant compared to Glu 300µM.

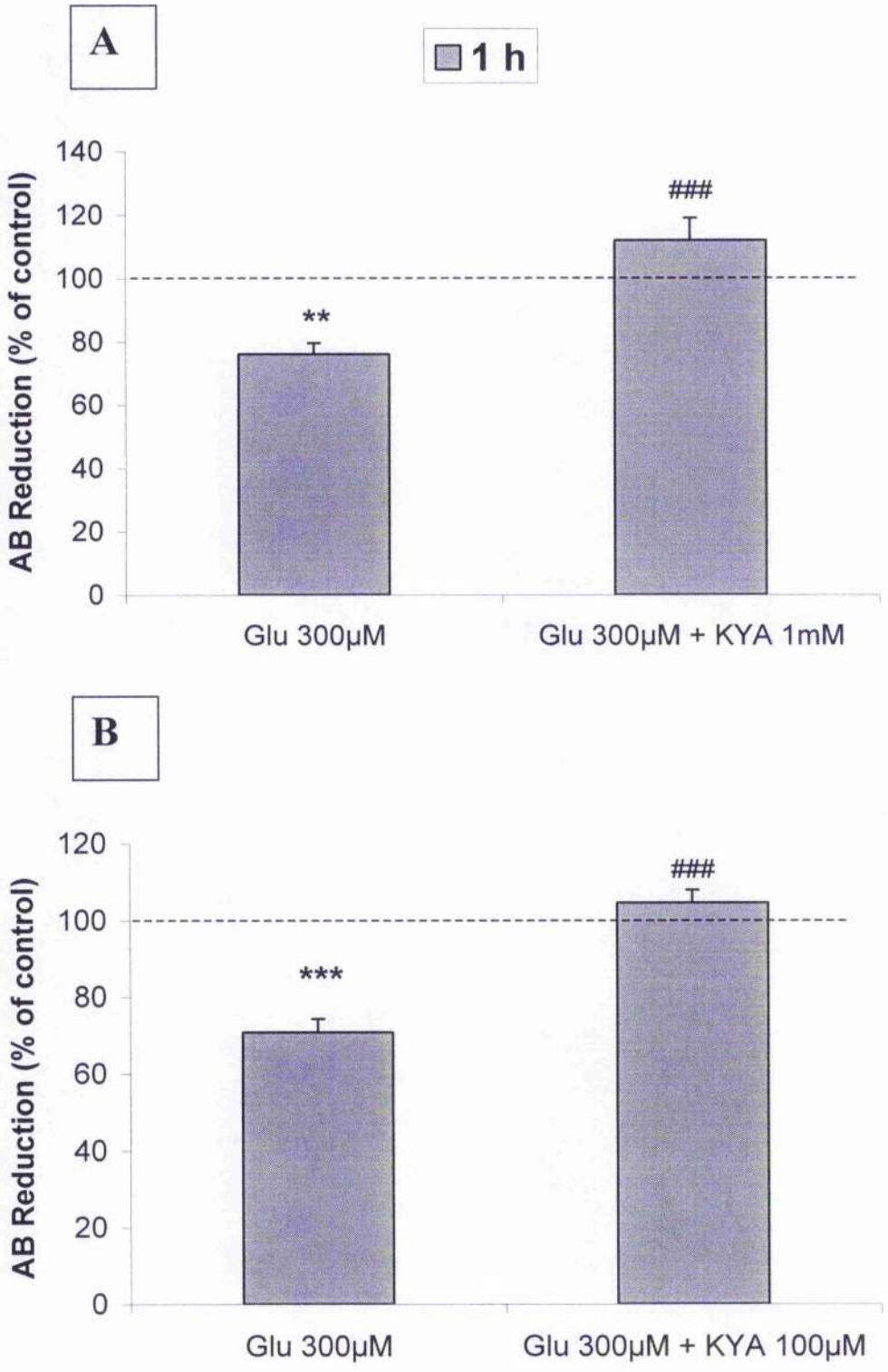


Figure 4.9. Histograms showing the blockade of the excitotoxic effects of glutamate (Glu 300µM) by the broad-spectrum glutamate receptor antagonist, kynurenic acid (KYA), at **(A)** 1mM, and **(B)** 100µM. Each column shows the mean ± SEM for n = 4 cultures. **P<0.01, ***P<0.001 compared to control; ###P<0.001 compared to Glu 300µM.

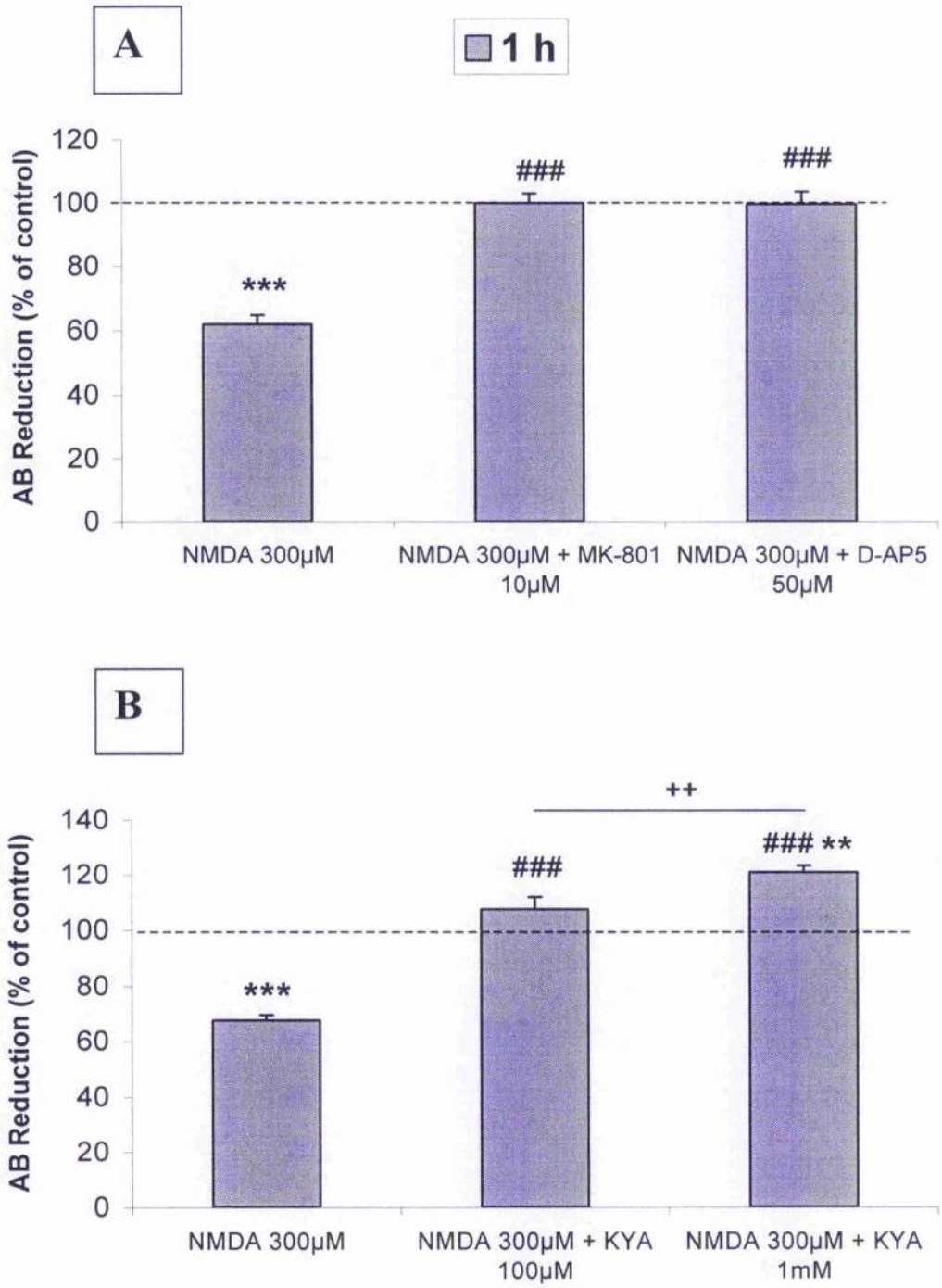


Figure 4.10. Histograms showing the blockade of the excitotoxic effects on CGNs of 1 h application of NMDA (300µM) by **(A)** the non-competitive NMDA receptor antagonist, MK-801 (10µM), and the selective NMDA receptor antagonist, D-AP5 (50µM) and **(B)** the broad-spectrum glutamate receptor antagonist, kynurenic acid (KYA) 100µM, 1mM. Each column shows the mean ± SEM for n = 3 cultures. **P<0.01, ***P<0.001 compared to control; ###P<0.001 compared to NMDA 300µM; ++P<0.01 for comparison between the effects of KYA 100µM and KYA 1mM on excitotoxicity induced by NMDA 300µM.

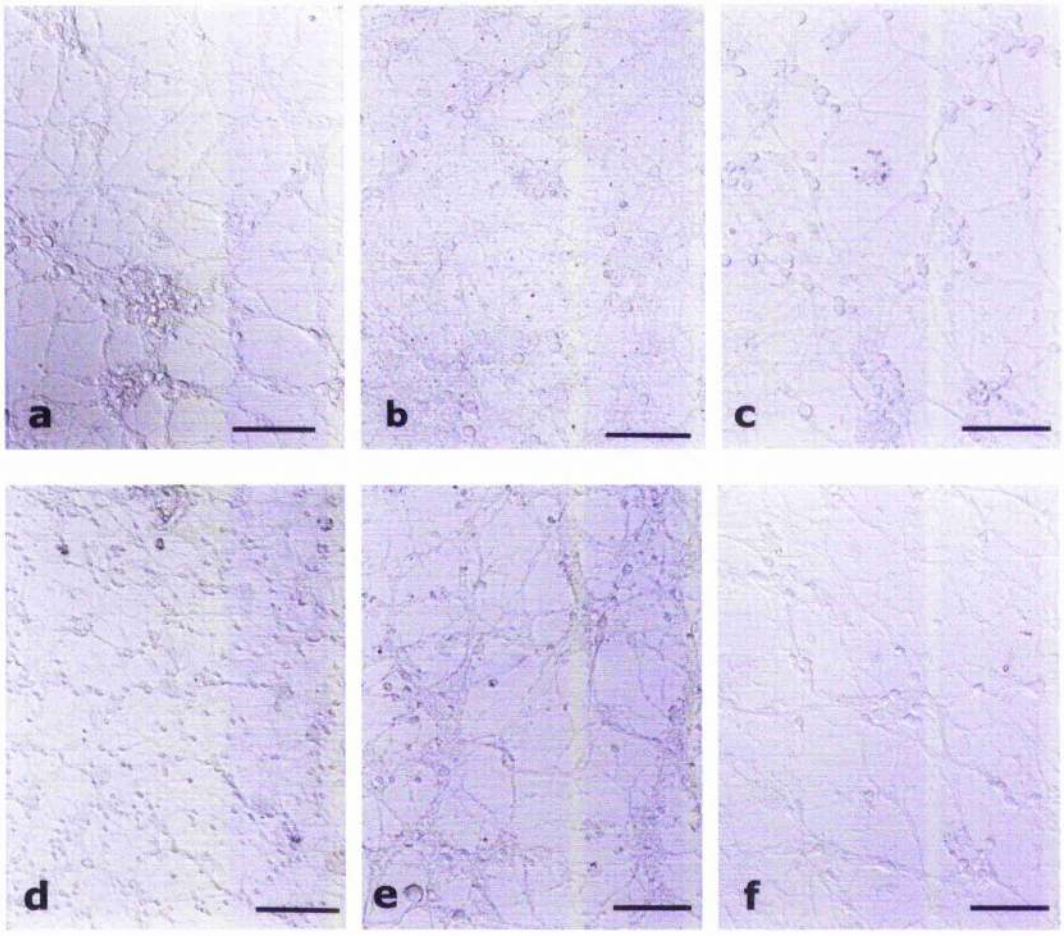


Fig. 4.11. Photomicrographs showing glutamate- and NMDA-induced morphological damage in cerebellar granule neurones and protection by some NMDA receptor antagonists. **(a)** Control **(b)** Glu 300μM **(c)** Glu 300μM + D-AP5 50μM **(d)** Glu 300μM + KYA 100μM **(e)** NMDA 300μM **(f)** NMDA 300μM + D-AP5 50μM. D-AP5 significantly improved glutamate-induced damage to both cell bodies and neurite outgrowth, while kynurenic acid reduced the damage by preserving cell number but not neurite outgrowth. Cultures that were treated with NMDA in the presence of D-AP5 retained a morphology that was identical to that of control cultures. Bar = 50μm.

4.4.2. Mitochondrial poisoning and NMDA receptor activation

In order to verify the extent of mitochondrial involvement in the damage and death of CGNs, the degree of sensitivity of these cultures to mitochondrial impairment was examined using the mitochondrial poison 3-nitropropionic acid (3-NPA). Following 1 or 6 h exposures, 3-NPA reduced CGN viability in a concentration-dependent manner, compared to the control ($P<0.01$, $n = 3$) (Fig. 4.12). While 3-NPA 10μM applied for 1 or 6 h had no effect, the damaging effects produced by each of the higher 3-NPA concentrations (100μM, 1mM) at 1 and 6 h were comparable. An examination of CGN morphology following these treatments showed profound loss of neurones and in some cases some pathological clumping together of cells (Fig. 4.13a-d). In an attempt to understand the potential mechanism(s) of 3-NPA-induced damage, the prospect of a possible involvement of NMDA receptor activation was investigated by testing the selective NMDA antagonist D-AP5 at 50μM against the damaging effect of 3-NPA. This concentration of D-AP5 had no effect on its own on basal viability (102.69% ± 1.00 of the control, $n = 4$). As shown in Fig. 4.14A, 3-NPA 1mM applied for 6 h reduced viability significantly to 59.58% ± 3.01 ($P<0.001$, $n = 3$). In the presence of D-AP5, this effect was completely abolished. Similarly, when tested against damage by another mitochondrial poison, potassium cyanide (KCN), D-AP5 raised viability from 62.85% ± 3.97 for KCN 1mM alone applied for 6 h ($P<0.001$, compared to control, $n = 4$) (Fig. 4.14B) to 89.75% ± 4.79, a level not significantly different from the control. The profound cell loss and damage to neurite outgrowths induced by this KCN concentration are as shown in Fig. 4.13 (a, e).

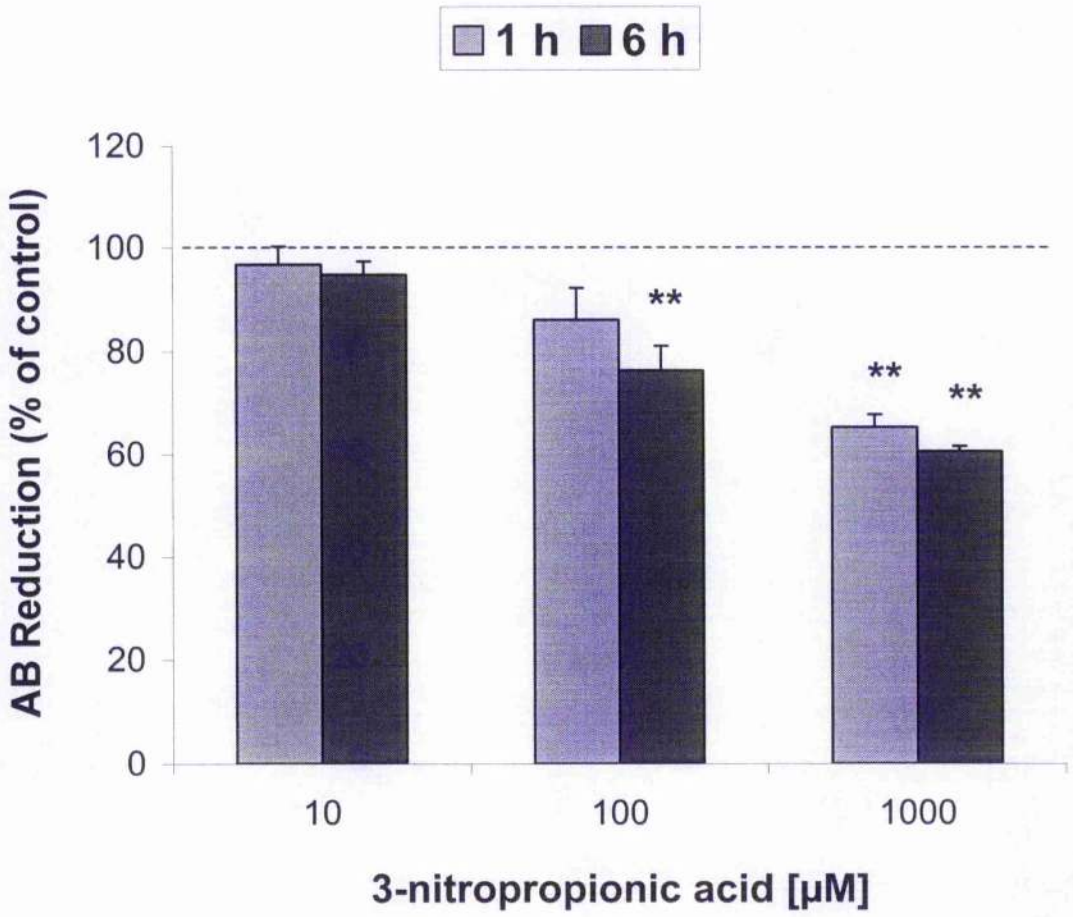


Figure 4.12. Histograms showing the dose-dependent effects on the viability of CGN cultures at 8 div of 1 and 6 h exposures to the mitochondrial poison, 3-nitropropionic acid (3-NPA). Each column shows the mean ± SEM for n = 3 cultures. **P<0.01 compared to control.

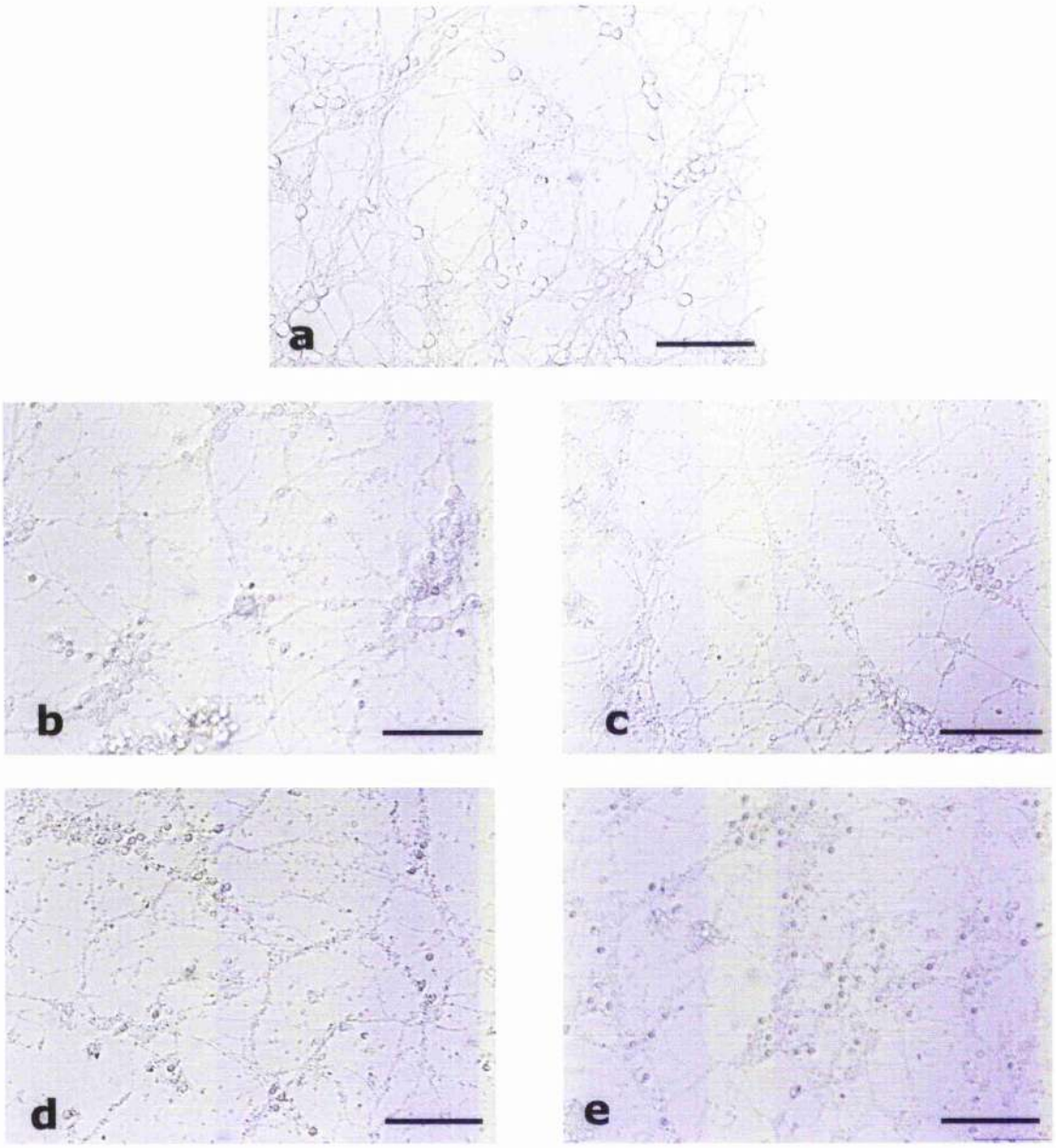


Figure 4.13. Concentration-dependent damaging effects of the mitochondrial poison 3-nitropropionic acid (3-NPA) and the damaging effect of another mitochondrial poison, potassium cyanide (KCN) at 1mM on the morphology of cerebellar granule neurones. **(a)** Control **(b)** 3-NPA 100μM (1 h) **(c)** 3-NPA 100μM (6 h) **(d)** 3-NPA 1mM (6 h) **(e)** KCN 1mM (6 h). Cells that were treated with the poisons **(b-e)** showed massive loss of cells, degeneration of neurite connections, and in some cases pathological clumping together of cells, in contrast to control cultures **(a)** with intact cell bodies and neurite connections. Bar = 50μm.

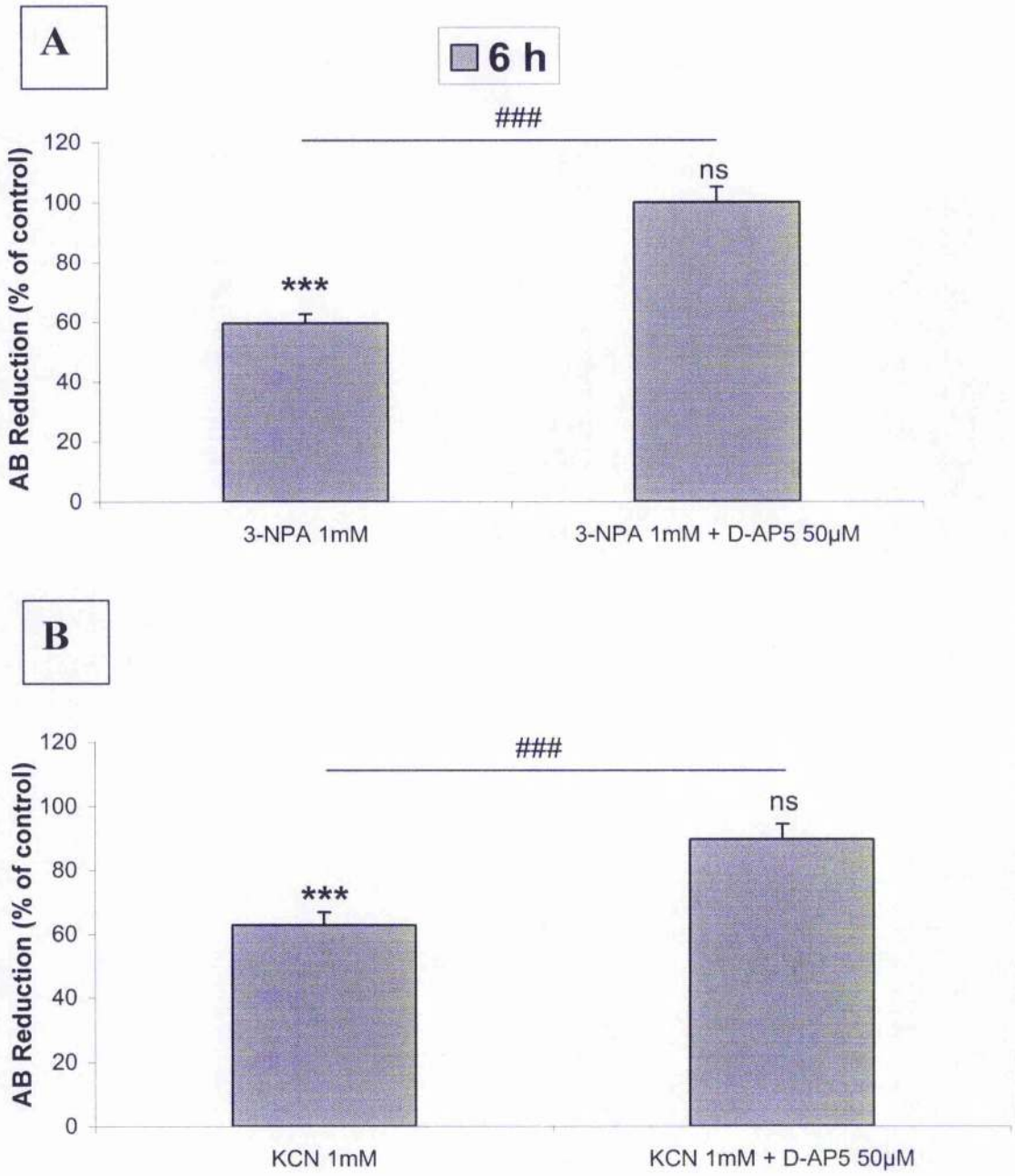


Figure 4.14. Histograms showing the effects of the selective NMDA receptor antagonist, D-AP5 (50µM), on the reduction in CGN viability induced by 6 h of exposures to the mitochondrial poisons **(A)** 3-nitropropionic acid (3-NPA) 1mM and **(B)** Potassium cyanide (KCN) 1mM. Each column shows the mean ± SEM for **(A)** n = 3 or **(B)** n = 4 cultures. ***P<0.001, ns = non-significant compared to control; ###P<0.001 compared to **(A)** 3-NPA 1mM or **(B)** KCN 1mM.

4.4.3. Mechanisms of glutamate-induced excitotoxic damage

The relative involvements of the induction of caspase-3, poly (ADP-ribose polymerase) and the process of membrane permeability transition in glutamate-mediated excitotoxicity were examined.

4.4.3.1. Role of caspase-3-mediated apoptosis

The caspase-3 inhibitor Z-DEVD at 40μM had no effect on its own on CGN viability. Exposure to glutamate 300μM for 1 h reduced viability significantly to 66.95% ± 3.15 of the control ($P<0.01$, n = 4) (Fig. 4.15). This glutamate effect was significantly attenuated (24.34%) in the presence of Z-DEVD 40μM to a viability level of 91.29% ± 7.57 ($P<0.01$ compared to glutamate alone), which was comparable to the untreated control.

4.4.3.2. Induction of membrane permeability transition (MPT)

The ability of the membrane permeability transition pore blocker cyclosporin A at 0.5, 1 and 10μM in preventing excitotoxic damage by glutamate was verified. When added alone, these concentrations gave viability values of 94.87% ± 6.92, 99.57% ± 11.24 and 97.63% ± 3.66, respectively (n = 3), which were not statistically different from the control. CsA at 0.5 and 1μM completely blocked the significantly reduced neuronal viability (69.97% ± 2.08, $P<0.001$, n = 4) (Fig. 4.16) resulting from Glu 300μM treatment, restoring levels to control values. Interestingly, CsA at 10μM also completely blocked the glutamate effect, but in this case improving viability to 131.35% ± 3.88, a level significantly higher than the control ($P<0.001$). When CsA at 1μM was tested against NMDA-induced reduction in CGN viability, it was equally capable of protecting the neurones against NMDA, raising this value to match the control at 103.48% ± 4.35 ($P<0.001$ vs. NMDA alone, n = 3), in contrast to NMDA 300μM alone which reduced viability to 64.44% ± 4.05 of the control ($P<0.001$).

4.4.3.3. Involvement of poly (ADP-ribose) polymerase (PARP)

Nicotinamide 1mM (a PARP inhibitor), while having no effect on its own, raised viability significantly ($P<0.01$) from 65.36% ± 1.50 for glutamate alone to 75.73% ± 2.38 when co-administered with glutamate for 1 h (n = 3) (Fig. 4.17A). The very potent PARP inhibitor DPQ at 10μM had no effect on basal neuronal viability when applied alone (n = 5) (Fig. 4.17B). Glu 300μM reduced viability to 60.76% ± 6.65 of the control ($P<0.01$). In the

presence of DPQ 10μM, this glutamate effect was significantly (P<0.01) attenuated (27.52%) to 88.28% ± 8.22, a level comparable to the control.

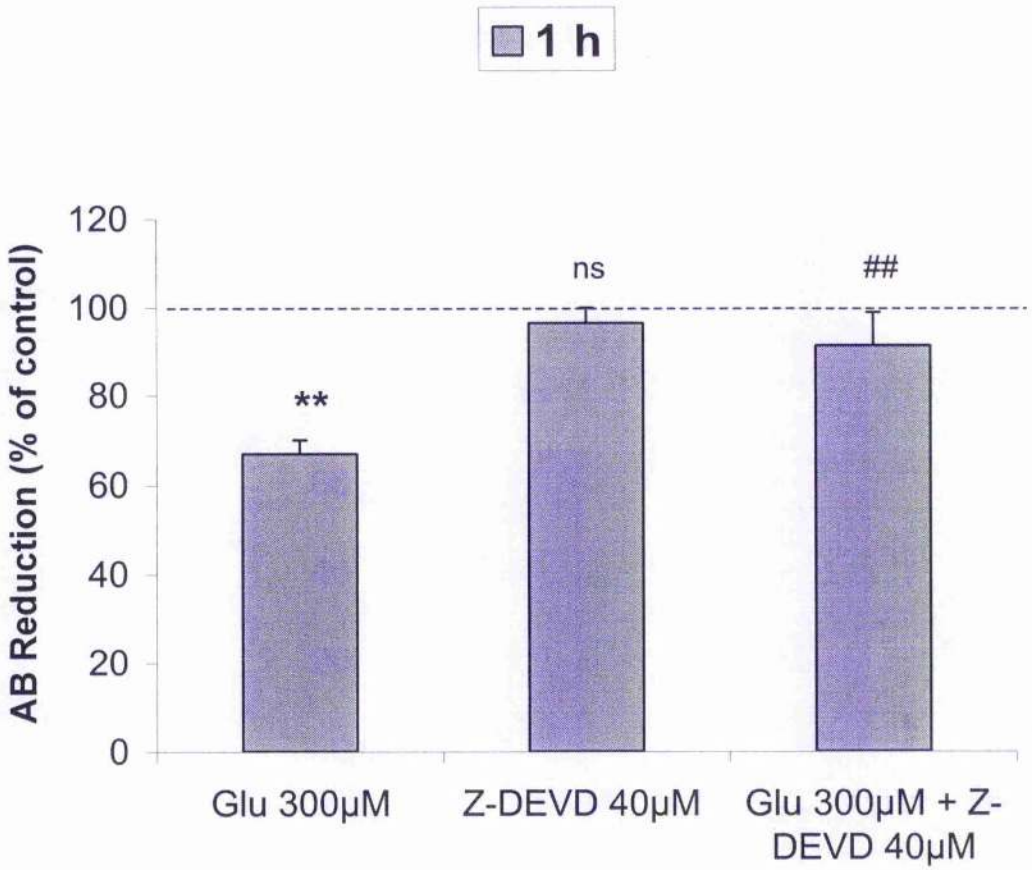


Figure 4.15. Histogram showing the effect of the caspase-3 inhibitor, Z-DEVD-fmk 40μM, on the neurotoxicity mediated by Glu 300μM in CGN cultures. Each column represents the mean ± SEM (n = 4 cultures). **P< 0.01, ns = non-significant compared to untreated control; ## P<0.01 for the protective effect of the inhibitor vs. cultures treated with Glu 300μM in the absence of the inhibitor.

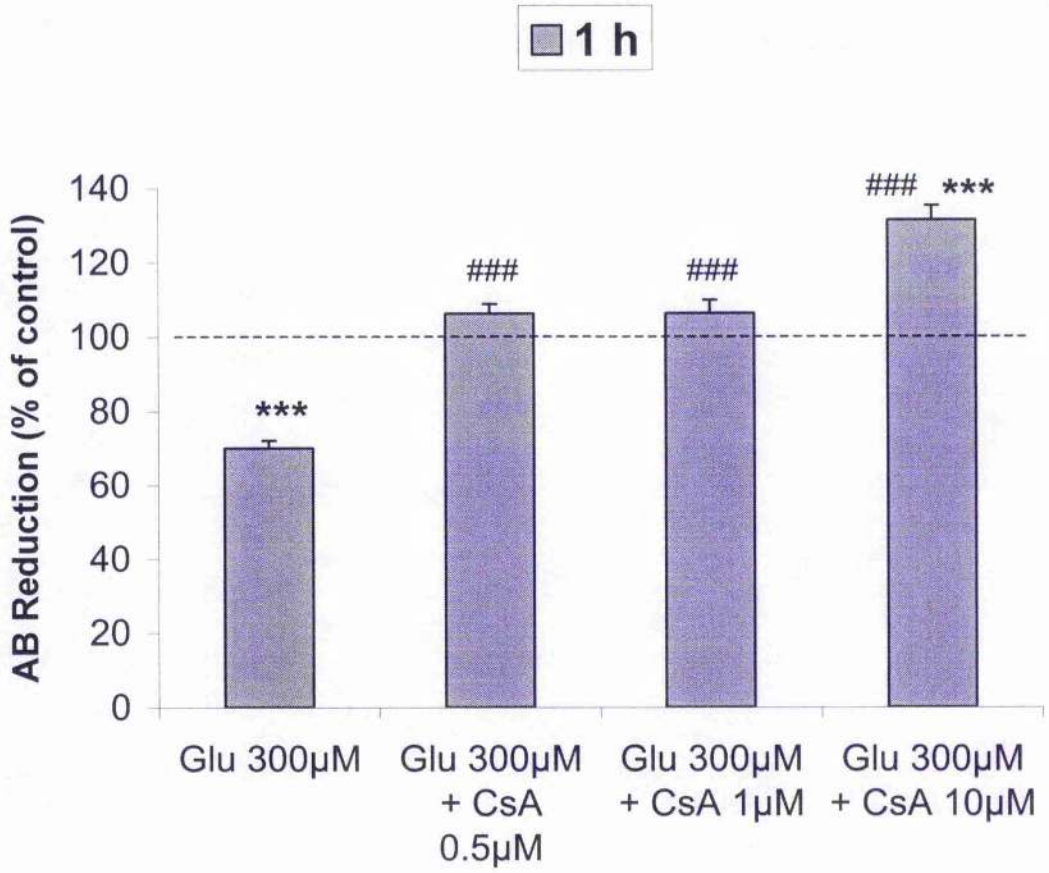


Figure 4.16. Histogram showing the effects of cyclosporin A (0.5, 1, 10µM), a membrane permeability transition pore blocker, on glutamate-mediated neurotoxicity in CGN cultures. Each column shows the mean ± SEM for n = 4 cultures. ***P<0.001 compared to control; ###P<0.001 compared to Glu 300µM.

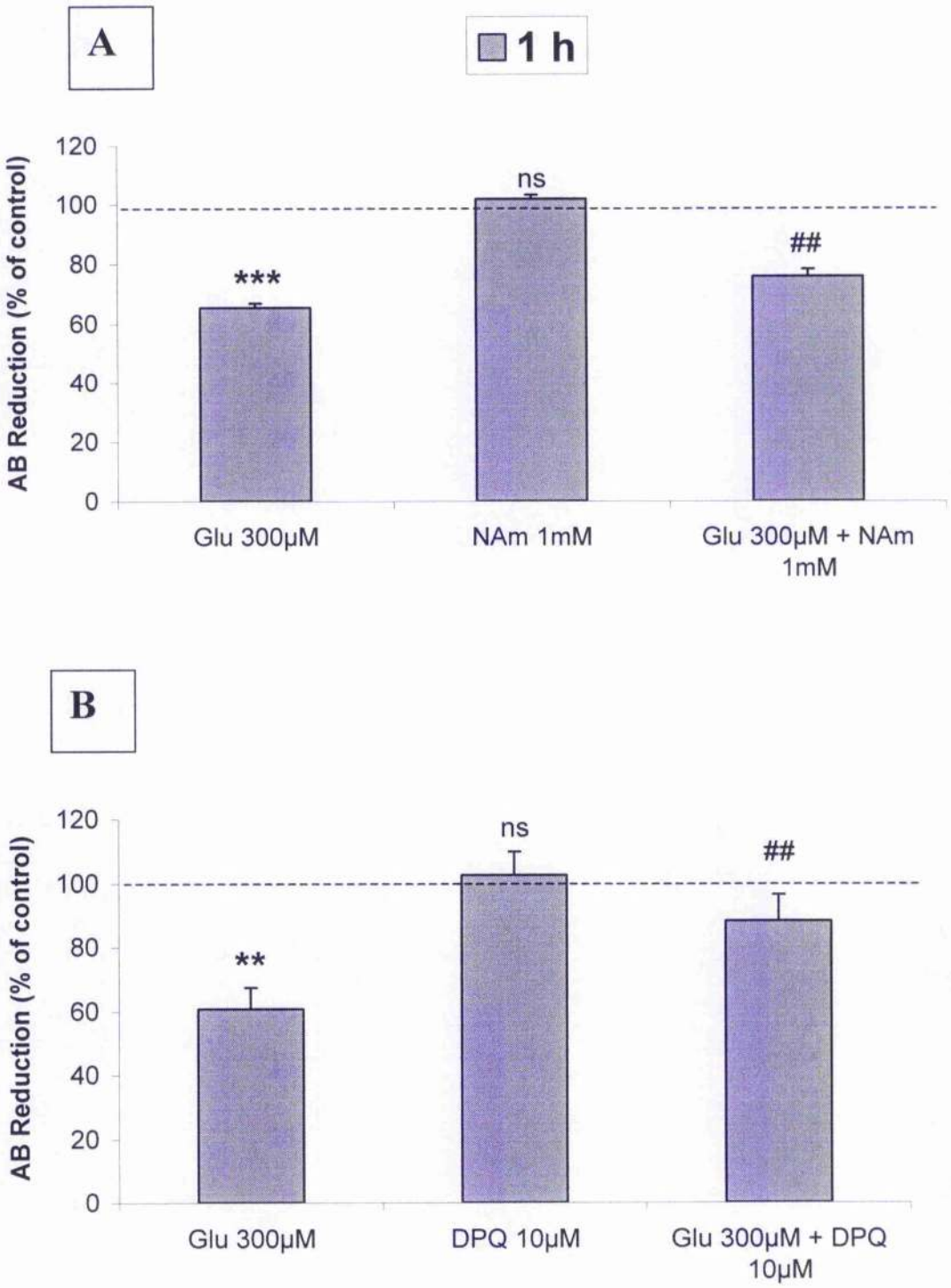


Figure 4.17. Histograms showing the effects on glutamate-mediated neurotoxicity in CGN cultures of the poly (ADP-ribose) polymerase (PARP) inhibitors **(A)** nicotinamide (NAm) 1mM and **(B)** DPQ 10µM. Each column shows the mean ± SEM for **(A)** n = 3 or **(B)** n = 5 cultures. **P< 0.01, ***P<0.001, ns = non-significant compared to control; ##P<0.01 compared to Glu 300µM.

4.4.4. Adenosine receptors and protection against glutamate-induced excitotoxicity

The roles of adenosine A_1 and A_{2A} receptor ligands in the modulation of glutamate-induced excitotoxic damage in the cerebellar granule neurones were examined.

4.4.4.1. A_1 receptor ligands and modulation of excitotoxic damage

The selective A_1 receptor agonist CPA at 100nM had no effect on basal viability. Application of glutamate 300μM reduced viability significantly to 66.02% ± 1.23 ($P<0.001$, n = 4) (Fig. 4.18A). Complete protection was afforded by CPA against this damage, raising viability to 95.16% ± 1.78 of the control. While the selective A_1 receptor antagonist DPCPX (100nM) did not have any effect on its own, it tended to exacerbate the neuronal damage induced by glutamate (n = 3) (Fig. 4.18B). Addition of glutamate 300μM alone decreased viability to 74.48% ± 4.59 ($P<0.001$). In the presence of DPCPX, the addition of glutamate lowered viability a further 8.34% to 66.14% ± 2.76, although the difference was not statistically significant.

4.4.4.2. A_{2A} receptor ligands and excitotoxic damage

Exposure to glutamate 300μM lowered neuronal viability significantly to 73.06% ± 3.99 ($P<0.05$, n = 4) (Fig. 4.19A). The selective A_{2A} receptor agonist, CGS21680 at 100nM neither had any significant effect on its own nor modified the damage caused by glutamate. On the other hand, although without effect on its own, the selective A_{2A} antagonist ZM241385 at 100nM significantly attenuated the damage caused by glutamate ($P<0.05$, n = 3) (Fig. 4.19B), restoring viability to 86.08% ± 8.02 from 62.19% ± 4.11 obtained in the presence of glutamate alone.

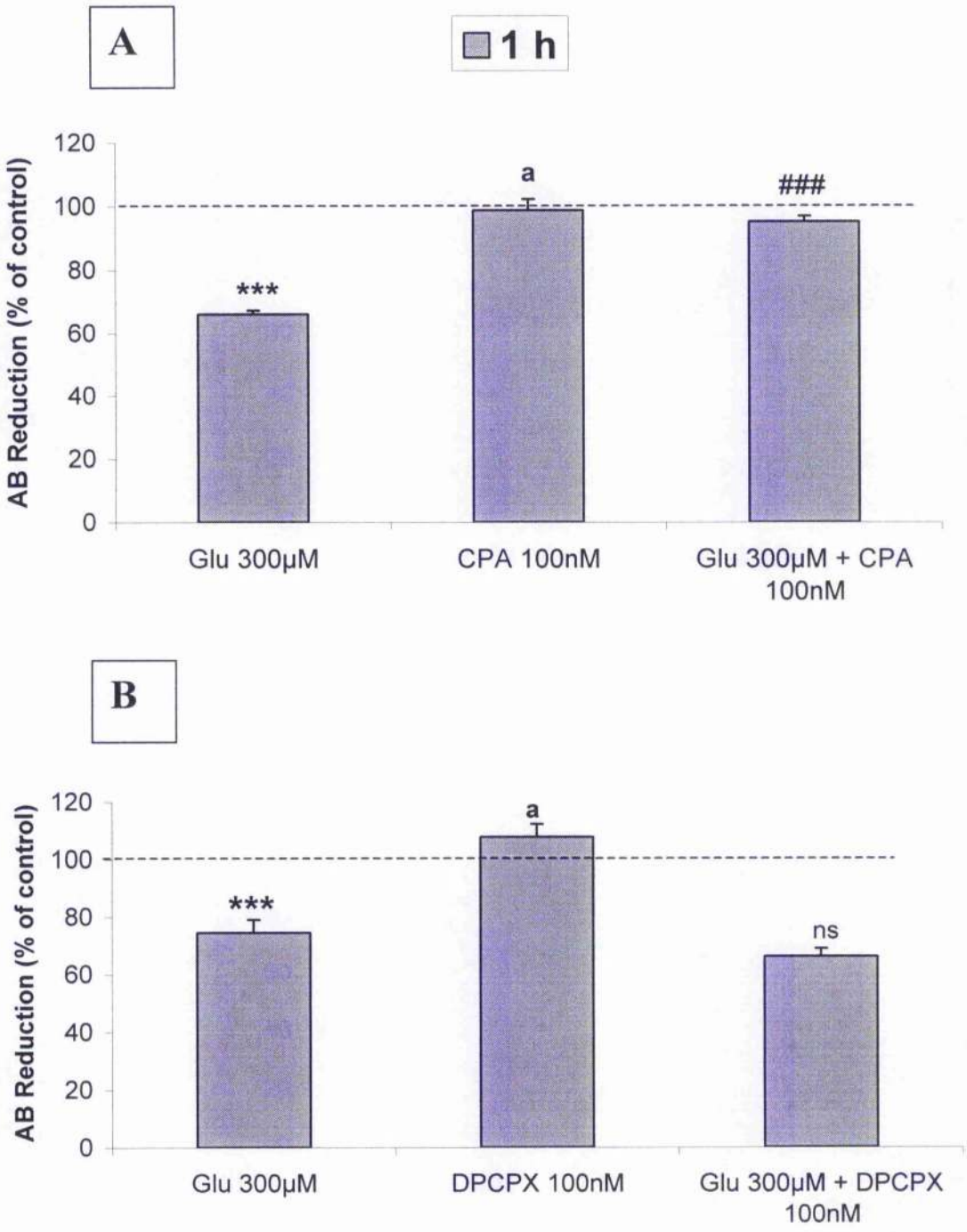


Figure 4.18. Histograms showing **(A)** the protective effect of the A_1 receptor agonist, CPA (100nM) and **(B)** the lack of significant effect of the A_1 receptor antagonist, DPCPX (100nM) against glutamate-induced reduction in the viability of CGN cultures. Each column represents the mean ± SEM for **(A)** n = 4 or **(B)** n = 3 cultures. ***P< 0.001, **a** = non-significant compared to control; ### P<0.001, ns = non-significant compared to Glu 300µM.

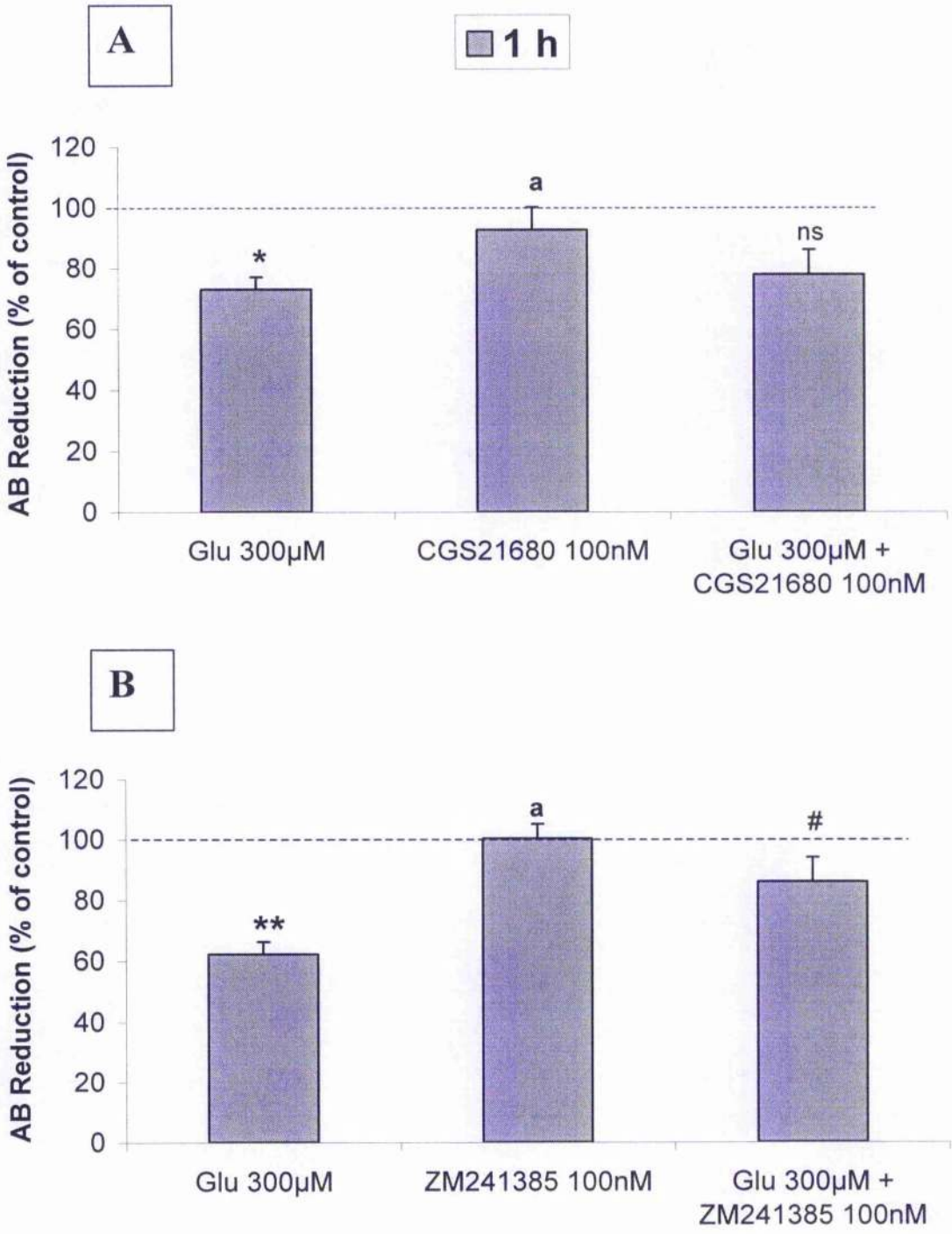


Figure 4.19. Histograms showing **(A)** the lack of significant effect of the A_{2A} receptor agonist, CGS21680 (100nM) and **(B)** the protective effect of the A_{2A} receptor antagonist, ZM241385 (100nM) against glutamate-induced reduction in the viability of CGN cultures. Each column represents the mean ± SEM for **(A)** n = 4 or **(B)** n = 3 cultures. *P< 0.05, **P< 0.01, **a** = non-significant compared to control; #P<0.05, ns = non-significant compared to Glu 300µM.

4.5. Reactive oxygen species (ROS) and neuronal viability

4.5.1. Hydrogen peroxide and the viability of cerebellar granule neurones

Cerebellar granule neurones were exposed to concentrations of H_2O_2 ranging from 10 to 400μM in full (serum-containing) medium for 0.25, 1, 3 and 6 h. In order to verify whether the duration of recovery could influence treatment outcomes, recovery durations of 6 and 24 h were employed. Cultures were all restored after treatment to full culture medium. 6 h of recovery was taken as the period immediately after treatment with H_2O_2 for which cultures were incubated with alamar blue (AB) before determining viability by plate reading. Recovery for 24 h was for the period allowed between the end of treatment and the determination of viability, including the last 6 h for which cultures were incubated with AB before plate reading. Fig. 4.20A shows the results obtained for 6 h of recovery while Fig. 4.20B illustrates the outcomes for 24 h of recovery. With a recovery period of 6 h, H_2O_2 reduced the viability of cerebellar granule neurones in a concentration- and time-dependent manner, compared to the controls and all effects were significant ($P<0.01$, $n = 4 - 7$). It was striking that even H_2O_2 10μM applied for as brief as 0.25 h induced a very significant (nearly 31%) reduction in viability to 69.45% ± 3.09 of the control. With a longer recovery period of 24 h, neuronal survival was significantly poorer ($P<0.05$ or $P<0.01$, $n = 4 - 7$). After short (0.25 h) exposures to H_2O_2 10 and 30μM, viability was still greater than 50%. However, following longer treatments - at all concentrations - the cultures showed practically no viability. Hydrogen peroxide caused progressive loss of cells and elimination of neurite processes (Fig. 4.21a-e), an effect that was more extensive with the longer (24 h) period of recovery (cf. 4.21a, b and c). The 24 h recovery interval was adopted for all subsequent experiments.

4.5.1.1. Prevention by catalase of H_2O_2 effects

The antioxidant enzyme catalase, at an activity of 100U/ml, was able to prevent completely the profound deleterious effects of H_2O_2 (30μM) when tested for 1 or 6 h ($n = 4$) (Fig. 4.22). At 1 h, it improved viability from 13.86% ± 3.04 for peroxide alone to 109.66% ± 7.61 ($P<0.001$) when combined with peroxide, and at 6 h from 11.98% ± 2.34 to 108.52% ± 5.17 ($P<0.001$). Catalase was able to improve significantly the loss of cells and neurite outgrowth induced by H_2O_2 (Fig. 4.21a, c and f), so that the morphology of H_2O_2-treated cultures in the presence of catalase was more similar to the control than to the H_2O_2-challenged phenotype. In addition to its protective action against peroxide, catalase was

also able to enhance significantly on its own the basal viability of CGN cultures to 130.73% ± 3.02 ($P<0.001$) and 125.00% ± 4.92 ($P<0.01$) of the control, for 1 and 6 h, respectively.

4.5.1.2. Aspirin (acetylsalicylic acid, ASA) and hydrogen peroxide

Catalase has been reported to induce cyclooxygenase-2 (COX-2) (Litvinov and Turpaev, 2004). Since aspirin blocks cyclooxygenases, its ability to modify the effect of H_2O_2 was examined. As shown in Fig. 4.23, aspirin at 1mM had no effect on basal viability. H_2O_2 (30μM) applied for 6 h lowered viability to 50.95% ± 7.68 of the control ($P<0.001$, $n = 4$). In the presence of ASA 1mM, H_2O_2 induced-damage improved nearly 11% to 61.82% ± 2.90 of the control, but this difference was not statistically significant.

4.5.1.3. Hydrogen peroxide (H_2O_2), copper and mannitol

Interactions between H_2O_2, copper (II) ion and mannitol were investigated for 1 and 6 h. Surprisingly, H_2O_2 (5μM) induced a significant ($P<0.05$) reduction in viability at 1, but not at 6 h ($n = 8$) (Fig. 4.24A). Copper (II) ion, generated from $CuSO_4$ (0.5mM), induced a time-dependent reduction of CGN viability, lowering it significantly at 6 h to 48.42% ± 9.23 of the control ($P<0.001$). Co-administration of H_2O_2 (5μM) and $CuSO_4$ (0.5mM) resulted in significant reduction of viability at 1 and 6 h to 59.63% = 9.26 ($P<0.01$) and 44.63% ± 8.98 ($P<0.001$) of the control. The reduction in the presence of the two agents at 1 h was not statistically different from the reduction in the presence of either of them alone, but at 6 h, reduction in viability by the two agents combined was significantly more than the reduction caused by H_2O_2 alone ($P<0.001$), though still not different from the effect obtained in the presence of $CuSO_4$ alone. The cultures became shrunken after treatments with the two agents, with extensive loss of outgrowths (Fig. 4.25a-c).

Through the Fenton reaction, the hydroxyl radical is generated by H_2O_2 in the presence of a transition metal like copper or iron. Mannitol is known to scavenge the formed hydroxyl radical, thus preventing its damaging effect. However, the current results contrasted with this expectation, as mannitol manifested a tendency to potentiate rather than alleviate the effect of co-administration of peroxide and copper. Mannitol at 50mM had no effect on its own on neuronal viability ($n = 4$) (Fig. 4.24B). Hydrogen peroxide and $CuSO_4$ when added together reduced viabilities at 1 and 6 h to 78.13% ± 2.47 ($P>0.05$) and 48.39% ± 10.79 ($P<0.001$), respectively. In the presence of mannitol, these values were further reduced to 59.21% ± 12.40 and 28.99% ± 7.29 ($P>0.05$ compared to damage by a combination of

H_2O_2 and $CuSO_4$ in the absence of mannitol). This further decrease in viability induced by mannitol was consistent with its exacerbation of morphological damage by H_2O_2 and copper (II) ion in these cultures (Fig. 4.25a, c, d).

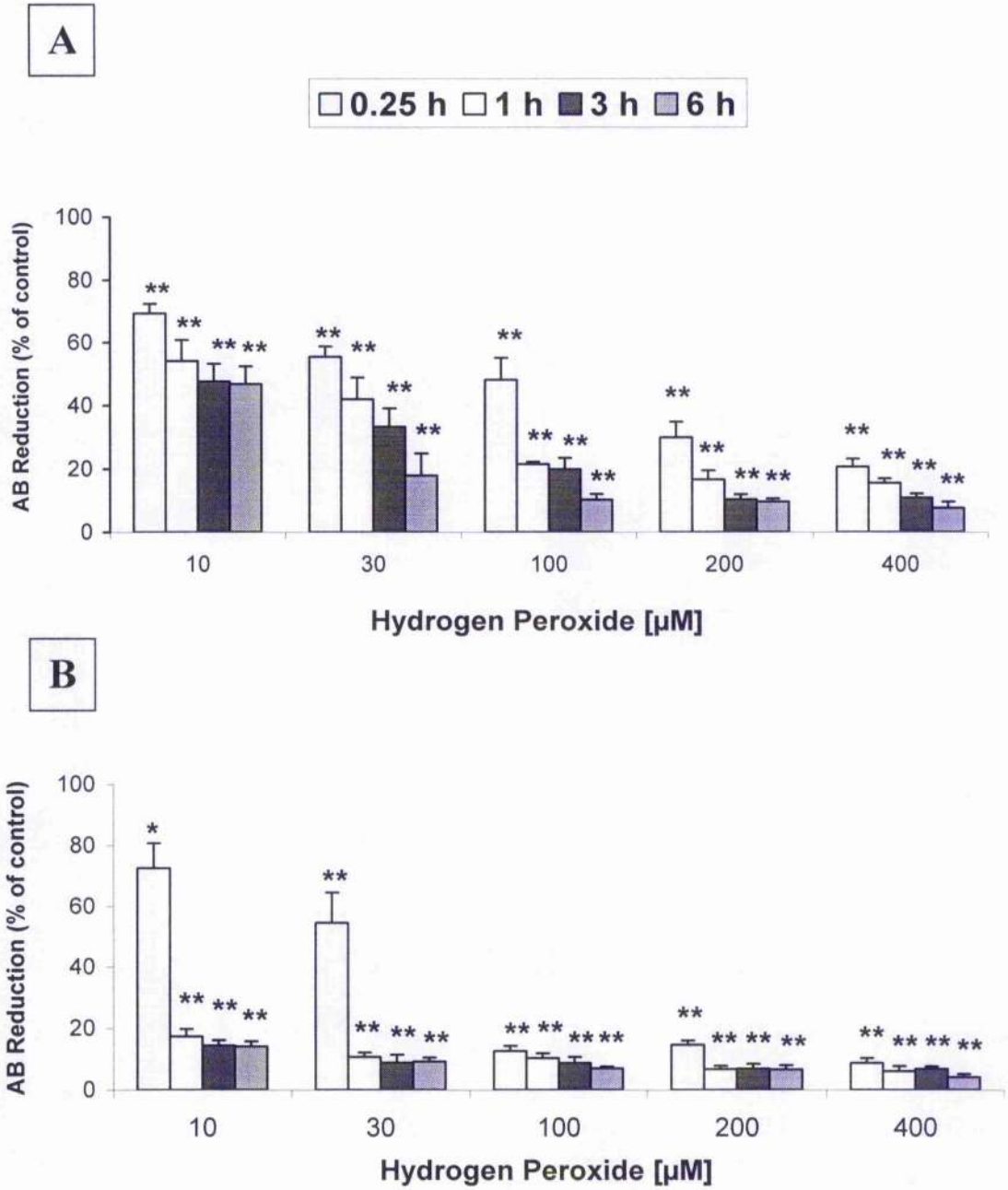


Figure 4.20. Histograms showing the effects of hydrogen peroxide (10µM - 400µM) on CGN cultures for 0.25, 1, 3 and 6 h exposures at 8 div when viability determination was done **(A)** 6 h and **(B)** 24 h after the end of treatment period. The 6 h recovery period was the duration immediately following treatment for which alamar blue was incubated with the cultures before determination of viability, while the 24 h recovery period was the duration between the end of treatment and the determination of viability, inclusive of the final 6 h during which alamar blue was incubated with the cultures before plate reading. Each column represents the mean ± SEM for n = 4 - 7 cultures. *P<0.05, **P< 0.01 compared to untreated control.

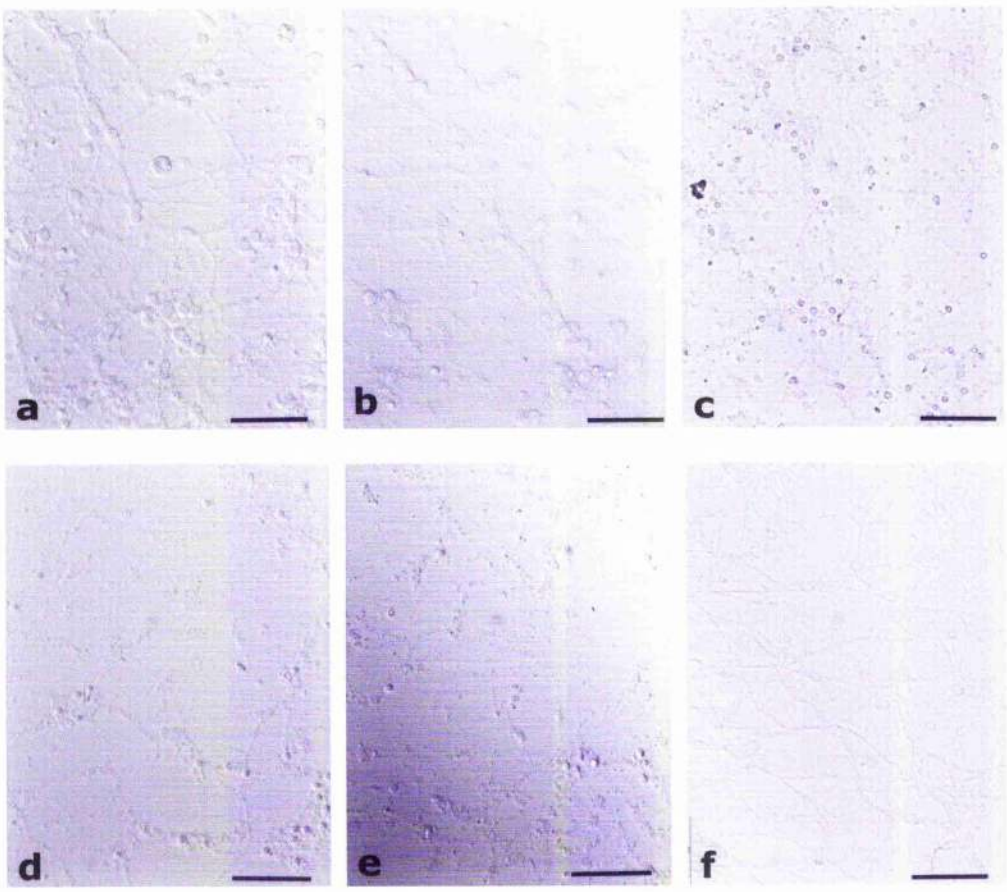


Figure 4.21. Photomicrographs showing the concentration- and time-dependent neurotoxic effects of hydrogen peroxide (H_2O_2) on the morphology of cerebellar granule cells when recovery duration was 24 h (except otherwise stated), and the protection by catalase. **(a)** Control **(b)** H_2O_2 10μM (3 h) with 6 h recovery **(c)** H_2O_2 10μM (3 h) **(d)** H_2O_2 30μM (1 h) **(e)** H_2O_2 30μM (6 h) **(f)** H_2O_2 30μM + CAT 100U/ml (6 h). Progressive loss of cells and neurite processes was evident in H_2O_2-treated cultures **(b-f)**. Damage was more pronounced with 24 h of recovery **(c)** than with 6 h **(b)**. In the presence of catalase, the phenotype of H_2O_2-treated cultures **(f)** was more similar to that of control cultures **(a)** than to that of cultures treated with H_2O_2 alone **(e)**. Bar = 50μm.

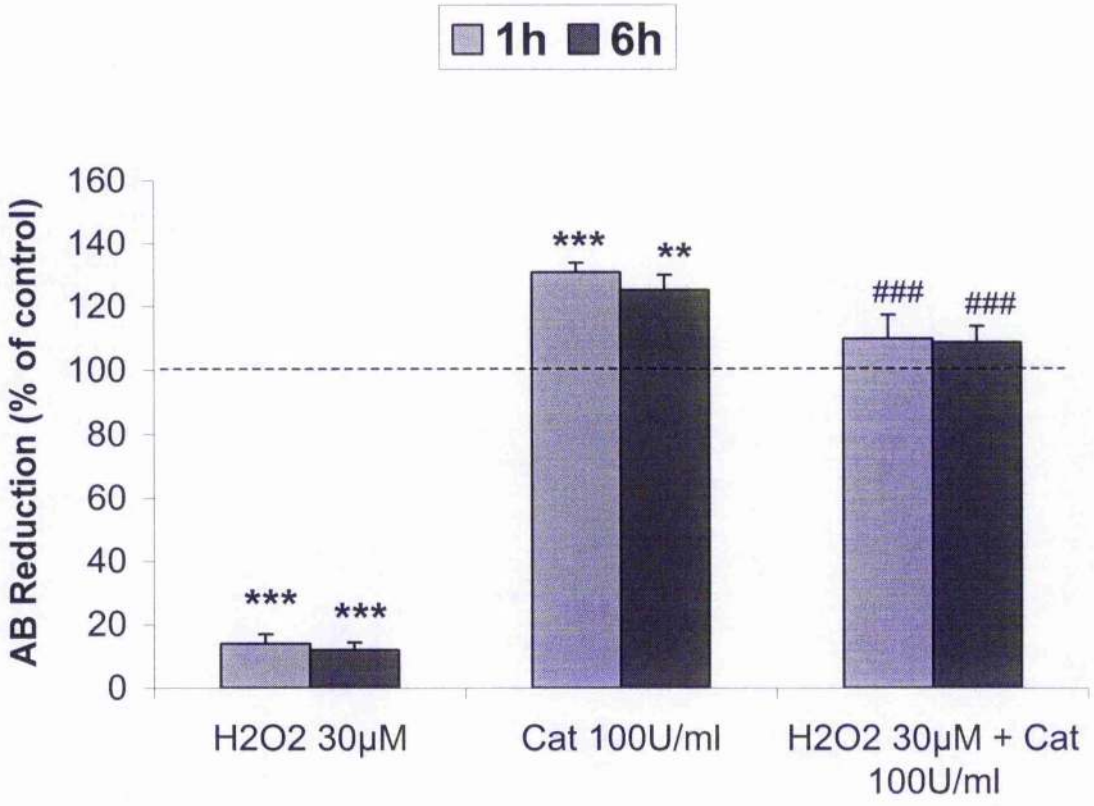


Figure 4.22. Histograms showing the effects of catalase 100U/ml on the viability of CGN cultures and the hydrogen peroxide-induced cytotoxicity in these cultures. Each column shows the mean ± SEM for n = 4 cultures. **P<0.01, ***P<0.001 compared to control; ###P<0.001 compared to corresponding H_2O_2 30µM.

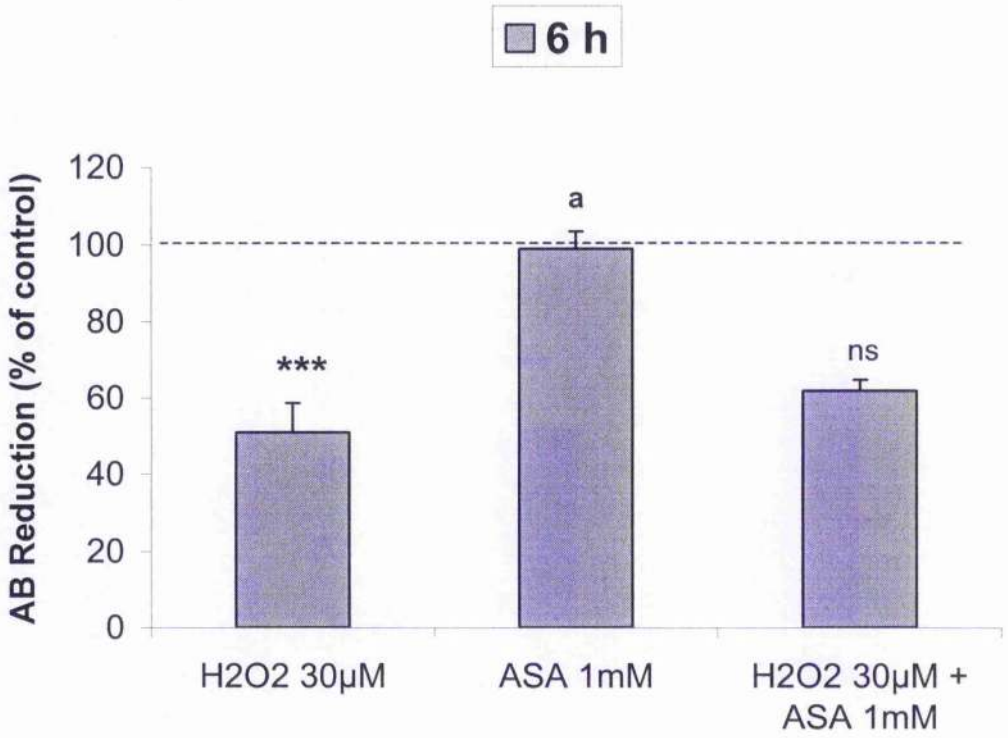


Figure 4.23. Histograms showing the effects of aspirin (ASA) 1mM on hydrogen peroxide-induced cytotoxicity in CGN cultures. Each column shows the mean ± SEM for n = 4 cultures. ***P<0.001, **a** = non-significant compared to control; ns = non-significant compared to H_2O_2 30µM.

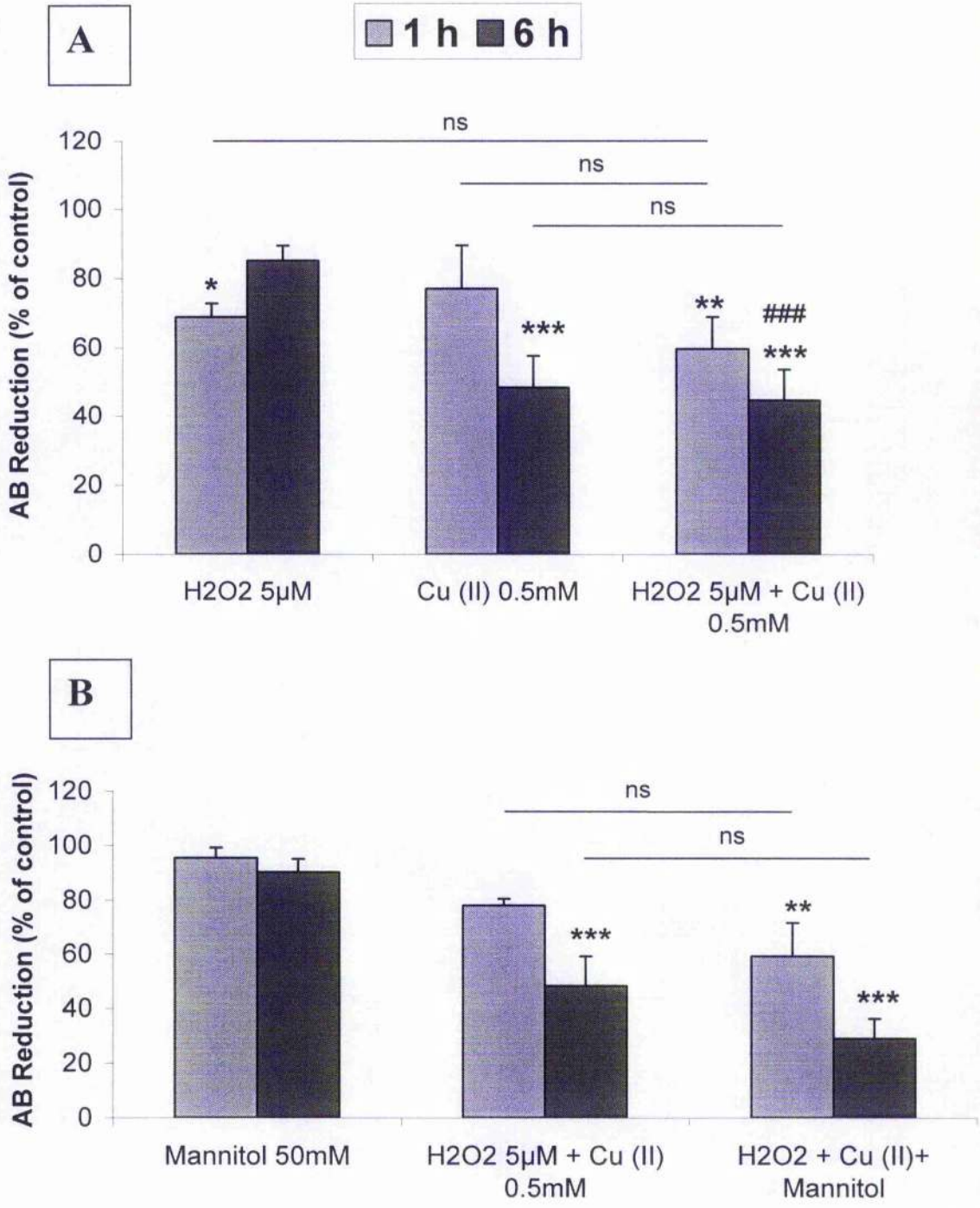


Figure 4.24. Histograms showing the effects on the viability of CGN cultures of **(A)** hydrogen peroxide (5µM) and copper (II) ion (0.5mM), and **(B)** hydrogen peroxide (5µM) and copper (II) ion (0.5mM) in the presence of mannitol 50mM. Each column shows the mean ± SEM for **(A)** n = 8 or **(B)** n = 4 cultures. *P<0.05, **P<0.01, ***P<0.001 compared to control; ###P<0.001 compared to H_2O_2 5µM, ns = non-significant.

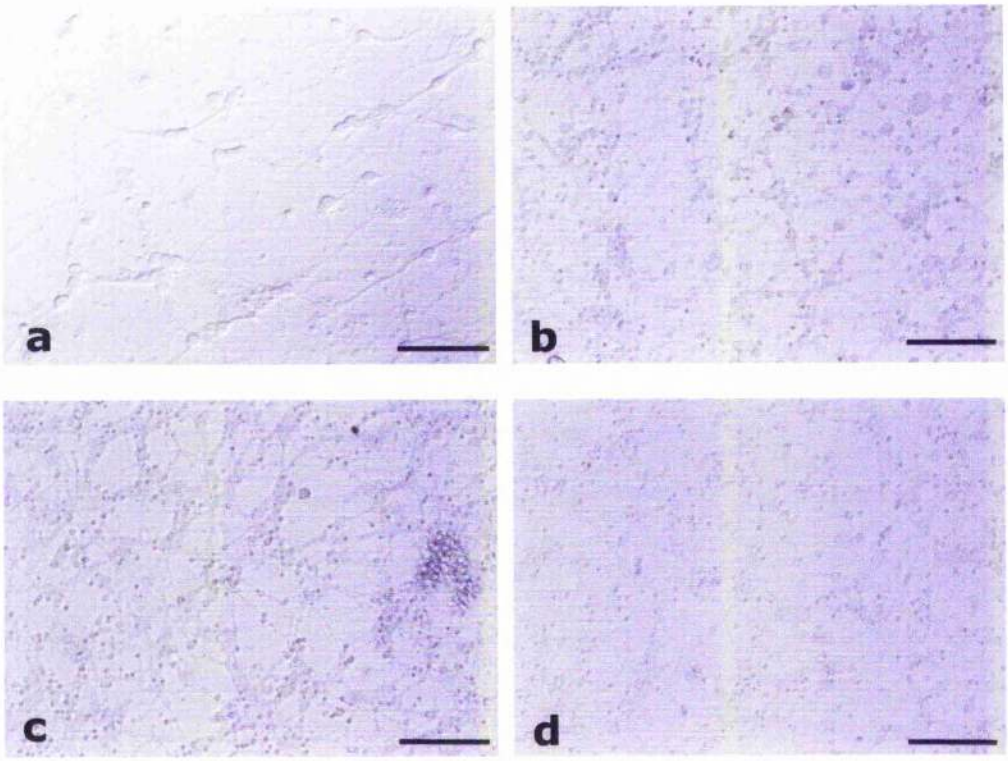


Figure 4.25. Morphological effects of simultaneous addition of hydrogen peroxide and copper (II) ion for 1 or 6 h on the cerebellar granule neurones and the exacerbation of this damage in the presence of the hydroxyl radical scavenger mannitol. **(a)** Control **(b)** H_2O_2 5μM + Cu^{2+} 0.5mM (1 h) **(c)** H_2O_2 5μM + Cu^{2+} 0.5mM (6 h) **(d)** H_2O_2 5μM + Cu^{2+} 0.5mM + Mannitol 50mM (6 h). Hydrogen peroxide and copper (II) ion caused significant damage to neuronal morphology when applied together for 6 h **(c)**, but the damage was more profound in the presence of mannitol **(d)**. Bar = 50μm.

4.5.2. Mechanisms of H_2O_2-induced neuronal damage in the CGNs

The involvements of caspase-3, PARP and the event of membrane permeability transition (MPT) in the H_2O_2-mediated damage and death of CGNs were examined.

4.5.2.1. Induction of caspase-3 and PARP

Basal CGN viability in the presence of the caspase-3 inhibitor Z-DEVD-fmk (40μM) did not differ from the control at 0.25 or 1 h, with values of 96.63% ± 2.59 and 103.17% ± 1.58, respectively (n = 4). Similarly, the PARP inhibitor DPQ (10μM) had no effect when applied on its own, giving viability values of 102.57% ± 4.51 and 103.53% ± 4.47 (n = 4) at 0.25 and 1 h. At H_2O_2 10μM, 0.25 and 1 h exposures reduced viability significantly to 77.89% ± 3.45 and 73.09% ± 7.72 of the control, respectively ($P<0.01$, n = 4) (Fig. 4.26A). While Z-DEVD-fmk failed to alleviate these effects, DPQ elicited significant improvements (comparable with the controls) to 91.88% ± 4.13 ($P<0.05$) and 101.57% ± 3.42 ($P<0.01$) of the control. A higher H_2O_2 concentration of 100μM decreased viability to 32.57% ± 3.33 ($P<0.001$) at 0.25 h and to 6.07% ± 1.59 ($P<0.001$) at 1 h (n = 4) (Fig. 4.26B). Z-DEVD alleviated the peroxide damage at 0.25 h to 55.81% ± 1.61 ($P<0.001$) whereas with DPQ, the damage was improved to 58.85% ± 3.16 ($P<0.001$). However, neither of these agents was able to improve the damage caused by 1 h exposure to H_2O_2 100μM.

4.5.2.2. Alleviation of H_2O_2 damage by cyclosporin A

Cyclosporin A (CsA) when added to the CGN cultures at 0.5 and 10μM for 1 h had no significant effects (92.29% ± 5.03 and 93.97% ± 3.05 of the control, respectively; n = 3). At 1 h, the H_2O_2-mediated reduction of CGN viability to 67.35% ± 3.77 of the control ($P<0.001$, n =3) (Fig. 4.27A) was potentiated in the presence of CsA 0.5μM to 55.62% ± 2.37 ($P<0.05$), but improved in the presence of CsA 10μM to 88.05% ± 5.32 ($P<0.01$). Exposure to 100μM of peroxide for 1 h lowered viability very significantly to 12.04% ± 0.63 of the control ($P<0.001$, n = 3) (Fig. 4.27B). Cyclosporin A at 0.5μM had no effect on this damage but at 10μM ameliorated it to 28.51% ± 5.87 of the control ($P<0.01$).

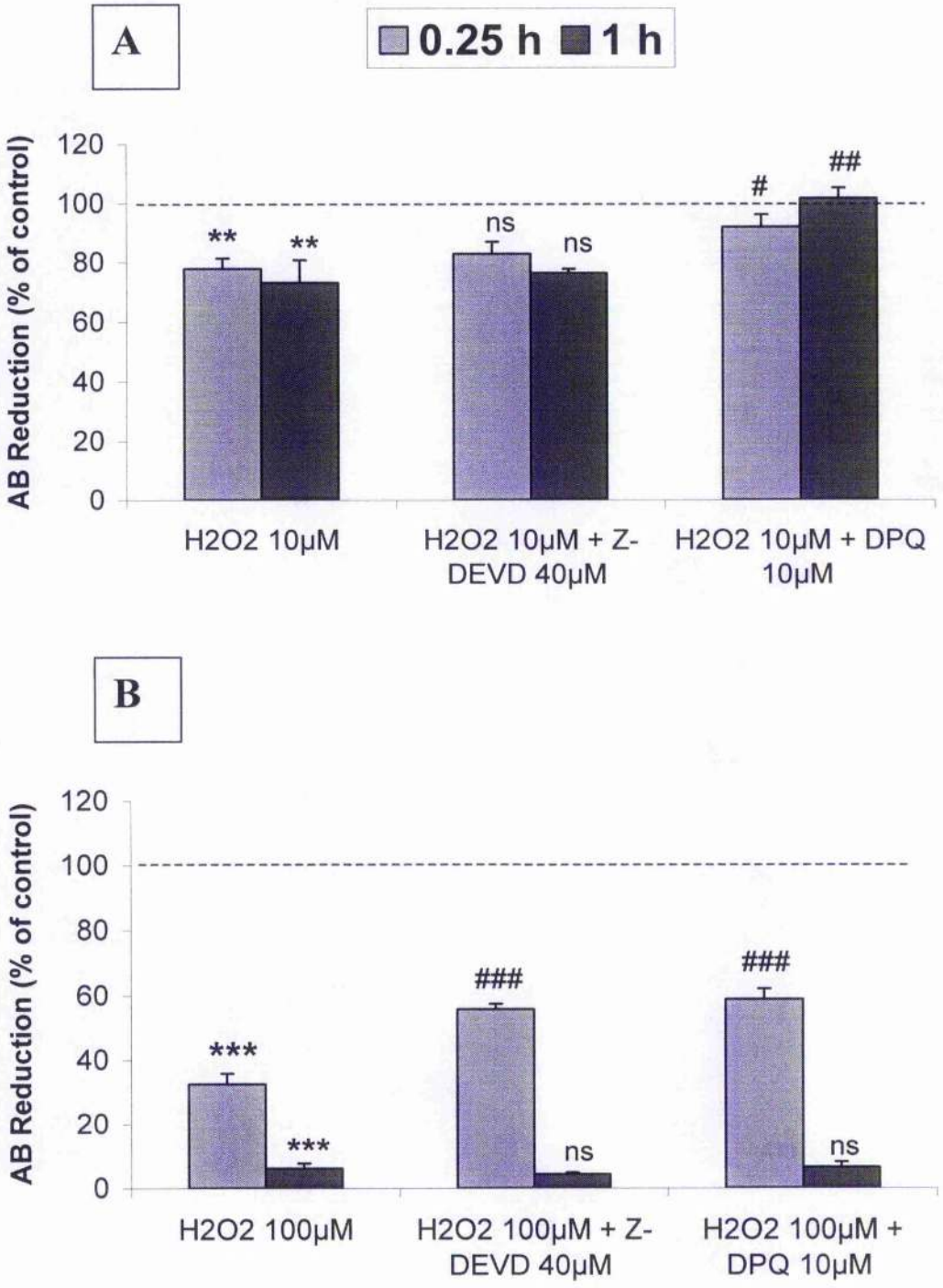


Figure 4.26. Histograms showing the effects of the caspase-3 inhibitor, Z-DEVD-fmk (40µM), and the PARP inhibitor, DPQ (10µM), on the neurotoxicity mediated by **(A)** H_2O_2 10µM and **(B)** H_2O_2 100µM in CGN cultures. Each column represents the mean ± SEM for n = 4 cultures. **P< 0.01, ***P< 0.001 compared to control; #P<0.05, ##P<0.01, ###P<0.001, ns = non-significant compared to corresponding H_2O_2.

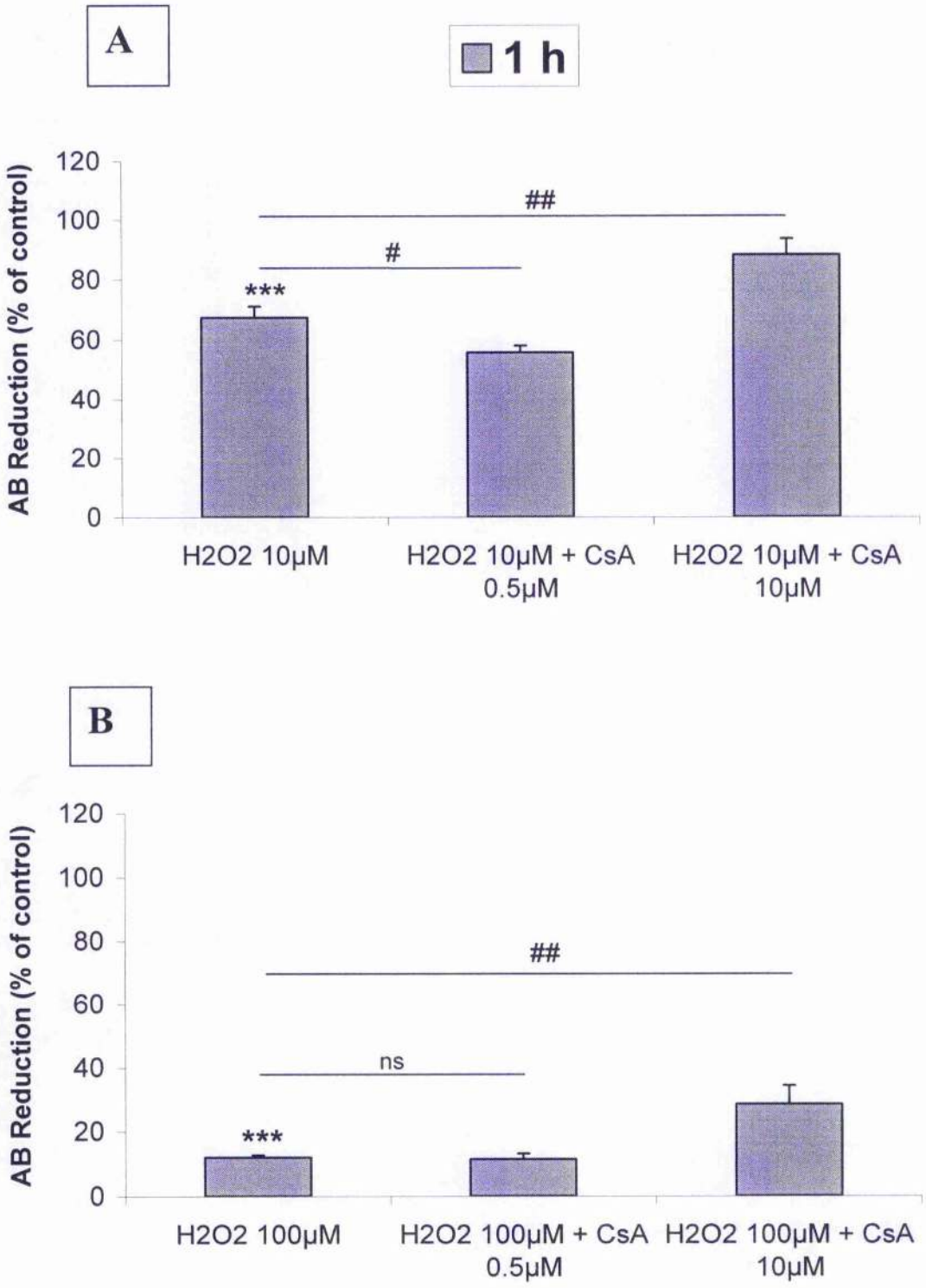


Figure 4.27. Histograms showing the effects of the membrane permeability transition pore blocker, cyclosporin A (0.5, 10µM), on the neurotoxicity mediated by 1 h application of **(A)** H_2O_2 10µM and **(B)** H_2O_2 100µM in CGN cultures. Each column represents the mean ± SEM for n = 3 cultures. ***P< 0.001 compared to untreated control; #P<0.05, ##P<0.01, ns = non-significant.

4.5.3. Adenosine receptor-mediated protection against oxidative damage by H_2O_2

The roles of A_1 and A_{2A} adenosine receptors in modulating oxidative damage by hydrogen peroxide in the CGNs were verified using ligands at these receptors. None of the ligands had any significant effect on viability when applied alone and each of them was tested against H_2O_2 for 1 h. 2-chloroadenosine (2-ClA) 1μM failed to modify the effect of hydrogen peroxide (30μM) that reduced viability to 31.22% ± 9.72 of the control ($P<0.001$, n = 3) (Fig. 4.28).

4.5.3.1. A_1 receptor ligands

Hydrogen peroxide 30μM decreased viability significantly to 36.02% ± 1.68 of the control ($P<0.001$, n = 3) (Fig. 4.29A). In the presence of the selective A_1 receptor agonist CPA (100nM), this damage was significantly attenuated ($P<0.001$) to 73.34% ± 3.53. On the other hand, the significant lowering of viability by H_2O_2 10μM to 60.09% ± 10.03 of the control ($P<0.01$) was unaltered in the presence of the selective A_1 receptor antagonist DPCPX (100nM) (n = 3) (Fig. 4.29B).

4.5.3.2. A_{2A} receptor ligands

A reduction in CGN viability to 51.67% ± 6.89 of the control ($P<0.001$) was induced by H_2O_2 10μM (n = 3) (Fig. 4.30A), which remained unchanged in the presence of the selective A_{2A} receptor agonist CGS21680 100nM. The selective A_{2A} receptor antagonist ZM241385 improved viability significantly ($P<0.05$) to 57.69% ± 13.42 of the control when present with H_2O_2 30μM (n = 3) (Fig. 4.30B), compared to a value of 34.10% ± 0.53 of the control ($P<0.001$) in the presence of H_2O_2 30μM alone.

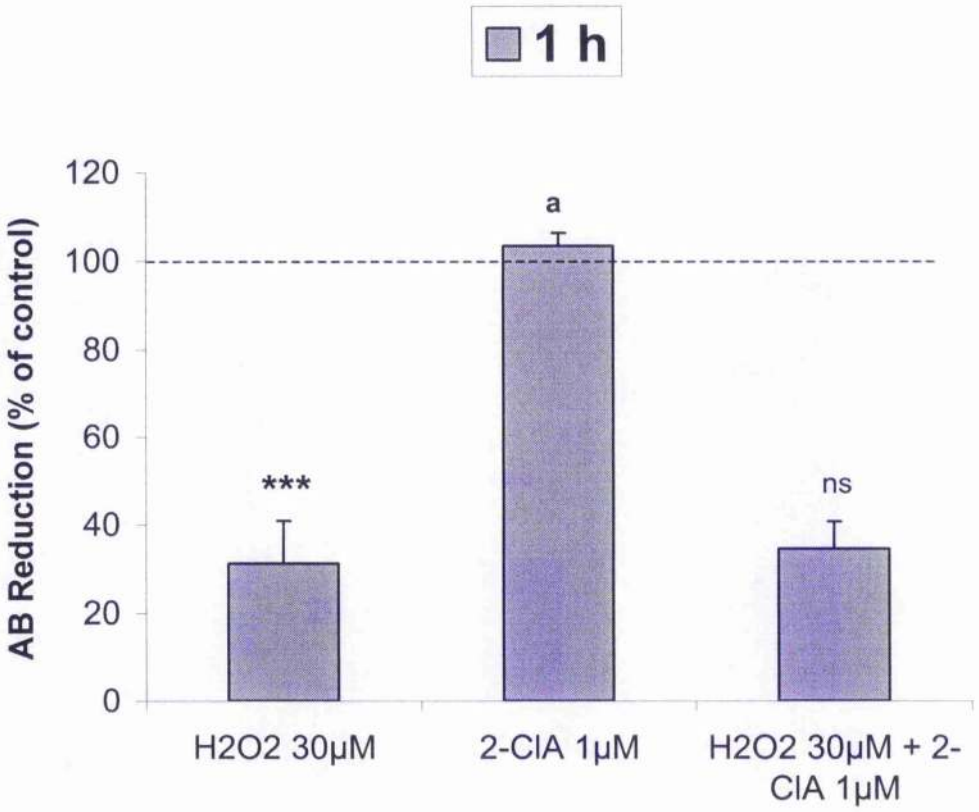


Figure 4.28. Histogram showing the lack of effect of 2-chloroadenosine (2-ClA) 1µM on the neurotoxicity mediated by H_2O_2 30µM in CGN cultures. Each column represents the mean ± SEM for n = 3 cultures. ***P< 0.001, **a** =non-significant compared to control; ns = non-significant compared to H_2O_2 30µM.

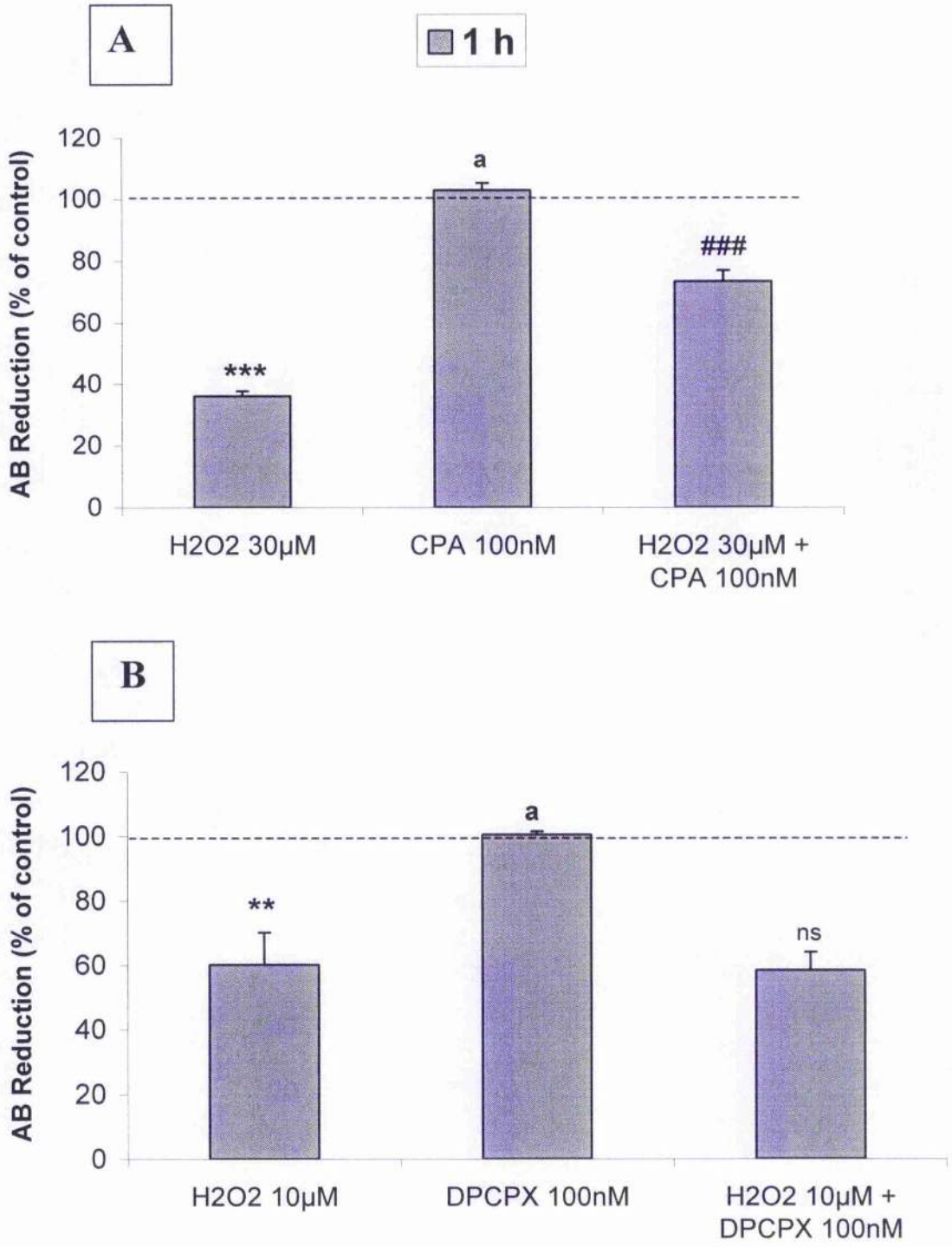


Figure 4.29. Histograms showing **(A)** the protective effect of the selective A_1 adenosine receptor agonist, CPA (100nM) and **(B)** the lack of effect of the selective A_1 adenosine receptor antagonist, DPCPX (100nM) against the neurotoxicity mediated by H_2O_2 in CGN cultures. Each column represents the mean ± SEM for n = 3 cultures. **P< 0.01, ***P< 0.001, **a** = non-significant compared to control; ###P<0.001, ns = non-significant compared to H_2O_2 alone.

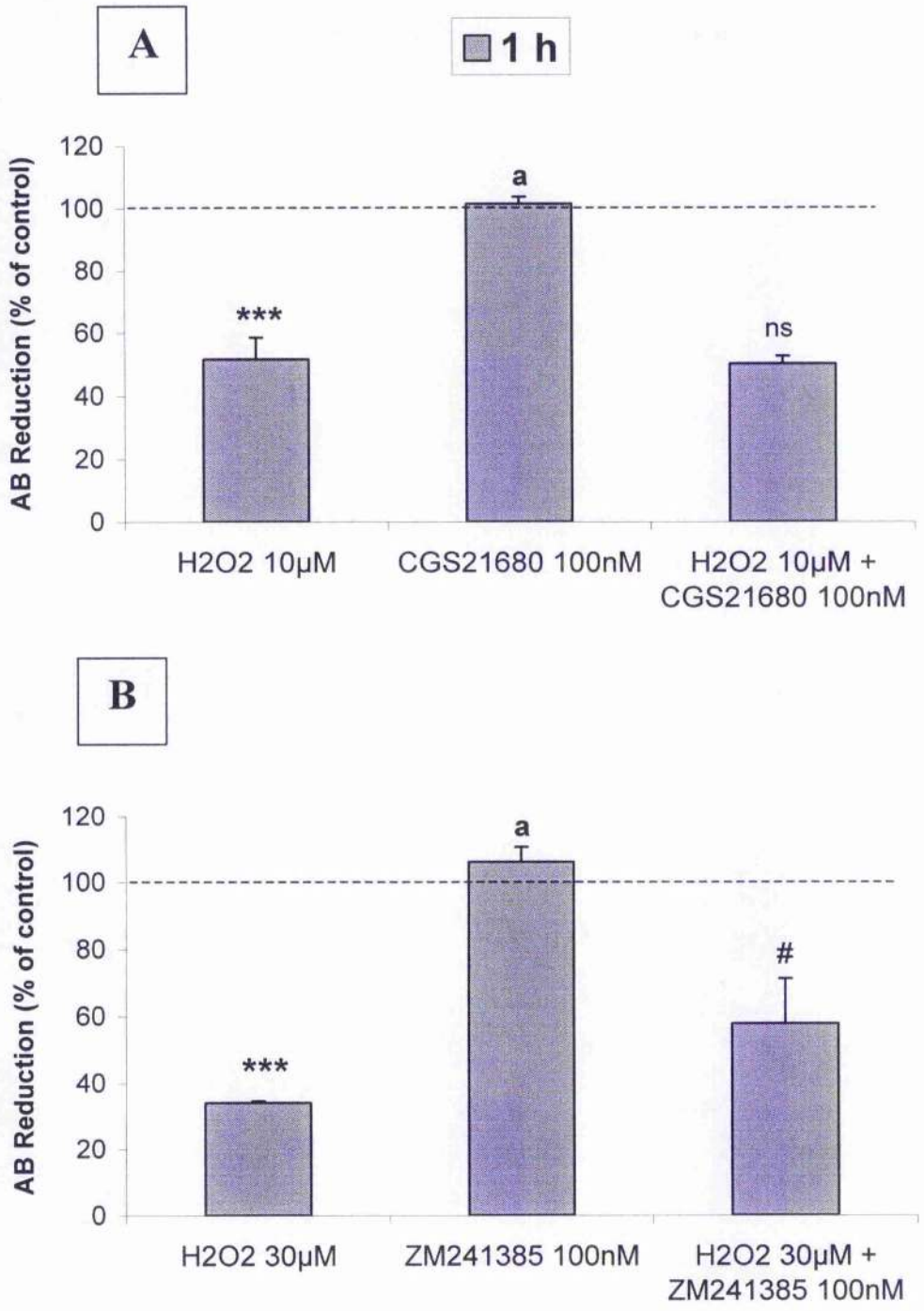


Figure 4.30. Histograms showing **(A)** lack of effect of the selective A_{2A} adenosine receptor agonist, CGS21680 (100nM) and **(B)** the protective effect of the selective A_{2A} adenosine receptor antagonist, ZM241385 (100nM) against the neurotoxicity mediated by H_2O_2 in CGN cultures. Each column represents the mean ± SEM for n = 3 cultures. ***P< 0.001, **a** = non-significant compared to control; #P<0.05, ns = non-significant compared to H_2O_2 alone.

4.5.4. Effects of some other inducers of oxidative stress

The kynurenine pathway metabolite 3-hydroxykynurenine (3-HK) is known to be a potent inducer of oxidative stress. Its effect on the viability of CGNs was therefore examined. The effect of the superoxide-generating system of xanthine (X) and xanthine oxidase (XO) was also investigated.

4.5.4.1. 3-hydroxykynurenine (3-HK) and neuronal viability

3-hydroxykynurenine reduced the viability of CGNs in a concentration-dependent manner ($P<0.01$, $n = 4$) (Fig. 4.31). At 1μM, 3-HK had no effect. Reduction following 6 h of exposure to 3-HK 10μM was significantly more than was obtained after 1 h of exposure to the same concentration (51.20% ± 2.96 vs. 88.71% ± 5.47; $P<0.001$), whereas exposure to 100μM or 1mM of the kynurenine elicited comparable reductions in viability at 1 and 6 h. The effects of 3-HK on CGN viability were consistent with the damage it inflicted on the morphology of the cultures (Fig. 4.32). The number of cells and neurites progressively dwindled with increasing concentrations of 3-HK or durations of exposure to it.

4.5.4.2. Mixture of xanthine (X) and xanthine oxidase (XO)

Exposure to xanthine (X) 100μM alone for 1 or 6 h had no significant effect on CGN viability ($n = 3$) (Fig. 4.33A). In contrast, the application of xanthine oxidase (XO) 0.02U/ml alone produced a time-dependent reduction in viability. At 6 h, XO lowered viability significantly to 47.51% ± 6.24 of the control ($P<0.001$). The mixture of X and XO further reduced viabilities at 1 and 6 h to 20.07% ± 8.59 and 13.58% ± 0.96 of the control, respectively ($P<0.001$). The reduction by X/XO mixture was significantly more than the reduction by XO alone, whether at 1 or 6 h ($P<0.001$). The mixture also damaged significantly the cell bodies and neurite outgrowths of the neuronal cultures (Fig. 4.34a, e, f). In order to confirm that the observed effect of XO was solely due to its enzymatic activity, the effect of combining X and heat-inactivated XO was determined. Heat inactivation was achieved by heating in a water bath at 60°C for 5-7 min. The results (Fig. 4.33B) showed that following heat inactivation, XO was no longer able to elicit any reduction in CGN viability, whether on its own or when combined with xanthine for 6 h ($n = 4$).

4.5.4.3. Effects of superoxide dismutase (SOD) and catalase (CAT) on damage by X/XO

The nature of the reactive oxygen species (ROS) produced by the mixture of X and XO in our experimental system was verified using the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Cultures were exposed for 6 h. Xanthine 100μM induced an increase in CGN viability to 116.93% ± 1.23 of the control ($P<0.05$, n = 4) (Fig. 4.35A), whereas xanthine oxidase 0.02U/ml lowered viability to 45.41% ± 10.25 ($P<0.001$). When combined, xanthine and xanthine oxidase induced a significant lowering of viability to 15.18% ± 1.24 ($P<0.001$), which was significantly more than the damaging effect of XO alone ($P<0.001$). Superoxide dismutase SOD 250U/ml had no effect on its own on basal viability. In the presence of SOD, the damaging effect of the mixture of xanthine and xanthine oxidase was unaltered.

The effects of catalase on the X/XO system are detailed in Fig. 4.35B (n = 4). Xanthine 100μM alone had no effect, xanthine oxidase reduced viability to 55.04% ± 3.04 ($P<0.001$), and the mixture of X and XO lowered viability to 29.76% ± 11.51 ($P<0.001$). The effect of X/XO was significantly more than the effect of XO alone ($P<0.01$). In the presence of catalase - which showed no effect on its own - the reduction in viability induced by a mixture of xanthine and xanthine oxidase was abolished, with viability restored to a level comparable to the control (114.87% ± 0.75).

4.5.4.4. Xanthine oxidase alone

In view of the observation that exposure to xanthine oxidase alone produced a significant reduction in the viability of CGNs, the possible mediation of this damage by ROS was examined (Fig. 4.36). Exposure of CGNs to xanthine oxidase 0.02U/ml alone reduced viability significantly to 54.56% ± 2.81 of the control ($P<0.001$, n = 4), while its morphological effects included extensive degeneration of neuronal cell bodies and neurite extensions (Fig. 4.34a, c, d). The xanthine oxidase inhibitor, allopurinol (AP) at 100μM, while having no effect on its own (99.66% ± 2.78 of the control), was able to attenuate very significantly ($P<0.001$) the effect of xanthine oxidase, improving viability to 89.43% ± 3.16, a level not significantly different from the control. When tested against XO, SOD at 250U/ml produced no significant improvement. However, catalase at 250U/ml completely prevented the damaging effect of XO ($P<0.001$), improving viability significantly beyond the control level to 120.11% ± 1.20 ($P<0.001$ compared to control).

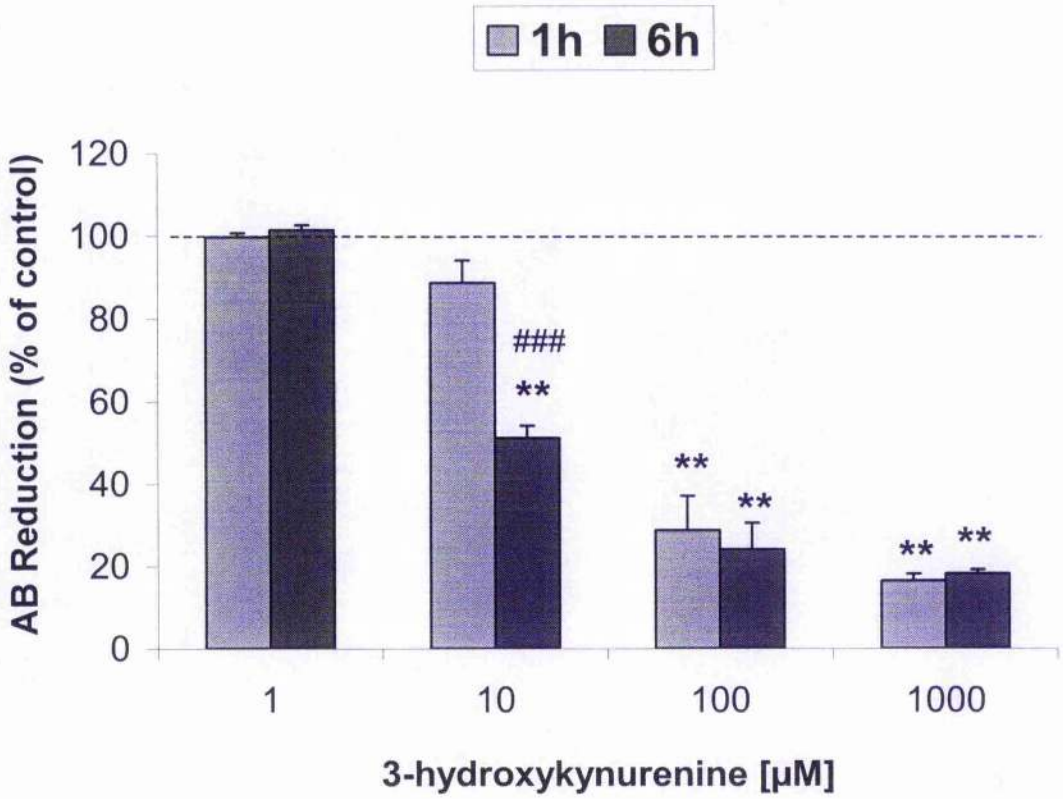


Figure 4.31. The effects of the kynurenine pathway metabolite, 3-hydroxykynurenine (3-HK) (1µM - 1mM), applied for 1 or 6 h, on the viability of cerebellar granule neurones at 8 div. Each column represents the mean ± SEM for n = 4 cultures. **P< 0.01 compared to control; ###P<0.001 for comparison of the effects of 3-HK 10µM at 1 and 6 h.

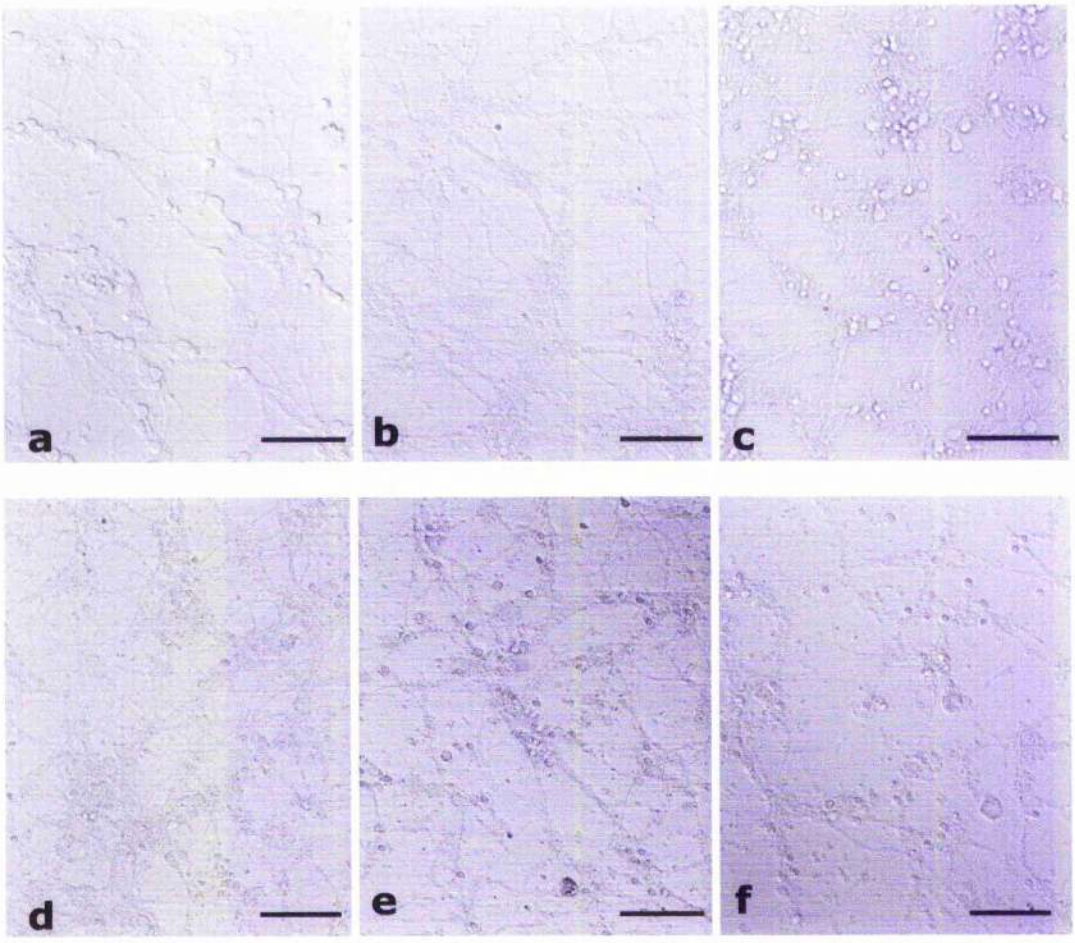


Figure 4.32. Photomicrographs showing concentration- and time-dependent damage to CGN morphology induced by 3-hydroxykynurenine (3-HK). **(a)** Control **(b)** 3-HK 10μM (1 h) **(c)** 3-HK 10μM (6 h) **(d)** 3-HK 100μM (1 h) **(e)** 3-HK 100μM (6 h) **(f)** 3-HK 1mM (6 h). There was greater damage (loss of cells and neurite outgrowth) with increasing concentrations of 3-HK **(c, e, f)** or with longer exposures **(b** and **c; d** and **e)**. Bar = 50μm.

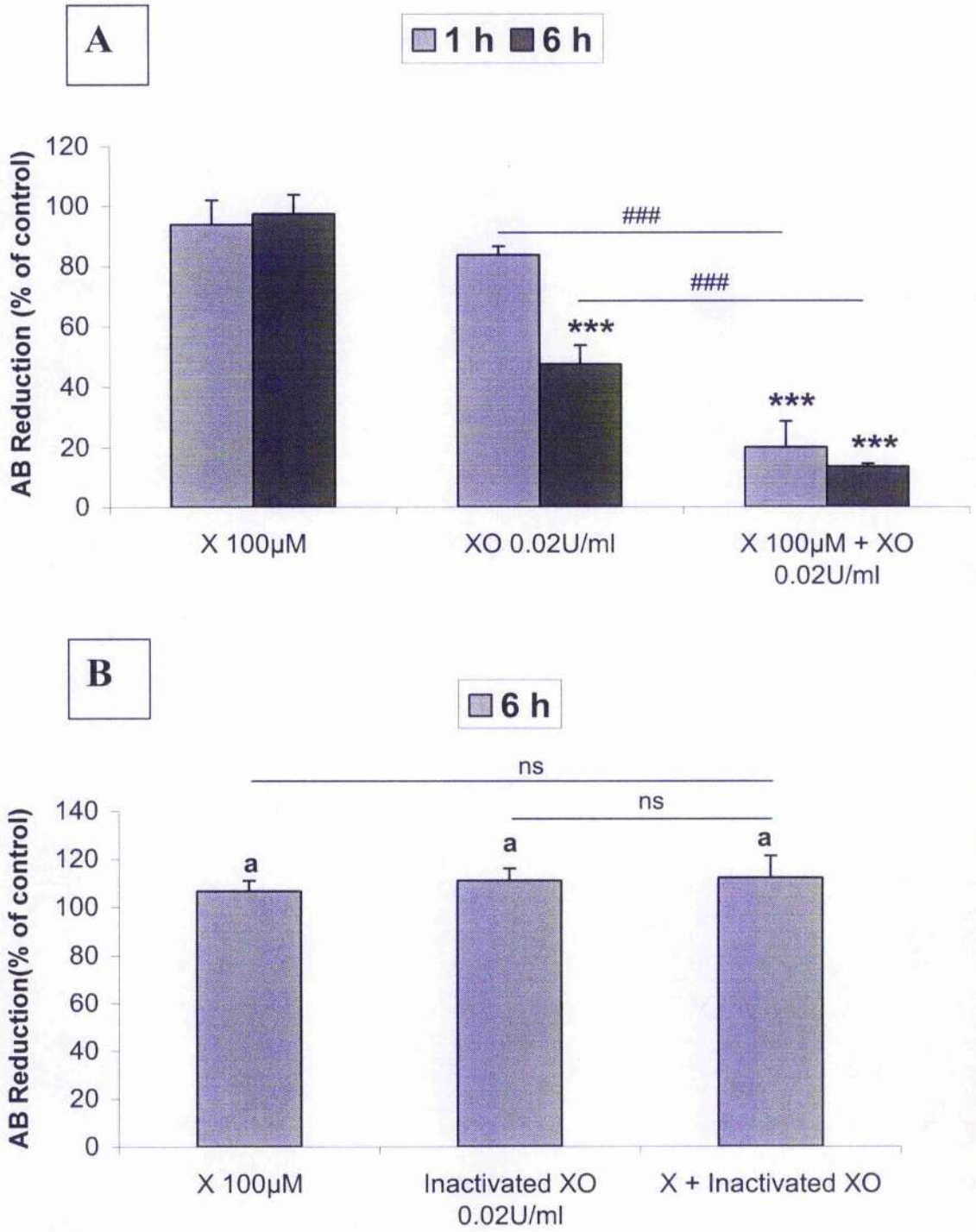


Figure 4.33. Histograms showing **(A)** the effects of xanthine (X) 100µM and xanthine oxidase (XO) 0.02U/ml and **(B)** the lack of effects of xanthine (X) 100µM and heat-inactivated xanthine oxidase (XO) 0.02U/ml on the viability of CGNs when each of them was applied alone or in combination for 1 or 6 h at 8 div. Each column represents the mean ± SEM for **(A)** n = 3 or **(B)** n = 4 cultures. ***P< 0.001, **a** = non-significant compared to control; ###P<0.001, ns = non-significant.

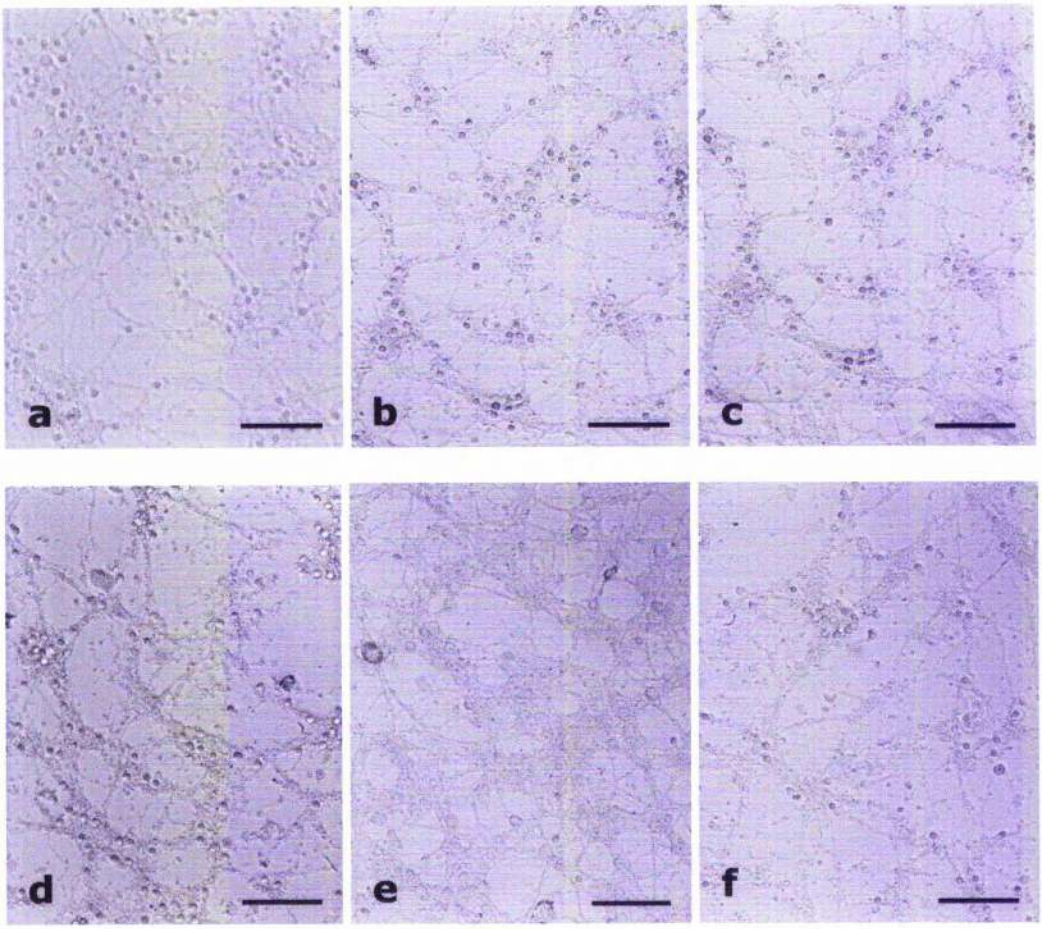


Figure 4.34. Photomicrographs showing the effects of xanthine (X), xanthine oxidase (XO) and their combination (X/XO) on the morphology of cerebellar granule neurones. **(a)** Control **(b)** X 100μM (6 h) **(c)** XO 0.02U/ml (1 h) **(d)** XO 0.02U/ml (6 h) **(e)** X/XO (1 h) **(f)** X/XO (6 h). Note the damage to cell bodies and neurite outgrowth in cultures treated with xanthine oxidase alone (6 h) **(d)** or with xanthine and xanthine oxidase (X/XO) for 1 or 6 h **(e, f)**, compared to control cultures **(a)**. Bar = 50μm.

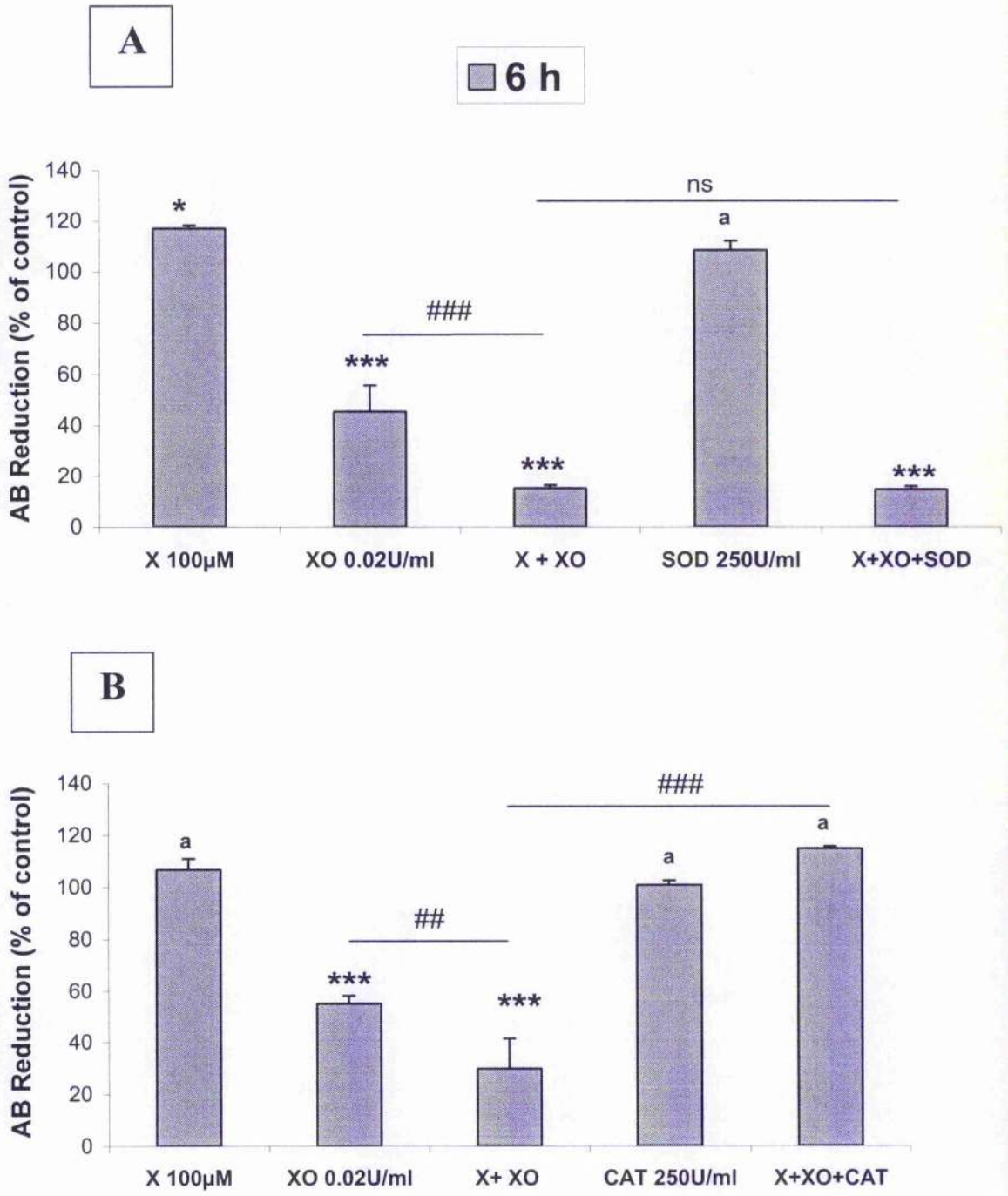


Figure 4.35. Histograms showing **(A)** the inability of the antioxidant enzyme superoxide dismutase, SOD 250U/ml and **(B)** the ability of the antioxidant enzyme catalase, CAT 250U/ml to prevent the damaging effect on CGN cultures of the mixture of xanthine (X) 100µM and xanthine oxidase (XO) 0.02U/ml. Each column represents the mean ± SEM for n = 4 cultures. *P<0.05, ***P<0.001, **a** = non-significant compared to control; ##P<0.01, ###P<0.001, ns = non-significant.

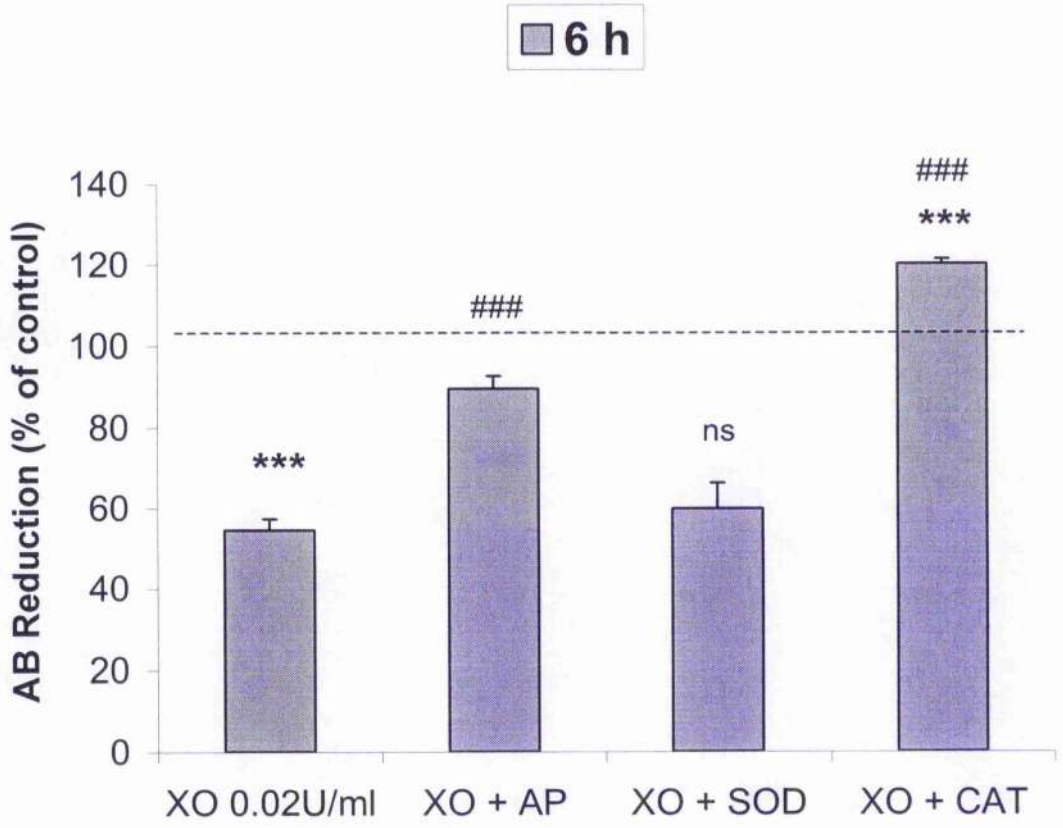


Figure 4.36. The effects of the xanthine oxidase inhibitor, allopurinol (AP) 100μM, and the antioxidant enzymes superoxide dismutase (SOD) 250U/ml and catalase (CAT) 250U/ml on the damage to CGN cultures induced by xanthine oxidase (XO) 0.02U/ml. Each column represents the mean ± SEM for n = 4 cultures. ***P<0.001 compared to control; ###P<0.001, ns = non-significant compared to XO 0.02U/ml.

4.5.5. Nitrosative stress and the viability of cerebellar granule neurones

In order to determine whether the effects of ROS such as hydrogen peroxide on the viability of CGNs are shared by reactive nitrogen species (RNS) such as nitric oxide at comparable concentrations, the extent of nitrosative stress-induced damage achievable with the nitric oxide (NO) donor, S-nitroso-*N*-acetyl-penicillamine (SNAP), was assessed (Fig. 4.37). At 10μM, SNAP induced no change in viability with up to 6 h of exposure, but with 24 h of exposure, it elicited a significant increase in viability to 116.29% ± 7.80 of the control ($P<0.05$). In contrast, at 100μM or above, SNAP produced a concentration- and time-dependent damaging effect on CGN viability ($P<0.01$, $n = 4$), which correlated with progressive loss of cells and extensive degeneration of neurite processes (Fig. 4.38a-d). The influences of the nitric oxide synthase (NOS) inhibitor L-NAME and the guanylate cyclase inhibitor ODQ on the damage induced by SNAP were examined. When added alone, L-NAME at 1 and 10mM produced no statistically significant effect, with values of 100.55% ± 2.13 and 113.80% ± 8.43 of the control, respectively ($n = 4$). Similarly, ODQ at 10 and 100μM had no effect, giving values of 99.29% ± 3.25 and 100.41% ± 3.24 of the control ($n = 4$). Exposure to SNAP 100μM for 24 h reduced viability significantly to 45.80% ± 4.28 of the control ($P<0.001$, $n = 4$) (Fig. 4.39A). In the presence of L-NAME 1mM, there was no improvement of the SNAP-induced damage (49.98% ± 5.43 of the control). However, in the presence of L-NAME 10mM, the SNAP-induced damage was significantly improved to 93.47% ± 0.82 ($P<0.001$), a level comparable with the control. The guanylate cyclase inhibitor ODQ, whether at 10 or 100μM, produced no significant effect on SNAP-induced damage of the CGNs that lowered viability to 40.90% ± 4.60 of the control after 24 h of exposure ($P<0.001$, $n = 4$) (Fig. 4.39B). Morphological assessment showed that L-NAME (10mM) significantly improved SNAP-induced damage to neuronal cell bodies and neurite extensions (Fig. 4.38a, b, e), while ODQ (100μM) lacked such restorative effect (Fig 4.38a, b, f).

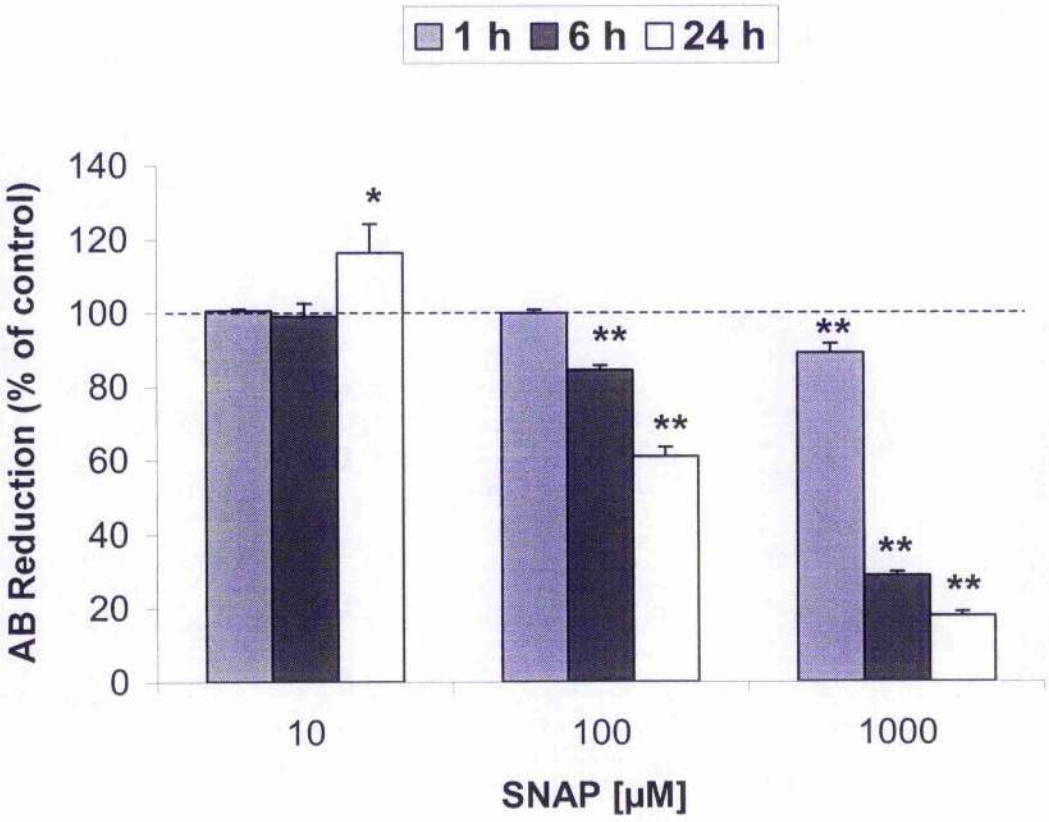


Figure 4.37. Histograms showing the effects of the nitric oxide (NO) donor, S-nitroso-*N*-acetylpenicillamine (SNAP) (10µM - 1mM), on the viability of cerebellar granule neurones for 1, 6 or 24 h of exposure. Each column shows the mean ± SEM for n = 4 cultures. *P<0.05, **P<0.01 compared to control.

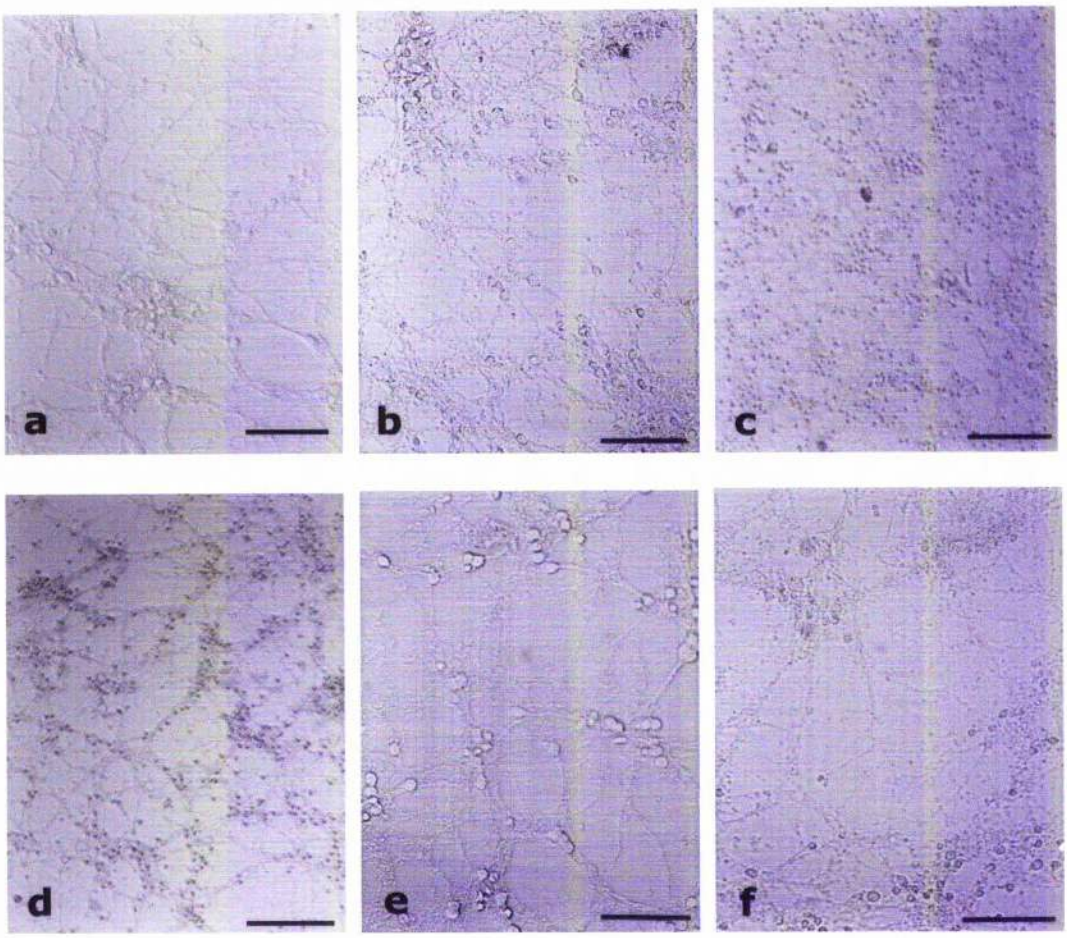


Figure 4.38. Photomicrographs showing the concentration- and time-dependent damaging effects of the nitric oxide-releasing compound, S-nitroso-*N*-acetylpenicillamine (SNAP), on the morphology of cerebellar granule neurones and the influences on this damage of the nitric oxide synthase (NOS) inhibitor, L-NAME, and the soluble guanylate cyclase inhibitor, ODQ. **(a)** Control **(b)** SNAP 100μM (24 h) **(c)** SNAP 1mM (6 h) **(d)** SNAP 1mM (24 h) **(e)** SNAP 100μM + L-NAME 10mM (24 h) **(f)** SNAP 100μM + ODQ 100μM (24 h). SNAP induced cell loss and extensive degeneration of neurite processes. In the presence of L-NAME, SNAP-induced morphological damage was significantly reduced **(a, b, e)**, restoring neuronal cultures to a phenotype closer to that of control cultures. ODQ had no effect on SNAP-induced damage to CGN morphology **(a, b, f)**. Bar = 50μm.

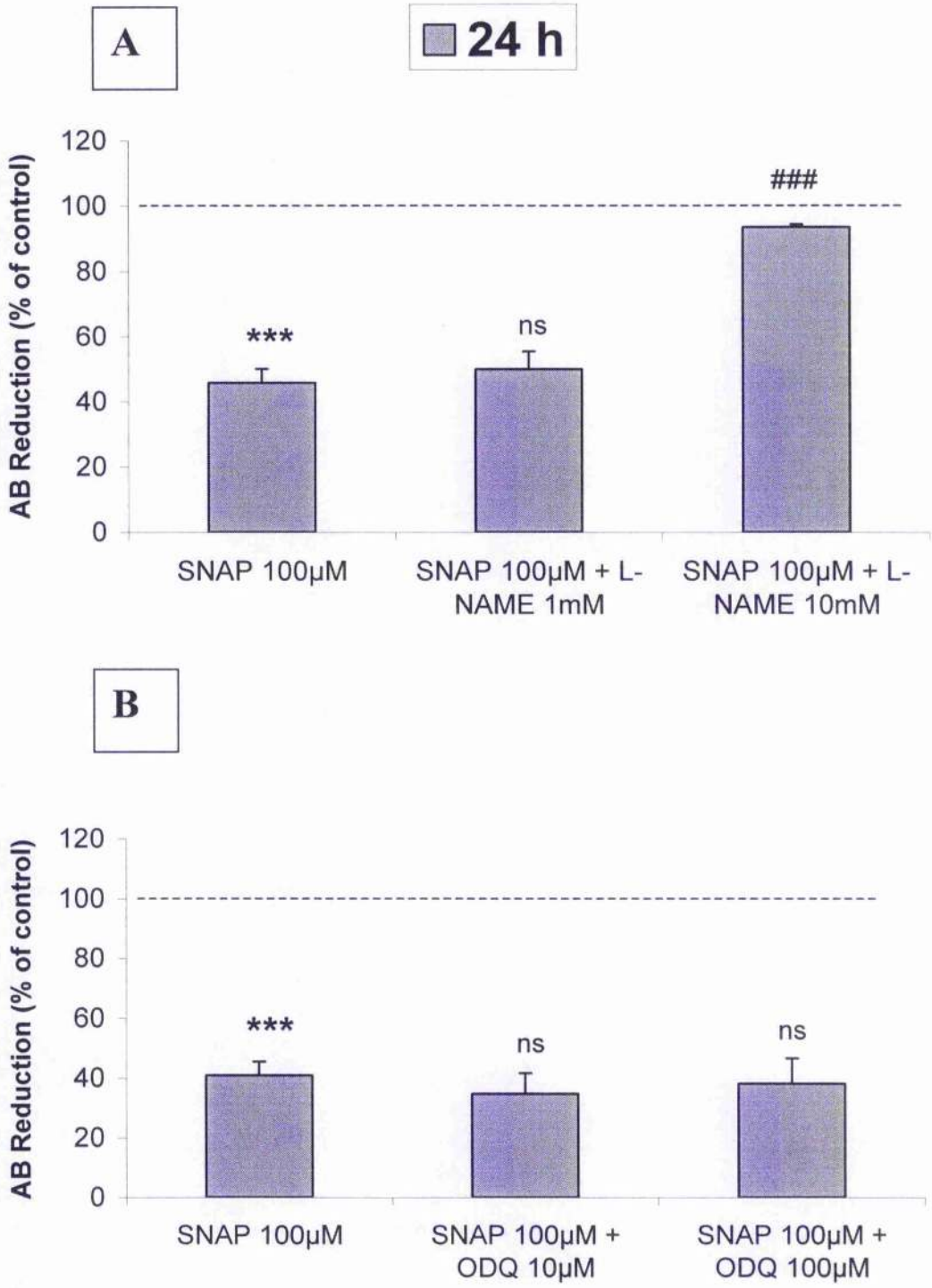


Figure 4.39. Histograms showing **(A)** the effect of the nitric oxide synthase (NOS) inhibitor, L-NAME (1mM, 10mM) and **(B)** the lack of effect of the guanylate cyclase inhibitor, ODQ (10µM, 100µM) on the SNAP-induced reduction in viability of CGNs. Each column shows the mean ± SEM for n = 4 cultures. ***P<0.001 compared to control; ###P<0.001, ns = non-significant compared to SNAP 100µM.

5. DISCUSSION

5.1. Culture purity and immunolabelling

Cerebellar granule neurones (CGNs) are frequently used in the study of mechanisms involved in apoptosis (Kalda & Zharkovsky, 1999) and in neuropharmacology for studying pathways of drug-induced apoptosis. They are easier to handle in microdissection, produce many more testable cultures (Cambray-Deakin, 1995), and have been used to assess the neurotoxic effects of glutamate (Slagsvold et al., 2000; Vaudry et al., 2003, Hou et al., 2006), kainic acid (Verdaguer et al., 2002; Smith et al., 2003), MPP^+ and colchicine (Gorman et al., 1999), which is why they have been chosen for neuronal investigations in this study. Cerebellar interneuron-enriched primary cultures are known to provide an almost pure population of a single type of live neurones (granule cells) (Levi et al., 1989). These cells are the most numerous neuronal population in the cerebellum. The CGN cultures used in this study were obtained from 8-day old Sprague-Dawley rat pups. This is a developmental stage at which cerebellar neurones have reached the differentiating (postmitotic) stage (Hertz et al., 1985). Cytosine arabinoside (Ara C) is an antimitotic agent for dividing cells that competitively inhibits the incorporation of 2' deoxycytidine into DNA (Smith & Orr, 1987; Wallace & Johnson, 1989). Cultures in this study were treated with Ara C (10μM) 20-24 h after passaging, as the addition of this compound to CGN cultures at such concentration and culture stage eliminates glial population of cells, since the glial cells are still in the mitotic phase. Other cerebellar neurones are eliminated by the process of differential centrifugation during the setting up of the cultures. With this standard procedure, the investigator is left with a homogeneous population (containing > 90% of granule cells), characterized as glutamatergic neurones (Schousboe et al., 1985). The concentration of Ara C used is established to be non-toxic to neurones, although Daniels & Brown (2002) reported that it may be toxic to CGNs after potassium withdrawal through p53-dependent apoptosis. Our study, however, maintained the cultures routinely in a high potassium concentration (25mM). Using the technique of immunocytochemistry, it was found that these cerebellar granule neuronal cultures showed strong immunoreactivity against the mouse anti-human beta-III isoform of tubulin monoclonal antibody, which is specific for neurones (cytoskeleton-staining) (Geisert & Frankfurter, 1989; Farina et al., 1999), thus confirming that the cultures were predominantly neuronal.

5.2. Alamar Blue reduction assay for the determination of cell viability

In this study, the relatively new Alamar Blue (AB) reduction method was used for the quantification of cell viability. Several methods are available for determining cell viability, including the trypan blue exclusion, neutral red uptake, crystal violet inclusion, Chromium-51 (^{51}Cr) release and the MTT (or XTT) assay (Nociari et al., 1998). As well as the MTT, trypan blue, calcein-AM, ethidium bromide, propidium iodide, fluorescein diacetate and lactate dehydrogenase (LDH) assays are also employed in excitotoxic studies (Lustig et al., 1992a; 1992b; White et al., 1996). However, there are several obvious advantages of the AB method over many of the other methods as follows:

1. AB assay allows the choice of detection method, as it can be read either fluorometrically or colorimetrically. Measurements in this study have been made colorimetrically (dual-wavelength) using the ELISA plate reader (DYNEX Technologies, USA). The chosen wavelengths were 540 and 595nm.
2. The dye is soluble, thus making any subsequent process of extraction unnecessary, unlike the MTT assay in which the generated formazan has to be solubilized before plate reading is possible.
3. No centrifugation is required, because the method is appropriate for either suspension or attached (monolayer) cells.
4. Fewer steps are required. Therefore, it is time saving and easily adaptable to automation.
5. AB is stable over a long period of time and could be stored (in the dark, as it is light-sensitive) for 12 months at room temperature and 20 months at 2 - 8°C. AB dye is also stable in culture medium, which is highly desirable for investigations involving continuous cell growth monitoring, kinetic studies, and incubation time of days.
6. It is relatively non-toxic to cells and is therefore less likely to interfere with normal metabolism, thereby allowing precious cells to be reused for further investigations (as the assay is performed under sterile conditions). This has the economic benefit of saving cost, unlike methods such as the MTT, which necessitate killing the cells (O'Brien et al., 2000).
7. It is non-radioactive and non-toxic to personnel and therefore considered safe and disposable with less regulation.

The claim that AB is non-toxic to cells in culture was confirmed in this study. Whether in MC3T3-E1 or cerebellar granule cultures, the viability of cultures previously treated with AB was not different from the viability of AB-naive cultures when quantification was done using trypan blue exclusion or even the dye itself.

The active ingredient in this dye is 7-hydroxy-3H-phenoxazin-3-one-10-oxide (O'Brien et al., 2000). These authors showed that the identity of AB is resazurin (blue and non-fluorescent), which is reduced to resorufin (pink and fluorescent) and further reduced to hydroresorufin (uncoloured and non-fluorescent). Cellular activity (likely to be by oxygen consumption through metabolism) reduces the oxidized alamar blue. The dye works on the basis that the internal environment of proliferating or metabolically active cells is more reduced than that of non-proliferating or metabolically inactive cells. Specifically, the ratios of NADPH/NADP, FADH/FAD, FMNH/FMN, and NADH/NAD increase during proliferation and AB can be reduced by all of these intermediates. The reduction is accompanied by a measurable shift in colour (Nociari et al., 1998). Because it can be reduced by cytochromes, AB substitutes for molecular oxygen as an electron acceptor while tetrazolium salts such as MTT are reduced by the above-mentioned intermediates but not by cytochromes (O'Brien et al., 2000). Acceptance of electrons from these compounds changes AB from the original, oxidized, indigo blue, non-fluorescent state to the reduced, pink, fluorescent state. In order to measure AB reduction colorimetrically, the absorbance is usually measured at two wavelengths because the absorption spectra of the oxidized (blue) form and the reduced (red) form overlap. The standard wavelengths are 570 and 600nm, where the reduced and oxidized forms absorb, respectively, but filter combinations at wavelengths other than these have been confirmed to be equally acceptable (e.g., 540 and 630nm; 565 and 610nm), as long as the necessary corrections are made, based on a formula given by the manufacturers (as explained in section **2.4** of this thesis). However, a second approach to the measurement of AB reduction is by fluorescence, which appears to be more sensitive than dual-wavelength colorimetry. In this case, excitation is made at 530-560nm and emission measured at 590nm. Several authors have cited the use of AB (see Ahmed et al., 1994; Stephen et al., 1999; Marcelo et al., 1998; Melissa et al., 1996; Page et al., 1993; Mershon et al., 1994; Harvey et al., 1995; Squatrito et al., 1995; White et al., 1996). While they are equally specific, the AB method has been found to require fewer cells and is more sensitive than the ^{51}Cr assay in evaluating cell-mediated

cytotoxicity (Nociari et al., 1998). There are also very small inter- and intra-assay variations with AB cytotoxicity assays, an observation that was confirmed in this study by the relatively small error bars in most of the data. In comparison with the LDH assay, AB is able to quantify cell death predominantly involving apoptosis more accurately than the LDH assay, as the loss of membrane integrity, which the LDH assay relies upon, is a late feature of apoptotic death (White et al., 1996). Another benefit of the AB assay in relation to the MTT assay arises from the fact that tetrazolium salts (MTT assay) could be reduced by superoxide that is generated during excitotoxic or other forms of cell death and thus give an erroneous indication of normal cell viability while cell death is occurring. This is because the reaction in which tetrazolium salts are reduced to their intensely coloured formazans occurs via the formation of a free radical intermediate, tetrazolinyl, in which only one electron has been transferred to the tetrazole ring. In fact, the reduction of nitroblue tetrazolium is the basis for a well-established indirect assay for superoxide dismutase (Marshall et al., 1995). The AB assay effectively does not harbour these potential artefacts.

In order to obtain the best results, however, factors such as cell density, incubation times, media composition and concentrations of the agents under investigation should be standardized or carefully selected. This process would avoid potential pitfalls such as over-reduction of AB to the colourless and non-fluorescent hydroresorufin. In this study, AB reduction varied directly and was well correlated with both cell density and the incubation period for the osteoblast and neuronal cultures used. Guided by these data, MC3T3-E1 cultures were therefore routinely seeded at a density of 1×10^5 cells/ml and incubated with AB for 4 h while CGN cultures were seeded at 1×10^6 cells/ml and incubated with AB for 6 h.

A possibility that the AB assay is sensitive to protein conditions in culture media has been raised (Gocgan et al., 1995). However, the manufacturers of the AB used in this study (alamarBlue™ from Biosource, Belgium) stated explicitly that the presence of 10% FCS in the culture medium has no effect on the results obtained with this AB when reduction is measured colorimetrically (as done in this study), whereas this level of serum causes some quenching of fluorescence. Assays were therefore carried out, either in serum-free medium or, as it was in most cases, in medium containing the same amount of FCS, especially for neuronal cultures whose viability could be significantly impaired with prolonged (> 1 h) culturing in serum-free medium. In addition, our use of the dye was for an endpoint measurement of cytotoxicity,

which has been observed as more valuable than its use as a kinetic measure for monitoring cell growth (O'Brien et al., 2000).

5.3. MC3T3-E1 cell line: a suitable *in vitro* model for osteoblastic development

The MC3T3-E1 cell line has been used in this work. It was derived from newborn calvariae and has been previously shown to exhibit osteoblast-like characteristics after repeated passages (Quarles et al., 1992). Parameters of the osteoblast phenotype which this cell line expresses include type 1 collagen synthesis, alkaline phosphatase (ALP) activity and nodular extracellular matrix mineralization resembling woven bone (Sudo et al., 1983). This evidence was further strengthened by Quarles et al. (1992) when they showed that these cells in culture display a temporal sequence of development that was characterized by distinct proliferative and differentiated stages. Alkaline phosphatase is a common marker of differentiation in bone and has been hypothesized to be involved in the process of mineralization, though its precise physiological role has not been delineated (Henthorn, 1996). To characterize the cells, therefore, a developmental profile of ALP activity from 4 div to 17 div was obtained in this study by measuring the conversion by ALP of the colourless p-nitrophenylphosphate to the yellow p-nitrophenol in alkaline solution at 405nm wavelength. The effects of a combination of ascorbate and β-glycerol phosphate (switching) on the proliferation and differentiation of these cells were also examined. In this regard, these supplements were added after three days in culture, which implied that a developmental stage of N day(s) post-switch corresponded to (N + 3) days *in vitro* (div). The addition of these supplements increased proliferation significantly. Similarly, the supplements induced increased differentiation in the MC3T3-E1 osteoblast cultures that reached significance 7 days after switching (days post-switch) (10 div) and onwards, as measured by ALP activity. In fact, at 11 days post-switch, ALP activity was more than four-fold higher in switch than in non-switch cultures. Interestingly, this phenomenon was not cell density-dependent, as a similar pattern of changes in ALP activity was obtained with a three-fold higher density. Our observations are consistent with earlier reports (Quarles et al., 1992). The initial stage of development of the cells is characterized by cell proliferation and high levels of type 1 collagen expression, biosynthesis and secretion, but the cells remain undifferentiated as evidenced by low levels of alkaline phosphatase activity. There is also an inability to assimilate effectively newly synthesized collagen into extracellular

matrix and mineralization is absent (Stein et al., 1996). In fact, it has been suggested that ascorbic acid stimulates osteoblast proliferation through its effects on the synthesis of collagen or some related matrix protein that interacts with cell surface adhesion receptors to cause the stimulation of proliferation (Harada et al., 1991). Another report by Franceschi & Iyer (1992) showed that the earliest effects of ascorbic acid were to stimulate type 1 procollagen mRNA and collagen synthesis (24 h after ascorbate addition), followed by induction of ALP (48-72 h) and osteocalcin (96-144 h) mRNAs. However, in their work, Quarles et al. (1992) argued that ascorbate does not stimulate type I collagen gene expression in MC3T3-E1 cells but is absolutely required for deposition of collagen in the extracellular matrix. During days 1-9 *in vitro*, subconfluent cultures are seen displaying rapid increases in cell number and high rates of DNA synthesis (Quarles et al., 1992). The next developmental stage, which occurs 10 days after plating, is characterised by downregulation of replication and expression of differentiated osteoblast functions. Growth rates reach a plateau and DNA synthesis becomes negligible. At this time, increases in alkaline phosphatase activity- a characteristic of the osteoblast phenotype- and acquisition of specialized bone function consisting of collagen deposition into an extracellular matrix occur. This was the same stage from when the switch cultures in this study began to exhibit a significantly greater level of ALP activity than their non-switch counterparts. With regard to ascorbate and β-glycerol phosphate, Quarles et al. (1992) observed that when these agents are absent, MC3T3-E1 cells attain growth arrest and express low levels of alkaline phosphatase at day 10 (as was the case with our non-switch cultures) but fail to express a fully differentiated osteoblast phenotype as revealed by the inability to form and mineralize extracellular matrix. They noted that ascorbate facilitates the expression of osteoblast phenotype in these cells without affecting the timing of maturation. When ascorbate is added to the culture medium, significant increases occur in cellular ALP activity in differentiated cells, but the time course of osteoblast phenotype development as defined by cessation of mitogenic growth and the onset of ALP expression is unaffected. β-glycerol phosphate acts synergistically with ascorbate to stimulate further collagen accumulation and ALP activity in post-mitotic, differentiated osteoblast-like cells. Quarles et al. (1992) also found, as did this study, that the expression of ALP was time- but not density-dependent. This expression was evident only at the onset of growth arrest at about day 9 and was enhanced 2-fold by ascorbate and β-glycerol phosphate. The current work, however, has shown the

possibility of a greater than 2-fold enhancement of ALP activity by the switching supplements. Many agents having a trophic influence on the MC3T3-E1 cell development are known to increase the expression of ALP, as measured by the extent to which the enzyme can release free p-nitrophenol (4-nitrophenol) from p-nitrophenylphosphate (4- nitrophenylphosphate) (Yada et al., 1994; Suzuki et al., 1998; Genever & Skerry, 2001). This shows ALP is involved in mineralization by MC3T3-E1 cells. There is now evidence for a role of p38 MAP kinase in the regulation of ALP expression during osteoblastic cell differentiation (Suzuki et al., 2002). A final phase of MC3T3-E1 maturation starts about day 20, characterized by matrix calcification linked with progressive increases in extracellular matrix accumulation and ALP activity (Stein et al., 1996). These cells are therefore representative of immature osteoblasts which undergo a temporal program consistent with osteoblast differentiation in stages analogous to *in vivo* bone formation, thus making the MC3T3-E1 cell line as used in this and other studies a suitable model of osteogenic development *in vitro*.

5.4. Differential responses of osteoblast and neuronal cultures to culture conditions

Responses of MC3T3-E1 cultures that have or have not been exposed to a number of culture conditions including levels of serum, serum withdrawal and supplementation with potassium or glutamine were examined in this study. The process of switching by the addition of ascorbate and β-glycerol phosphate initiated differences in responsiveness to serum, which varied depending on the concentration of serum in the culture medium. In culture medium containing a low FCS concentration (1%), non-switch cultures exhibited a higher level of viability compared to switch cultures. The reverse was the case at the highest FCS concentration used (20%). No such differences in viability were found at 10% FCS, which justifies the usual concentration for culturing these and most other cells. In general, serum at 10 or 20% concentration (v/v) promoted survival better than at 1%. However, complete withdrawal of serum from these cells for up to 1 day in culture did not provoke any significant impairment in their viability and even with longer days of withdrawal (2 or 3 days), reduction in viability, though significant, was no more than 18%, and the process of switching did not influence this outcome. In fact, the hardy nature of these cultures was further revealed by an insignificant (6.8%) reduction in viability obtained when they were maintained for 10 div without changing the medium, compared with parallel cultures replenished every 2-3 days

until 10 div. No difference was found when cultures were fed every 2-3 days by replacing half of the culture medium as opposed to replacing the complete medium. It is possible that the mild but significant reduction in viability after 3 days of serum withdrawal was due to apoptosis, as it has been reported that MC3T3-E1 cells undergo apoptosis in response to serum withdrawal or addition of TNF-α. This effect was shown by TUNEL labelling and DNA fragmentation (Jilka et al., 1998). A readily observed feature of serum deprivation in cultures is an increase in the level of lipid peroxidation, possibly partly due to the absence of serum components that might provide an anti-oxidant function, e.g., ascorbate, urate, caeruloplasmin, etc. Acute serum deprivation is also known to lead to profound effects on the permeability of the cell membrane to Na^+ and K^+ which may be related to increased levels of lipid peroxidation (Hopp et al., 1987). Chang et al. (2004) observed that in postnatal cortical neurones, transfected serum response factor (SRF) exerted a dose-dependent protection against the effect of trophic withdrawal downstream of extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol-3 kinase (PI3 K) signalling, which are known to be activated by brain-derived neurotrophic factor (BDNF) (Hetman et al., 1999).

With regard to potassium supplementation, 10mM K^+ for 10 div elicited a trophic effect, especially in the switch cultures; 25mM was without effect, while 50mM was potentially toxic. The addition of glutamine to a final concentration of 2mM induced significant and comparable trophic effects in the switch and non-switch cultures. A continued depolarization with 30-60mM KCl inhibits glutamate release in MG63 cells (in the presence of extracellular Ca^{2+}) and in MC3T3-E1 cells grown for at least 7 div in osteogenic conditions. However, it does not have any effect on MC3T3-E1 cultures grown without osteogenic ascorbate or β-glycerol phosphate or on these cultures when grown with the osteogenic supplements for up to 5 div (Genever & Skerry, 2001). Therefore, high potassium could impair osteoblastic viability of well-differentiated MC3T3-E1 cultures by reducing the trophic effect of glutamate. Overall, our data are consistent with these previous findings and additionally have furnished culture standards that could be adopted in the routine culture of this and possibly other osteoblastic cell lines.

Further to this, the relevance of this research was again emphasized by considering the relative fastidiousness of cerebellar granule cultures in comparison with MC3T3-E1 cultures when the former were grown under similar conditions. While 1 h of serum withdrawal had no effect on

CGN viability, 6 h of serum withdrawal was profoundly detrimental to their survival, quite unlike the case with MC3T3-E1 cultures. This reinforces the previous realization that the survival of neuronal cultures depends critically on trophic factors including antioxidants present in serum that could prevent the induction of lipid peroxidation. Equally damaging to CGN viability was the lowering of the potassium concentration from 25mM to 5mM for 2 days (6 to 8 div). This is most likely due to apoptotic cell death, as cultured CGNs are known to undergo spontaneous apoptosis if grown in physiological KCl concentrations (5mM) but survive in the presence of depolarizing concentrations of KCl (25mM) (Xifro et al., 2005). The authors found that neuroprotection mediated by this high KCl concentration was BDNF-independent but mediated by a mitogen-activated protein kinase- (MAPK) and phosphatidylinositol-3 (PI3) kinase-dependent inhibition of caspase-3. During their postnatal migration from the external to the internal granule cell layer, cerebellar neurones require excitatory inputs from mossy fibres. Consequently, neurones which receive the excitatory inputs survive and differentiate, while those which fail to do so die by apoptosis (Williams & Herrup, 1988; Wood et al., 1993). This requirement is mimicked *in vitro* by maintaining CGN cultures in the presence of a depolarizing concentration of KCl (e.g., 25mM) (Gallo et al., 1987), providing a reason why these cultures are routinely cultured in a high concentration of KCl, often 25mM. Alternatively, the CGN cultures could be maintained in the presence of exogenous trophic agents such as NMDA (Gallo et al., 1987; Balazs et al., 1988), or BDNF (Lindholm et al., 1993). The presence of 25mM potassium or NMDA in CGNs does induce a trophic effect, which includes a specific regulation at the mRNA level of the enzymes involved in the glutamate neurotransmitter synthesis, such as aspartate aminotransferase (AAT) and phosphate-activated glutaminase (PAG) (Caballero-Benitez, 2004). The protection by NMDA against caspase-mediated apoptosis of cultured CGN is dependent on activation of tyrosine kinases and PI3 kinase but, unlike the protection by high KCl concentration, is also BDNF-dependent (Xifro et al., 2005). In contrast, however, some other authors have argued that it is caspase-independent mechanisms that are involved, as caspase inhibitors do not inhibit apoptotic death of cerebellar neurones induced by low K^+ concentrations, although they block caspase activity and DNA fragmentation (Miller et al., 1997). These discrepancies are easily resolved in the light of current evidence that the process of apoptosis involves both caspase-dependent and -independent mechanisms. Furthermore, apoptosis induced by low K^+

in CGNs is associated with reduced interaction between CREB-binding protein (CREB is cAMP/calcium response element-binding protein) and the ubiquitously expressed transcription factor, NF-κB. There are five NF-κB proteins in mammalian cells, namely p50, p52, p65 (RelA), RelB and c-Rel. Yalcin et al. (2003) showed that in normal neurones, p65 interacts with the transcriptional co-activator, CREB-binding protein (CBP). However, the decrease in transcriptional activity of NF-κB caused by low K^+ is accompanied by a reduction in the interaction between p65 and CBP, which causes hyperphosphorylation of CBP. It has also been suggested that neuronal apoptosis following serum and potassium deprivation in CGNs may be due to inappropriate control of the cell cycle, as roscovitine, an antitumour drug that inhibits cyclin-dependent kinases Cdk1, Cdk2 and Cdk5, showed a significant neuroprotective effect in CGNs deprived of these supplements (Verdaguer et al., 2004). Another Cdk inhibitor, flavopiridol, at therapeutic dosage almost completely prevented colchicine-induced apoptosis in CGNs (Jorda et al., 2003). One other possible mechanism by which the reduction of K^+ could trigger apoptosis in the CGNs is by partial closure of L-type Ca^{2+} channels with a consequent drop of intracellular concentrations of this cation (Vitolo et al., 1998). Valencia & Moran (2001) suggested that the superoxide anion could also be responsible for the apoptotic death of CGNs induced by potassium deprivation. Interestingly, adenosine and ADP were shown to prevent apoptosis caused by lowering the K^+ concentration from 25mM to 5mM in cultured rat CGNs (Vitolo et al., 1998).

5.5. Glutamate and NMDA receptors

5.5.1. Glutamate and the viability of neurones

The neurotoxic actions of glutamate and NMDA were demonstrated in this study in a dose-dependent manner. The absence of serum from the culture medium during post-treatment recovery, which lasted for at least 20 h, enhanced the neurotoxicity of glutamate, but not of NMDA. This enhancement was best demonstrated with glutamate at 10 and 100μM. The presence of a high level of glucose in culture medium during post-treatment recovery was unable to compensate for the absence of serum. It is possible that the absence of serum imposed additional stress on the cultures, thus potentiating the excitotoxic effect of glutamate, since it was shown earlier in this study that serum withdrawal from CGN cultures for just 6 h

resulted in more than 30% reduction in basal viability. However, the reason why a similar occurrence of serum deprivation-induced potentiation of neurotoxicity was not found with NMDA is not clear. Again, at comparable concentrations, glutamate induced greater damage to the viability of CGNs than did NMDA. One possibility is that glutamate was able to activate both the NMDA and non-NMDA receptors - all of which may contribute to cell death - to cumulatively induce a higher level of death, whereas NMDA was a specific agonist activating only the NMDA population of glutamate receptors. This, however, may not be the case, as blockade of the non-NMDA receptors by CNQX completely failed to attenuate the damaging effect of glutamate, whereas both the non-competitive and competitive NMDA receptor agonists, MK-801 and D-AP5, respectively, were able to protect significantly against this effect of glutamate, with D-AP5 restoring viability to a level comparable to that of control cultures without glutamate. This suggests that the neurotoxic action of glutamate in the CGNs, at least in our culture system, is attributable mainly and most clearly to the overactivation of NMDA receptors. The NMDA receptor has several modulatory sites sensitive to a variety of agents (Watkins et al., 1990; Stone & Addae, 2002), including those with redox properties. For example, the receptor is known to require glycine for its activation (Johnson & Ascher, 1987) and of late, the critical role of the amino acid D-serine to the activation of NMDA receptor has also been espoused (Mustafa et al., 2004). All these may add to the complexity of the NMDA receptor in mediating neurotoxicity. The likelihood of NMDA receptor desensitization in this paradigm is also suspect, as 100μM and 1mM NMDA each caused less damage than a mid-way concentration of 300μM. It is therefore possible that while 100μM NMDA was not sufficient to significantly overactivate the receptor in order to mediate toxicity, 1mM was so high a concentration that it desensitized the receptor. Should this be the case, a concentration of 300μM would then just be adequate to overactivate the receptor and lead to significant neurotoxicity- without inducing desensitization- in a way that shows it is equipotent with a comparable concentration of glutamate at this receptor.

Glutamate receptor-mediated neurodegeneration can now be separated into two distinct forms, acute and delayed, which can be distinguished from each other based on the time course and ionic dependence of neuronal degeneration (Kato et al., 1991; Choi et al., 1987). Acute neurotoxicity is typified by neuronal swelling in the presence of agonist, leading to osmotic lysis. Removing from the medium Na^+ and Cl^-, two ions responsible for the massive influx of

water into the cell through the open Glu-gated channels, can prevent this. On the other hand, delayed neurotoxicity which is caused by NMDA (and, in most cases, KA receptor agonists) occurs over hours after a brief exposure to a high concentration of agonist or prolonged exposure to a low concentration of agonist (Kato et al., 1991; Choi et al., 1987; Rothman et al., 1987). Blockade of acute neurotoxicity does not prevent delayed NMDA- or KA-induced neurotoxicity, indicating that this form of glutamate receptor-mediated neurodegeneration does not depend on acute toxicity. In fact, delayed neurotoxicity *in vitro*, which more closely resembles glutamate receptor-mediated neurodegeneration *in vivo*, has been successfully dissociated from neuronal excitation (Choi et al., 1987; Rothman et al., 1987). Calcium elevation may not need to be prolonged to elicit neuronal damage (Michaels & Rothman, 1990) and, depending on the type of neurone, increase in intracellular calcium may be mediated by both voltage-dependent calcium channels and NMDA receptor-gated Ca^{2+} influx (Frandsen & Schousboe, 1993). In our case, since the quantification of viability following 1 h exposure to glutamate or NMDA was carried out at least 20 h post-treatment, it is reasonable to conclude that the data reflect contributions to neuronal cell death from both events of acute and delayed neurotoxicity. In the event of a stroke, it is known that excitotoxic brain lesions initially cause primary destruction of brain parenchyma, but subsequently result in secondary damage of neurones several hours after the insult. It is this secondary damage that is responsible for most of the volume of the infarcted area and the loss of brain function (Skaper, 2003). In secondary neuronal damage, a major component is the migration of macrophages and microglial cells towards the sites of injury, where they are known to produce massive quantities of toxic cytokines and oxygen radicals (Skaper, 2003).

In relation to neuroprotection, the role of kynurenic acid (KYA) as a glutamate receptor antagonist was confirmed in this study. Even at a low concentration of 100μM, it completely abolished the neurotoxic effects of glutamate and NMDA. Kynurenic acid is an antagonist at both the NMDA and non-NMDA receptors, acting at the strychnine-resistant glycine-sensitive site of the former. It is able to antagonise glutamate receptor activation in rodents and primates (Stone & Perkins, 1984). Endogenously, it is produced in the brain, though synthesized more effectively in the periphery (Scharfman & Goodman, 1998). Research over the last decade has furnished massive evidence that changes in the levels of endogenous KYA can affect NMDA receptor function *in vivo*, thus modifying excitotoxic vulnerability, neurophysiological activity

and behaviour (Coyle, 2006). Kynurenic acid is able to cross the blood-brain barrier (Scharfman & Goodman, 1998, Salvati et al., 1999) and has been used as a lead compound in the development of compounds with potential therapeutic use (Stone & Darlington, 2002) in the treatment of strokes, epilepsy (Rowley et al., 1993; Nichols & Yielding, 1993) and neurodegenerative disorders, following observations that its glutamate antagonist property is responsible for its ability to prevent brain damage from anoxia (Simon et al., 1986) and ischaemia (Germano et al., 1987, Salvati et al., 1999).

5.5.2. Glutamate and osteoblast viability

In contrast to the highlighted observations of glutamate neurotoxicity, no toxicity resulting from glutamate or NMDA exposure was demonstrated in the osteoblast cultures, even at concentrations as high as 1mM, which are profoundly neurotoxic. It is now known that osteoblasts express the whole range of glutamate receptors that display the same electrophysiological characteristics as their neuronal counterparts. In fact, the NMDA-type glutamate receptors expressed by primary rat osteoblasts have the same electrophysiological characteristics as neuronal receptors (Gu et al., 2002). They also express glutamate transporters and the exocytotic machinery required for complete glutamate release. In addition, there is evidence for targeted vesicular glutamate exocytosis in osteoblasts (Bhangu et al., 2001) and the mechanisms of calcium handling are similar to those in neurones (Skerry & Genever, 2001). Therefore, the presence of glutamate release, together with functional receptors on bone cells, raises the possibility that excessive glutamate could also induce damage in bone tissue. However, results from this study render this rationalization largely improbable. Significant increases in cell numbers were observed when 100μM and 1mM glutamate were applied to osteoblast cultures for 1 h and 15 min respectively. Similarly, NMDA 100μM and 1mM induced increases in cell proliferation when applied for up to 3 h. In fact, 20 h exposure to glutamate or NMDA at 4 div resulted in significant trophic effects for concentrations up to 10mM. These findings demonstrate a trophic rather than a toxic role for glutamate in bone. Our data are consistent with previous reports using either cell lines or primary cultures of osteoblasts. Excitotoxic effects have not been demonstrated to date in bone cells. The observation that glutamate release parallels ALP activity (Bhangu et al., 2001) suggests that, once MC3T3-E1 cells differentiate, they express a phenotype resembling that of

glutamate-releasing neurones. The anticonvulsant drug, riluzole, has been reported to inhibit glutamate release and ALP activity and to induce, at higher concentrations, apoptosis in MC3T3-E1 cells, suggesting a trophic role for glutamate in development. In addition, it has been shown that addition of exogenous glutamate (50μM-1mM) promoted osteoblast survival under serum-free conditions and increased the survival rates of human osteoblasts exposed to TNF-α and IFN-γ, providing an evidence for an intrinsic synaptic-like glutamatergic signalling network in bone (Genever & Skerry, 2001). However, according to these authors, 10mM glutamate significantly reduced viable osteoblast numbers. This study exposed the osteoblast cultures to glutamate 10mM only at 4 div and found no toxic but trophic effect. Unlike in the CNS, glutamate release is negatively regulated by voltage-dependent calcium entry, since a continued depolarization with 30-60mM KCl inhibits glutamate release in osteoblastic cells (Genever & Skerry, 2001). The finding in our study that 50mM, but not 10 or 25mM K^+, had a tendency to be toxic to the osteoblast cells is therefore consistent with this report, further supporting the evidence of a trophic role for glutamate, the inhibition (by high potassium) of which removes the effect, resulting in decreased viability.

However, with regard to the action of glutamate in osteoblast cells, our data point to a fundamental line of research that is worth investigating further. This arises from the finding that the application of glutamate, NMDA, or the antagonists MK-801 and D-AP5 in combination with hydrogen peroxide (H_2O_2) tended to be more toxic than the application of peroxide alone. The role of glutamate in disease states in bone characterized by elevated levels of free radicals or ROS such as H_2O_2 therefore demands clarification. In support of these identified concerns, it has been reported that a similar glutamatergic (NMDA) receptor ligand, quinolinic acid, can be excitotoxic to hippocampal neurones at concentrations not injurious to neurones by itself in the presence of donors of nitrosative and oxidative stress (Behan & Stone, 2002). It is therefore possible that, in the presence of oxidative or nitrosative stress, these mechanisms occur in osteoblasts too, thus causing glutamatergic ligands to have a toxic action on MC3T3-E1 cells.

5.5.3. Downstream effectors of glutamate toxicity

It is now known that the neuronal damage that occurs following conditions like stroke is due to the massive release of glutamate from neurones and glia, rather than to the immediate

hypoxia or ischaemia itself (Obrenovitch & Urenjak, 1997), a role that has also been demonstrated for glutamate in neurodegenerative diseases like Alzheimer's, Parkinson's and Huntington's diseases (Coyle & Puttfarcken, 1993). Excessive accumulation of glutamate in the synaptic cleft or the overactivation of its receptors, especially the NMDA receptors, leads to increased intracellular concentration of free calcium, which is able to activate several pathways, including the calcium-dependent nitric oxide synthase (NOS), which generates the free radical nitric oxide (NO) from the amino acid L-arginine. The calcium-dependent activation of phospholipase A_2 (PLA_2) also results in the production of arachidonic acid (AA), which yields superoxide radical through its subsequent metabolism by lipoxygenases and cyclooxygenases to form eicosanoids (Chan & Fishman, 1980). Arachidonic acid and oxygen radicals enhance the release of glutamate and inhibit its inactivation by uptake through glial transporter processes, thus promoting a vicious cycle (Pellegrini-Giampietro et al., 1988; Williams et al., 1989). In addition, under conditions of energy failure and elevated calcium levels, xanthine dehydrogenase could be converted by proteases such as calpains to xanthine oxidase (McCord, 1985), which can in turn yield superoxide radical through its conversion of accumulated hypoxanthine and xanthine to uric acid (Halliwell & Gutteridge, 1989). The dismutation of superoxide radical by superoxide dismutase (SOD) yields H_2O_2. Through the Fenton reaction, H_2O_2 in the presence of transition metals like iron or copper could yield the very reactive hydroxyl radical (Halliwell & Gutteridge, 1989). Besides, NMDA receptor agonists can cause ATP depletion and elevation of AMP within hours, and a concurrent increase in lactic acid (Retz & Coyle, 1982) causes the resulting acidic conditions to promote the release of Fe^{2+} from its cellular stores, thus yielding the hydroxyl radical from H_2O_2 through the Fenton reaction (Halliwell & Gutteridge, 1989). Reaction between NO and superoxide produces peroxynitrite anion which is also a very toxic ROS and can decompose to $^{\cdot}OH$ (Beckman et al., 1990).

The mitochondria act as low-affinity, high-capacity "sinks" to sequester excess calcium in order to maintain its homeostasis (Sapolsky, 2001). However, after a while, a combination of excessive levels of calcium and generated ROS (oxidative stress) overwhelms the capacity of the mitochondria, leading to calcium deregulation and subsequently neuronal damage and death through apoptotic or necrotic pathways involving mediators such as caspases, apoptosis inducing factor (AIF) and the nuclear enzyme, poly (ADP-ribose) polymerase (PARP). The

mediation of glutamate-induced neuronal damage by oxidative stress is now a subject of intensive research and it is now widely accepted that oxidative stress is a causal, or at least an ancillary, factor in the neuropathology of several adult neurodegenerative disorders and conditions such as stroke, trauma and seizures (Coyle & Puttfarcken, 1993) in which excessive extracellular levels of glutamate or the overstimulation of its receptors has been implicated. Our study therefore set out to investigate the effects of oxidative stress induced by ROS and glutamate on CGNs and MC3T3-E1 cells, using the parameter of cell viability as end-point.

5.6. Oxidative stress-induced modulation of cellular viability

The effects of oxidative stress on the viability of MC3T3-E1 osteoblast and CGN cultures were examined using H_2O_2, xanthine/xanthine oxidase (X/XO) mixture and ROS-generating kynurenines- 3-hydroxyanthranilic acid (3-HAA) and 3-hydroxykynurenine (3-HK).

5.6.1. Hydrogen peroxide-induced oxidative stress

Oxidative stress results from excessive production of ROS to a degree that overwhelms the antioxidant defence capacity of a cell (Sapolsky, 2001). It induces significant damage by altering cellular structure and function of biomolecules, including proteins, lipids, and nucleic acids. In neurones, oxidative stress induced by ROS mediates cellular damage by glutamate. Since glutamate is equally released in osteoblasts where its receptors are functionally expressed, the effects of ROS on MC3T3-E1 osteoblast-like cells and cerebellar granule neurones were investigated. Hydrogen peroxide was the main ROS used in this study, even though the effects of free radicals generated by the X/XO mixture and kynurenines (3-HAA, 3-HK) were also investigated at a later stage. Even though H_2O_2 is not very reactive unless its concentration builds up significantly in living tissues, it is very important to the elucidation of pathologies associated with elevated levels of ROS for a number of reasons. It is known that H_2O_2 is produced by almost all cell types and has the profound ability to diffuse within and across cells, unlike superoxide, which is unable to traverse membranes. This ability results from its stable nature, causing H_2O_2 to target a wide range of intracellular and extracellular sites. In addition, in the presence of a transition metal like iron or copper, H_2O_2 produces the hydroxyl radical, which is the most reactive and toxic of the ROS. In the present study, H_2O_2 applied to the 10 div cultures at concentrations up to 100μM showed no deleterious effect on

the osteoblast cultures. In fact, when these cultures were exposed to H_2O_2 concentrations up to 50μM at an earlier developmental stage of 4 div, significant increases in viability, suggesting increased proliferation, were obtained. It is known that exogenously added superoxide and H_2O_2, as active oxygen species, stimulate the expression of early growth-regulated genes such as c-*fos* and c-*jun,* and activate protein kinases distinct from protein kinase C, which suggests that these ROS might function as mitogenic stimuli through signalling pathways common to natural growth factors (Burdon, 1995). The observation that superoxide (or its dismutation product, H_2O_2) are released by cells either constitutively, in the case of tumour cells, or following cytokine stimulation has led to speculation that they serve as an autocrine growth stimulation system or as a means of intercellular communication (Burdon, 1995).

However, in this study, the application of 200μM or above of peroxide for at least 1 h at 10 div elicited significant reduction in osteoblast viability, which was both concentration- and time- dependent. Morphological changes as assessed by phase contrast microscopy correlated well with degrees of reduction in viability, with progressive loss of cells and the rounding up of the remaining cells as peroxide concentrations or durations of exposure were increased. These observations led us to the conclusion that low concentrations of H_2O_2 are generally stimulatory to the growth of MC3T3-E1 cells, while higher concentrations (>200μM) have deleterious effects leading to cell death. This is consistent with previous studies which reported inhibited differentiation of MC3T3-E1 cells (Mody et al., 2001), and with studies of other cell types where H_2O_2 proved toxic (Burdon, 1995; Li et al., 2003; Demelash et al., 2004).

When bone cultures were treated with 200μM of peroxide for 1 h, increasing their recovery duration from 4 to 24 h resulted in a slightly improved viability while treatment for 6 h with the same concentration and lengthening of recovery duration led to a worsening of survival. On the other hand, with a higher concentration of 500μM, raising the duration of recovery after treatment for 1 or 6 h did not affect viability outcome. This observation justified our choice of 4 h recovery duration and a working concentration of 500μM for most subsequent experiments.

Whether H_2O_2 elicited damage directly or indirectly in the MC3T3-E1 cultures was examined. Catalase (CAT) is an enzyme known to catalyse the breakdown of H_2O_2 to water and oxygen, while superoxide dismutase (SOD) converts the superoxide anion to hydrogen peroxide and

oxygen. They are both naturally occurring antioxidant enzymes. Catalase completely prevented the deleterious effects of H_2O_2 at all the concentrations used, while SOD was unable to modulate significantly H_2O_2 effects. The heat-inactivated catalase also completely failed to prevent damage due to peroxide. Taken together, these observations suggest that the observed damage inflicted on the osteoblast cells was due to a direct action by H_2O_2 and that the prevention of this damage was entirely due to the enzymatic action of catalase and not attributable to some other protein that may be present in the "cocktail."

Interestingly, both CAT and SOD induced some mitogenic effect when applied alone. This could imply that MC3T3-E1 cells generate a continuous level of ROS sufficient to restrain cell proliferation. This seems unlikely however, in view of the stimulatory effects of low concentrations of exogenous peroxide. An alternative explanation may be due to effects of CAT and SOD that are not dependent on their metabolism of ROS, for example, the depletion of local metal concentrations below the levels which limit cellular proliferation.

Catalase has been reported to induce COX-2 expression in smooth muscle cells (Chen et al., 1998), macrophages (Jang et al., 2004), microglia (Jang et al., 2005), and hippocampal neuronal cells (Choi et al., 2001), but the mechanisms of induction of COX-2 expression by catalase in osteoblast and neuronal cells are not clear. Extracellular catalase also induced COX-2, IL-8, and stromelysin in primary human chondrocytes (Litvinov &Turpaev, 2004). Whereas COX-1 is constitutive in most cells, COX-2 is inducible by various cytokines, endotoxins, growth factors, or tumour promoters (Baigent & Patrono, 2003), thus demonstrating its prime importance in inflammation and cell proliferation. A new acetaminophen-sensitive isoform, COX-3, has recently been identified mainly in the brain (Chandrasekharan et al., 2002; Shaftel et al., 2003). Constitutively expressed COX-2 has been reported in certain regions of the brain, reproductive tissues, kidney, and thymus (Baigent & Patrono, 2003; Rocca et al., 1999). The possibility that the induction of COX-2 modulates the prevention by catalase of H_2O_2-induced damage was therefore examined using acetylsalicylic acid (aspirin, ASA), a non-specific COX inhibitor. Aspirin had no effect on H_2O_2-mediated osteoblastic damage, indicating that catalase cytoprotection against oxidative damage by peroxide may not be related to its COX-2-inducing potential. However, in contrast to our observation, Petersen et al. (2005) found that low concentrations of NSAIDs or ASA caused a significant protection against H_2O_2-induced apoptosis in human lens epithelial cells whereas

higher concentrations were toxic. This could imply a substantial degree of tissue specificity in ASA-mediated protection against oxidant injury.

Our work and that of others have shown that a combination of ascorbate and β-glycerol phosphate (which we have referred to as switching) increases the proliferation and differentiation of MC3T3-E1 osteoblast cells (Quarles et al., 1992; Harada et al., 1991; Fatokun et al., 2006). Since the more differentiated osteoblast cultures manifest a phenotype resembling the *in vivo* situation more closely, the sensitivities of switch (i.e., the more differentiated) and non-switch (i.e., the less differentiated) cultures to oxidative damage by H_2O_2 were compared. Switch cultures generally tended to be more sensitive to peroxide damage than non-switch cultures, implying that differentiation in osteoblasts may increase sensitivity to oxidative damage. Whether this is related to a developmental change in the level or nature of antioxidant defence systems is not clear and is therefore worth investigating. A similar observation was reported in motor neuronal cell lines: the fully differentiated forms (NSC34D) were more sensitive to low and high concentrations of nitric oxide than the less differentiated (NSC34) cells (Chodimella et al., 2005).

In comparison to the degree of MC3T3-E1 sensitivity to peroxide-mediated damage, cerebellar granule neurones (CGNs) were much more sensitive to this oxidant, to an extent that even 10μM H_2O_2 applied for just 15 min caused a significant reduction in the viability of CGNs, even though we are very cautious here of making conclusive generalizations, as immortal and stem cells could show intrinsic resistance to oxidative stress (Kondoh et al., 2005). The effects of peroxide on CGNs were both concentration- and time-dependent and morphological assessment showed that the degree of peroxide-induced phenotypic changes correlated positively with increasing concentrations of, or the durations of exposure to, the oxidant. Cells were grossly shrunken in appearance with their neuronal processes severely damaged after treatment with H_2O_2. Survival was poorer when the recovery duration was increased from 6 to 24 h, suggesting the initiation of secondary damage to cells that might have previously escaped the oxidative insult and a gradual progression towards complete demise of cells that were initially only partially buffeted. As was the case with osteoblast cells, catalase completely abolished the toxic effect of H_2O_2 on CGNs. Although not statistically significant, aspirin showed a tendency to improve damage caused by H_2O_2, which may imply a possible contributory role for the induction of COX-2 in the event of oxidative damage. This

may also serve to support the oxidative stress theory of glutamate excitotoxicity, as COX-2 contributes to NMDA-mediated neuronal cell death in primary cortical cell culture (Hewett et al., 2000), possibly through its induction of significant oxidative stress following overactivation of glutamate receptors sensitive to NMDA. Agents that are able to block COX-2 would therefore prevent intracellular accumulation of ROS and thus protect against NMDA receptor-mediated excitotoxicity. In fact, COX-2 inhibition has been found to protect cultured CGNs from glutamate-mediated cell death (Strauss & Marini, 2002). However, inhibition of PLA_2 or the subsequent metabolism of arachidonic acid by lipoxygenases, but not by cyclooxygenases, provides protection against glutamate toxicity (see Coyle & Puttfarcken, 1993). This may suggest that the lipoxygenase pathway compared to the cyclooxygenase pathway generates a substantially higher level of oxidants. Overall, the profound sensitivity of CGN cultures to H_2O_2 in this study strongly suggests that ROS are a major effector of glutamate-induced excitotoxicity in the CNS. The use of H_2O_2 as an inducer of damage could be a clinically important model of oxidative stress in neurodegenerative diseases. For example, in Alzheimer's disease, β-amyloid plaque builds up in the brain and causes intracellular accumulation of H_2O_2 (Behl et al., 1995). This work is therefore very relevant to investigation of mechanisms underlying neurodegeneration, with the ultimate aim of finding novel therapies for the associated conditions.

5.6.2. Effects of hydrogen peroxide in the presence of copper (II) ion

The generation of toxic hydroxyl radical by H_2O_2 in the presence of a transition metal like copper or iron through the Fenton reaction is well known (Mazzio & Soliman, 2003). In the MC3T3-E1 cells used in this study, a combination of H_2O_2 and Cu^{2+} (generated from $CuSO_4$) for 1 h resulted in significant reduction in viability, an effect that was more significant than the reduction obtained in the presence of either H_2O_2 or Cu^{2+} alone, suggesting that the hydroxyl radical may be involved in this further lowering of viability. In CGNs, a similar pattern was obtained, although this did not reach significance and so was less clear-cut. A puzzling conundrum, however, was the observation in both MC3T3-E1 and CGN cultures that, in the presence of mannitol, a known scavenger of hydroxyl radical (Khan et al., 2005) and which had no effect on its own on either culture, a combination of H_2O_2 and Cu^{2+} induced a considerably greater reduction in viability than was the case when these two agents were

combined without the scavenger. This phenomenon reached statistical significance with data from osteoblast cultures. A possible explanation of this strange effect is that the addition of mannitol at a concentration of 50mM could disturb ionic homeostasis, thus toppling isotonic balance in the cultures, which could have a net effect of further damaging cellular viability, especially in cells whose integrity has already been compromised through oxidative stress inflicted by the high level of reactive oxygen species such as hydrogen peroxide or even the hydroxyl radical, if it was at all produced through the reaction of hydrogen peroxide with copper.

5.6.3. Xanthine (X) and xanthine oxidase (XO)

The xanthine and xanthine oxidase (X/XO) system represents another free radical-generating system which has been used in many experimental studies as a source of superoxide radical. As explained earlier, XO is able to act on xanthine to produce superoxide radical through its conversion of accumulated hypoxanthine and xanthine to uric acid (Halliwell & Gutteridge, 1989). The ability of the X/XO mixture to modify osteoblastic and neuronal viability was therefore investigated.

No damaging effect was found after co-administration of neurotoxic concentrations of X (100μM) and XO (0.02U/ml) to MC3T3-E1 cells for up to 24 h. Instead, the application of X or X/XO for 24 h produced a significant increase in viability, while XO had no effect at any time point. This seems to conflict with an earlier report of significant induction of oxidative stress in these cells by X/XO (25μM/25mU/ml) (Mody et al., 2001), although it was intracellular ROS production that these authors measured and not the direct effect of the generated ROS on MC3T3-E1 viability. In view of their resistance to oxidative damage, increased levels of ROS in these cells may be difficult to interpret as correlating directly with damage to their viability. The same report showed a significant decrease in ALP activity by X/XO in these cells.

In a striking contrast to what was found with the MC3T3-E1 cultures, the application of similar concentrations of X and XO to CGNs resulted in a profound reduction in viability. Xanthine alone had no effect in most cases, while the application of xanthine oxidase alone resulted in a significant time-dependent lowering of viability. Application of X and XO produced a time-dependent reduction in CGN viability significantly greater than was found

with XO alone. Heat-inactivation of XO led to a complete loss of its enzymatic activity, as the inactivated enzyme had no effect when applied alone or in combination with xanthine, implying that the xanthine oxidase-induced effects were solely due to its enzymatic activity. The nature of the ROS generated by the X/XO mixture was examined using superoxide dismutase (SOD) and catalase (CAT). Surprisingly, SOD completely failed to rescue the lowering of viability induced by X/XO while CAT offered complete protection against the viability-lowering effect of X/XO on CGNs. These data strongly suggest that the damage to neuronal viability by the X/XO mixture, at least in CGNs, could be entirely mediated by hydrogen peroxide under certain experimental conditions and not by the superoxide radical as is commonly the case. Further to this, in view of the ability of XO to impair CGN viability on its own, mediation of this damage by ROS was also examined. The lowering effect of XO on CGN viability was significantly blocked by the XO inhibitor, allopurinol, which restored viability to a level comparable to the control, suggesting that the effect was initiated by XO itself. An attempt to establish whether ROS generation was involved in this phenomenon and if so, the nature of the ROS, revealed that, similar to what was found with the X/XO mixture, H_2O_2, but not superoxide radical, mediated the damage by XO, as CAT was effective in completely preventing this damage, restoring viability to beyond control level, while SOD was entirely incapable of modulating the damage. It is therefore reasonable to conclude that sufficient levels of xanthine are present in the cerebellar granule neurones for exogenously added xanthine oxidase to act upon, resulting in the production of ROS such as hydrogen peroxide, which are capable of eliciting significant neuronal damage. Earlier works have implicated XO as a source of free radicals mediating damage by kainate in mouse CGNs, because allopurinol as well as a combination of SOD and CAT protected against kainic acid-induced toxicity in these cells (Dykens et al., 1987). In this context, protein kinase C (PKC) is thought to be a possible modulator through its effects on intracellular Ca^{2+}, as treatments with gangliosides which can inhibit PKC translocation attenuated KA-induced neurotoxicity in primary cultures of CGNs (Favaron et al., 1988).

5.6.4. Modulation of viability by kynurenines

The effects of two ROS-generating kynurenines on osteoblastic and neuronal viability were examined. 3-hydroxyanthranilic acid (3-HAA) generally had no significant effect on MC3T3-

E1 cultures with up to 24 h of exposure, except for a slight enhancement of viability when 100nM was applied for 1 h. On the other hand, 3-hydroxykynurenine (3-HK) at 1mM significantly and time-dependently damaged osteoblastic viability with 1 and 6 h of exposure, while lower concentrations had no effect. In view of this, the possible effects of 3-HK on the viability of CGNs were also investigated at the same time points. In this case, a significant damage to viability was evident with a much lower concentration of 10μM and the trend of damage was concentration- and time-dependent. 3-hydroxykynurenine is known to generate substantial levels of H_2O_2 and the nature of cell death by this kynurenine is essentially apoptotic whereas its metabolite, 3-HAA, is a far weaker neurotoxin (Okuda et al., 1998). It is therefore possible that the damage inflicted on MC3T3-E1 or CGNs by 3-HK in this study was due to the generation in either of these cell types of hydrogen peroxide, which we have shown to be toxic to these cells on its own. There is a general awareness of the intrinsically higher resistance to cytotoxic agents of cell lines compared to primary cultures. This seems to explain the reason for the lower sensitivity of MC3T3-E1 cultures to 3-HK, in view of the consideration that the differential sensitivities of these and CGN cultures to 3-HK-induced damage could be due to the previously established less vulnerability of MC3T3-E1 cells to hydrogen peroxide, which primarily mediates 3-HK toxicity. Our more extensive work on the toxicity of ROS has been focussed mainly on the effects of exogenously added H_2O_2, but it would be very interesting to confirm whether other ROS are involved in 3-HK toxicity in osteoblasts. This future investigation may further our understanding of this paradigm, especially in the light of the fact that most of the earlier reports have investigated the effects of 3-HK in the CNS and even so, articles such as that of Okuda et al. (1998), which examined the possible brain regional selectivity of 3-HK toxicity, have reported that, while the cortical and striatal neurones were the most vulnerable to 1-10μM concentration of 3-HK, hippocampal neurones were less sensitive and CGNs (whether from embryonic or postnatal rat brains) were totally resistant to these concentrations of 3-HK. It is notable that while the CGNs used in this study also proved completely insensitive to 1 μM 3-HK, they succumbed to damage by 10μM, the upper limit of the concentration range tested by Okuda et al. (1998). However, intermediate concentrations between 1 and 10μM have not been examined in this study. There is evidence that neurotoxicity of lower concentrations of 3-HK (10μM or below) is entirely dependent on cellular uptake processes involving Na^+-dependent amino acid transporters that

recognise as substrates large neutral amino acids like tryptophan, phenylalanine and leucine, but not acidic or basic amino acids. Pretreatment of striatal neurones with xanthine oxidase inhibitors such as allopurinol prevented intracellular accumulation of peroxide as well as cell death induced by these concentrations of 3-HK, indicating that the generation of ROS by 3-HK (1-10μM) occurs intracellularly through 3-HK interaction with xanthine oxidase. On the other hand, neurotoxicity of a higher concentration of 3-HK (100μM) is not dependent on uptake processes and does not show brain region selectivity, possibly because this high concentration undergoes non-enzymatic autoxidation in the extracellular compartment (Okuda et al., 1996; 1998).

The neuronal death processes activated by 3-HK and another kynurenine, quinolinic acid (QA) were compared by Chiarugi et al. (2001). NMDA receptor antagonists and NOS or PARP inhibitors reduced QA, but not 3-HK toxicity. On the other hand, scavengers of free radicals, caspase inhibitors and cyclosporin preferentially decreased 3-HK neurotoxicity.

The significance of 3-HK-induced damage to the pathology of Huntington's disease (HD) has been variously reported. Increased levels of 3-HK have been shown in the brain with several neurodegenerative disorders including HD (Pearson & Reynolds, 1992) and acquired immunodeficiency syndrome dementia (Sardar et al., 1995). When 3-HK at comparable concentrations in the brains with these disorders was applied to primary neuronal cultures, pronounced cell death was found (Okuda et al., 1996), which was attributable to H_2O_2 generated by 3-HK autoxidation. It is interesting to note that NADPH diaphorase-positive neurones are spared 3-HK toxicity, which mimicks the pathological features of HD brain. Therefore, taken together with earlier reports, the results of this current study of 3-HK further lend credence to its critical role in neurodegenerative pathologies, and argues that 3-HK-induced neurotoxicity may represent a suitable experimental model for the elucidation of mechanisms of neurodegeneration, with the potential benefit of aiding the discovery of novel therapies for the treatment of debilitating neurodegenerative conditions.

5.7. SNAP-induced nitrosative stress

The primary interest of this work was on oxidative stress. However, as nitric oxide (NO) is a member of the free radical family with diverse biological functions, both in the CNS and the periphery, its effects on the viability of MC3T3-E1 and CGN cultures were examined, in order

to determine whether NO induced damage through nitrosative stress in a way similar to the oxidative damage induced by H_2O_2, X/XO, or 3-HK. Endogenously, NO is generated through the action of nitric oxide synthase (NOS), of which there are three isoforms (nNOS, iNOS, and eNOS), on the amino acid L-arginine. However, experimental studies investigating the actions of NO use a number of NO donors including sodium nitroprusside (SNP), S-nitroso-*N*-acetylpenicillamine (SNAP), S-nitroso-*N*-acetylcysteine (SNAC), S-nitrosoglutathione (GSNO), nitroglycerine (NTG), the NONOates and 3-morpholinosydnonimine (SIN-1) (Nara et al., 1999; Volbracht et al., 2001; Shishido et al., 2003, Kennedy et al., 2006). The NO donor SNAP, a nitrosothiol derivative, has been used in this study. It is stable in storage (with EDTA added) and yields NO under physiological conditions, with reproducible results. Denitrosation of SNAP and the subsequent release of NO may also be due to catalysis by specific enzymes located at external vascular cell membranes (Kowaluk & Fung, 1990). Exposure of MC3T3-E1 cultures to SNAP at a concentration range of 10μM - 1mM for up to 24 h did not affect viability. When applied for 24 h, SNAP at 10μM resulted in a significant enhancement of baseline viability, possibly indicating NO-mediated cell proliferation. However, a very high concentration of SNAP (5mM) led to a complete demise of the osteoblast cultures. In an earlier study, osteoblast apoptosis resulted when NOS activity was induced and this was enhanced by pretreatment with TNF-α (Damoulis & Hauschka, 1997), suggesting NO-mediated cell death. A study by Chen et al. (2005) using SNP as the NO donor showed that the viability of MC3T3-E1 cells was only significantly affected when the donor was applied at 1.5mM for 16 h or more, or at 2mM for at least 8 h. They concluded that NO released from SNP can induce osteoblast insults and apoptosis, and the mechanism may involve the modulation of mitochondrial functions, intracellular ROS, and Bcl-2 protein. The study was an extension of an earlier one by the same group which showed that NO induces osteoblast apoptosis through the *de novo* synthesis of Bax protein (Chen et al., 2002). With regard to the CGNs, SNAP at 10μM (24 h) enhanced viability, similar to its effects on MC3T3-E1 osteoblast cultures, but significant lowering of viability began to be observed with SNAP at 100μM applied for 6 h. The toxic effects of SNAP were both concentration- and time-dependent. To investigate the possible involvements of NOS and NO-activated soluble guanylate cyclase (sGC) in the observed effects of SNAP, the NOS inhibitor L-NAME and the sGC inhibitor ODQ were employed. At 1mM, L-NAME had no effect on SNAP-induced

lowering of neuronal viability, but at a very high concentration of 10mM, L-NAME significantly protected against SNAP-induced damage, restoring viability to a level comparable to the control, indicating that the activation of endogenous NOS could be a critical factor in the event of neuronal damage elicited by exogenously added SNAP. However, since ODQ at 10 or 100μM completely failed to modulate this SNAP-induced damage, activation of sGC may not be critical to the damage, at least in CGNs. It has been observed that in CGNs, nitric oxide donors elicit apoptosis by a mechanism involving excitotoxic mediators, Ca^{2+} overload, and subsequent activation of caspases (Leist et al., 1997), though hippocampal neurones in organotypic slice culture have been reported to be highly resistant to damage by endogenous and exogenous nitric oxide (Keynes et al., 2004). Calpain inhibitors are known to prevent NO-mediated apoptotic death of CGNs exposed to nitric oxide donors (Volbracht et al., 2001).

To date, controversies remain as to whether this unstable diatomic radical NO causes neurodegeneration on its own. For example, whereas Dawson and co-workers (1992) demonstrated protection against NMDA neurotoxicity in tissue culture by treatment with NOS inhibitors or with reduced haemoglobin (indicating that NO could contribute to neurotoxicity), other investigators have not confirmed protection against NMDA- or glutamate-induced neurotoxicity by inhibition of NOS (Demerle-Pallardy et al., 1991; Pauwells & Leysen, 1992; Hewett et al., 1993). In addition, systemic treatment with NOS inhibitors was found to reduce (Nowicki et al., 1991) or exacerbate (Yamamoto et al., 1992) brain damage resulting from middle cerebral artery ligation, a lesion mediated by NMDA receptors, and NOS-expressing neurones are known to be resistant to both NMDA-mediated and NO-induced neurodegeneration, a phenomenon that could be explained by the protective effect of high concentrations of MnSOD in these cells (Inagaki et al., 1991).

According to Lipton and colleagues (1993), these disparities in the reported roles of NO in neurodegeneration could stem from the profound sensitivity of the NMDA receptor to redox changes: elevated levels of superoxide anion favour its reaction with NO to form the toxic peroxynitrite leading to neurotoxicity, while reducing conditions may support S-nitrosylation of the NMDA receptor thiol, thus downregulating the receptor and conferring protection against neurotoxicity. It is interesting to note that NO has recently been recognised as a novel and potent inhibitor of apoptosis (Figueroa et al., 2005). The nitric oxide donor SNAP was

able to oppose caspase-mediated apoptosis induced by serum deprivation in cortical neurones, although in a normally serum-supplemented medium, high doses of SNAP were neurotoxic in a caspase-3-dependent manner (Figueroa et al., 2005). Another mechanism by which NO could be toxic is through its enzyme inhibition in the mitochondrial respiratory chain (Beltran et al., 2000).

5.8. Effects of mitochondrial poisons

In view of the realisation that the MC3T3-E1 osteoblast cultures were substantially less sensitive to oxidative or nitrosative damage than the CGN cultures, an attempt to compare the sensitivities of these two cultures to mitochondrial poisoning was made. Potassium cyanide (KCN) and 3-nitropropionic acid (3-NPA) are two agents that are known to severely impair mitochondrial functioning in cells. Cyanide is a mitochondrial poison that inhibits cytochrome oxidase. 3-NPA inhibits irreversibly succinic dehydrogenase localized in the mitochondrial inner membrane (Alexi et al., 1998) and is also a respiratory inhibitor of mitochondrial electron transport chain complexes II and III and the tricarboxylic acid cycle (Alexi et al., 1998; Cavaliere et al., 2001). In addition, 3-NPA is an excitotoxin that has been shown to cause brain lesions similar to those of Huntington's disease. The results of this study showed that MC3T3-E1 cells were insensitive to KCN to an extent that even 10mM of the poison applied for up to 24 h produced no effect. The cultures were also insensitive to 3-NPA at 10μM applied for up to 24 h, or at 100μM and 1mM applied for up to 6 h. However, when 3-NPA was applied for 24 h at 100μM and 1mM, viability was reduced significantly by 33% and 57%, respectively.

In CGN cultures, 3-NPA at 10μM for up to 6 h had no effect on viability, but significant reductions in viability were obtained with 100μM applied for 6 h or 1mM applied for 1 or 6 h. This effect was concentration-dependent. Interestingly, the toxic effect of 3-NPA on the CGNs was abolished in the presence of the competitive NMDA receptor antagonist D-AP5, suggesting that the toxic effect of 3-NPA occurs through NMDA receptor activation. This is consistent with earlier observations. Wullner et al. (1994) and Lee et al. (2000) showed that 3-NPA toxicity in the striatum was mediated by NMDA receptor activation, possibly through the removal of the Mg^{2+} blockade commonly associated with the receptor. Similarly, Riepe et al. (1994) showed that the non-competitive NMDA receptor antagonist MK-801 mitigated

morphological lesions caused by 3-NPA. It has been confirmed that oxygen free radicals and peroxynitrite play an important role in the pathogenesis of 3-NPA neurotoxicity, as this deleterious effect was attenuated in copper/zinc superoxide dismutase transgenic mice (Beal et al., 1995). In fact, 3-NPA-induced lesion is used as a suitable model of Huntington's disease and there is evidence that NMDA receptor activation is involved in the degeneration of striatal neurones in Hungtington's disease (Beal et al., 1986; Albin et al., 1990). Agents that improve mitochondrial function or inhibit membrane permeability transition may eliminate increased caspase activation and cell death associated with enhanced NMDAR activity in HD (Zeron et al., 2004). In a related observation, KCN applied at 1mM caused profound damage to the viability of CGNs, and this was also prevented by D-AP5. A report by Patel et al. (1993) showed that the NMDA receptor mediated cyanide-induced cytotoxicity in hippocampal cultures, while Sturm et al. (1993) confirmed that adenosine attenuated KCN-mediated neuronal cell death through A_1-specific mechanisms. It is therefore possible that the protective effect of adenosine is due to inhibition of NMDA receptor activation. In CGNs, cyanide produces a Ca^{2+}-dependent generation of NO and ROS, which is initiated by NMDA receptor activation (Gunasekar et al., 1996). Cyanide-induced toxicity is therefore prevented by NMDA receptor blockade or inhibition of PKC (Rathinavelu et al., 1994; Pavlakovic et al., 1995). Pretreatment of granule cells with NS398, a COX-2 inhibitor, attenuated ROS generation by KCN, suggesting that the COX-2 pathway is an important route for AA metabolism and the production of ROS during cyanide exposure (Gunasekar et al., 1998). The overall results suggest that, while the MC3T3-E1 osteoblast cultures are largely insensitive to the mitochondrial poisons at concentrations that are known to elicit mitochondrial dysfunction, the CGNs do succumb to these mitochondrial poisons in a manner that critically involves a potent activation of the NMDA receptor. Based on this evidence, it could be hypothesized that osteoblast cultures may more likely follow the glycolytic pathway of metabolism, making them less reliant on mitochondrial oxidative phosphorylation for the generation of ATP, whereas neurones such as the CGNs depend mainly on mitochondrial oxidative phosphorylation for ATP production.

5.9. Mechanisms of oxidative and excitotoxic damage: apoptosis and necrosis

The contributions of apoptosis and necrosis to osteoblastic cell death resulting from H_2O_2-induced oxidative damage and to neuronal cell death following both glutamate-induced excitotoxic and H_2O_2-induced oxidative damage were examined. Investigation of apoptotic mechanisms focussed on caspase-3, the executive caspase in apoptosis, and the event of membrane permeability transition (MPT), which allows the release of cytochrome c from the mitochondria for the formation of the apoptosome that subsequently activates caspase-3. Even though it has been shown that staurosporine and hypoxia both cause activation of the protease in these cells (Chae et al., 2000; 2001), there is yet a paucity of studies addressing the role of caspase-3 induction in apoptosis in MC3T3-E1 osteoblast-like cell line. Demonstrating the critical and central role of caspase-3 in excitotoxic cell death of many neuronal cell types, Du et al. (1997) noted that activation of a caspase-3-related cysteine protease is required for glutamate-mediated apoptosis of cultured cerebellar granule neurones. Both *in vivo* and *in vitro* data suggest that glutamate stimulates caspase-3 induction in neurones (Gottron et al., 1997; Tenneti et al., 1998; Tenneti & Lipton, 2000; Hirashima et al., 1999; Thomas & Mayle, 2000; Han et al., 2002; Lok & Martin, 2002; Zhang et al., 2002). Furthermore, caspase-3-deficient mice show doubling of brain size, correlated with decreased apoptosis and premature death (Kuida et al., 1996) and pro-caspase-3 is cleaved into its active form during apoptosis in many cells, including cerebellar granule neurones (Du et al., 1997).

On the other hand, investigation of contributions from necrosis in this study was mainly concerned with poly (ADP-ribose) polymerase 1 (PARP-1), the nuclear enzyme that is known to be activated by necrosis-inducing stimuli and which may, in some cases, also be implicated in the non-caspase type of apoptotic cell death involving the apoptosis inducing factor (AIF).

5.9.1. Induction of caspase-3, membrane permeability transition (MPT) and poly(ADP- ribose) polymerase (PARP)

The agent Z-DEVD-fmk used in this study is an irreversible, cell-permeable caspase-3-selective inhibitor. Even though there have been reports that it is also capable of inhibiting caspases-6, -7, -8 and -10, this only occurs at much higher concentrations and the rank (decreasing) order of binding affinity is caspase-3 >> caspase-8 > caspase-7 > caspase-10 > caspase-6 (Bravarenko et al., 2006). Furthermore, while most earlier works using this agent as

a caspase-3 inhibitor were carried out at a concentration of around 100μM, the concentration used to achieve caspase-3 inhibition in this study was 40μM, less than one-half of what is commonly used. Cultures were pretreated with Z-DEVD-fmk for 1 h and maintained in culture medium containing the inhibitor for the entire period of recovery.

It was clear from the results that inhibition by Z-DEVD-fmk of caspase-3 activation protected, though partially, against oxidative damage in MC3T3-E1 cultures and also against oxidative and excitotoxic damage in CGN cultures. For the MC3T3-E1 cultures, the protective effect of caspase-3 inhibition was most clear-cut with H_2O_2 concentration of 500μM applied for at least 6 h. This suggests the involvement of caspase-dependent apoptosis in cell damage and death mediated by hydrogen peroxide in MC3T3-E1 osteoblast cultures. In CGN cultures, Z-DEVD-fmk did not protect against damage by 10μM H_2O_2 applied for up to 1 h. While it protected partially against damage by a higher concentration of H_2O_2 (100μM), it lacked any effect on damage by this same concentration when treatment was prolonged to 6 h. Interestingly, when tested for its ability to prevent excitotoxic cell death by glutamate, the inhibitor completely protected CGN cultures against cell death resulting from 1 h application of 300μM of glutamate. This most clearly indicates that glutamate-induced excitotoxic cell death occurs through caspase-mediated apoptosis and could be effectively prevented by inhibiting the induction of caspase-3. A number of studies using the glutamate analogue kainic acid (KA), however, concluded that caspase-3 may not play a major role in CGN death following high KA doses (Verdaguer et al., 2002; Smith et al., 2003). This could mean that the NMDA receptor-mediated death of CGNs could involve distinct death pathways different from the ones involved when other ionotropic receptors such as kainate receptors are the major targets of excitotoxicity-inducing ligands.

A critical step that precedes the induction of caspase-3, which has been investigated in this study, is the release of cytochrome c from the mitochondrial inner membrane. This occurs when a pore (membrane permeability transition pore) becomes opened through the process of membrane permeability transition (MPT). The released cytochrome c combines with Apaf-1 and procaspase-9 to form the apoptosome through which the processing of caspase-3 into its active form occurs. However, the release of cytochrome c is preceded by its dissociation from its binding to cardiolipin in the inner mitochondrial membrane, which is stimulated by ROS-mediated cardiolipin peroxidation (Fariss et al., 2005). Blocking permeability transition pore

should therefore prevent the release of cytochrome c and consequently the processing and induction of active caspase-3, thus preventing caspase-mediated apoptotic cell death. Cyclosporin A (CsA) is an immunosuppressant agent that is known to block the permeability transition pore. Its immunosuppressive action results from binding to mitochondrial cyclophilin, an intracellular immunophilin. The cyclosporin/immunophilin complex then inhibits the activity of the calcium-calmodulin-regulated phosphatase, calcineurin (protein phosphatase 2B), resulting in the accumulation of phosphorylated calcineurin substrates in the cell, including the transcription factor, nuclear factor of activated T cells (NF-AT), which is active only in the non-phosphorylated state (Ruiz et al., 2000; Canudas et al., 2004). This results in the blockade of interleukin-2 (IL-2) transcription (Liu, 1993). When used in this study at a range of 0.5 - 10μM, CsA dose-dependently blocked the viability-lowering effect of 6 h (but not 1 h) application of H_2O_2 (500μM) on MC3T3-E1 osteoblast cultures, but the blockade could not totally account for this damaging effect of peroxide. In CGN cultures, CsA at 10μM significantly blocked cell damage by H_2O_2 (10μM) applied for 1 h, although at 0.5μM, it paradoxically tended to potentiate damage by this concentration of H_2O_2. When tested against a higher concentration of H_2O_2 (100μM) applied for 1 h, CsA at 0.5μM had no effect, but at 10μM showed slight protection. On the other hand, the neuronal damage mediated by 300μM of glutamate was completely abolished by 0.5, 1, or 10μM of CsA, with viability rising significantly beyond control levels for CsA 10μM. These data further support the evidence for the involvement of apoptosis in oxidative death of osteoblasts and neurones and in neuronal excitotoxicity, indicating that, while the process of MPT may not fully explain cell death mediated by ROS such as H_2O_2, it (MPT) may be highly critical to the initiation of the cascade of events leading to delayed excitotoxicity by apoptotic mechanisms. Glutamate-induced apoptotic changes involve various mechanisms and biochemical events and it has been shown that mitochondria are central to glutamate-induced cell death (Montal, 1998). This could also be linked to the complete protection afforded by CsA against glutamate neurotoxicity in this study, as previous studies have shown that inhibition of calcineurin prevents the collapse of mitochondrial membrane potential and apoptotic cell death (Ankarcrona et al., 1995; Wang et al., 1999) and that CsA protects against glutamate neurotoxicity through calcineurin-dependent and -independent mechanisms (Brutovetsky & Dubinsky, 2000; Dawson et al., 1993; Ruiz et al., 2000). Nevertheless, data on the effects of

CsA in neuronal preparations are controversial. For example, it has been reported that CsA enhances rather than inhibits MPP^+-induced apoptosis in SH-SY5Y neuroblastoma cells (Fall & Bennett, 1998) and also increases the neurotoxic effect of NMDA in cortical cultures (McDonald et al., 1997). It can also induce apoptosis in mixed neuronal/glial cultures (McDonald et al., 1996). According to Canudas et al. (2004), CsA did not decrease the viability of primary cultures of rat CGN or induce apoptotic features but specifically enhanced the cytotoxicity and apoptosis induced by colchicine, through mechanisms possibly involving Cdk5. Chang & Johnson (2002) showed that the antiapoptotic effects of CsA are more pronounced in the presence of caspase inhibitors.

The release of cytochrome c from the mitochondria prior to the activation of caspases could be induced in a series of steps critically involving calcineurin. Calcineurin does dephosphorylate Bcl-X_L/Bcl-2-associated death promoter (BAD), a pro-apoptotic gene product that is then translocated to the mitochondria to dimerize with B-cell lymphoma/leukaemia-X_L (Bcl-X_L), a process that initiates apoptosis (Wang et al., 1996; 1999). It is this dimerization that induces the release of cytochrome c from mitochondria, and cytochrome c combines with apoptotic protease-activating factor-1 (Apaf-1) and pro-proteases to form the apoptosome, leading the way to cell death by apoptosis (Neame et al., 1998). Besides, the activator protein-1 (AP-1) transcription factor and the cellular counterpart of v-Jun (c-Jun) activation by its N-terminal kinase Jnk, especially Jnk-3 (selectively expressed in the CNS), have been found to be critical, as Jnk-3 knockout protects mice from excitotoxic apoptosis (Tabuchi et al., 1996; Yang et al., 1997a; 1997b). Glutamate-induced apoptosis can also activate the tumour suppressor gene p53 pathway and this can lead to cell death by activating a Bax-related pathway in neurones (Uberti et al., 1998). It therefore means that glutamate induces apoptosis by activating a number of processes that possibly converge into caspase activation. While the gene products that constitute all the targets for caspases may not yet be known, it is clear from the foregoing that caspases play a crucial role in activating cell death by apoptosis during excitotoxicity.

The level of activation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP-1) was also examined. Under normal conditions, PARP, especially PARP-1, is involved in the repair of DNA strands and breaks. However, overactivation of PARP-1, which mostly occurs following cellular insults, could adequately precipitate necrosis, characterized by large DNA fragmentation. It therefore means that prevention of PARP-1 overactivation should be a viable

protective strategy against necrotic cell death. There are a number of PARP inhibitors currently in use, one of which is the very potent DPQ (Takahashi et al., 1997), which has been used in this study at 10μM. It is recognised as a specific PARP-1 inhibitor (Czapski et al., 2004). Cultures were pretreated with DPQ for 1 h and maintained in culture medium containing the inhibitor for the entire period of recovery. Because nicotinamide (NAm) has also been reported to have some PARP-inhibiting potential (Tronov et al., 2002), its effects on oxidative and excitotoxic damage and death were equally examined. As was the case with caspase-3 inhibition, the significant but partial protective effects of DPQ inhibition of PARP-1 in MC3T3-E1 cultures were most clearly observed against 500μM of hydrogen peroxide applied for 6 h. Similarly, nicotinamide at 1mM displayed a significant but partial protection against the damaging effect of H_2O_2 (500μM) applied for 6 h, while its lower concentration (30μM) had no effect on this damage. With regards to CGN cultures, DPQ protected against damage by 10μM H_2O_2 applied for up to 1 h and 100μM applied for up to 15 min. The excitotoxic effect of glutamate (300μM) in CGN cultures was prevented by DPQ to the extent of restoring viability to a level comparable to the control. Nicotinamide at 1mM was also able to confer significant protection against this excitotoxic damage by glutamate. It is possible that nicotinamide rescued the cells in our study only from the necrotic component of the overall H_2O_2-induced cell death, as a report by Tronov et al. (2002) showed that nicotinamide protected resting lymphocytes exposed to hydrogen peroxide from necrosis but not from apoptosis. They argued that the inhibition of DNA repair by NAm (possibly by inhibiting PARP) inhibits the necrotic pathway, thereby enhancing H_2O_2-induced apoptotic death of lymphocytes. However, the protective effect of nicotinamide may be tissue- or insult-specific, as Lin and co-workers (2004) found no protective effect when the compound was tested against glutamate/NMDA toxicity in murine CGNs whereas nicotinamide can exert significant protective effect against striatal lesions induced by the mitochondrial toxin malonate (Beal et al., 1994). Overall, this investigation into mechanisms of oxidative and excitotoxic cell death has furnished a number of very important clarifications. First, it showed that oxidative damage by H_2O_2 in osteoblasts and neurones could involve simultaneous activation of apoptotic and necrotic pathways, neither of which could singly explain the induced cell death in both cell types. Second, excitotoxic cell death involves substantial activation of apoptotic and necrotic pathways, although we have not ruled out the possibility that PARP-1-mediated death in this

paradigm could be intricately linked to apoptosis inducing factor (AIF), which is localized to the intermembrane space of the mitochondria and known to mediate caspase-independent apoptosis. Overactivation of PARP-1 could induce the translocation of AIF, first into the cytosol, and ultimately into the nucleus, where, due to its own lack of intrinsic endonuclease activity, it recruits downstream nucleases such as cyclophilin A and endonuclease G in order to elicit DNA damage leading to apoptotic cell death (Cande et al., 2002; Wang et al., 2002; Cande et al., 2004; Dawson & Dawson, 2004). Overactivation of PARP has been previously observed in neuronal excitotoxicity (Leist et al., 1997). Since the translocation of AIF and its subsequent DNA damage precede the release of cytochrome c from the mitochondria and thus the activation of caspase-3, we propose that the event of membrane permeability transition which heralds cytochrome c release occurs downstream of AIF-induced damage in delayed glutamate-induced neurotoxicity. This could explain why inhibitors of caspases are not effective in certain types of apoptotic death, since the recruitment of AIF itself could effectively induce cell death much before caspase activation is achieved. Energy failure and free radical generation also contribute to caspase-independent neuronal death (Lang-Rollin et al., 2003).

However, despite all these reports, the subject of whether cell death by glutamate-induced excitotoxicity is apoptotic or necrotic is still a matter of intense investigation. For example, Kure et al. (1991) concluded excitotoxicity to be an active suicide process (apoptosis or programmed cell death), since endonuclease and mRNA synthesis inhibitors prevented cell death. On the other hand, workers like Dessi et al. (1993) contended that excitotoxicity in granule neurones is non-apoptotic, because protein synthesis inhibitors and an endonuclease inhibitor, aurintricarboxylic acid, could not prevent cell death in these cells, an observation similar to that of Chihab et al. (1998) who showed that, though glutamate could not induce cell death in neurones less than 6 days old, it was able to induce necrosis, but not apoptosis, at 13 days. In their own work, Ankarcrona et al. (1995) reported glutamate-induced neuronal death as a succession of necrosis and apoptosis, depending on mitochondrial function. An attempt to resolve these obvious disparities could consider some of the points earlier mentioned to conclude that excitotoxic cell death may be far from identical in every neuronal type, due to the high diversity in the expression, localization and function of glutamate receptor subtypes and second messenger systems. Moreover, even though it is a common assumption that cells

die by necrosis when they are exposed to severe insults and by apoptosis when they are subjected to mild trauma (Cheung et al., 1998; Banasiak et al., 2000), excitotoxicity may be an exception to this rule. For instance, Gwag et al. (1997) showed that cultured murine cortical neurones exposed to low concentrations of NMDA, AMPA and kainate showed the classical morphological features of necrosis. In newborn rats, excitotoxic activation of NMDA and non-NMDA glutamate receptors causes neuronal death, with phenotypes ranging from the apoptotic to the necrotic (see Martin et al., 1998). The authors described in their review "structurally different forms of dying cells: a classic apoptotic form; a vacuolated form, similar to the endocytic-autophagic type, and a classic necrotic form". When they examined the progression of excitotoxin-induced neuronal apoptosis in the newborn brain, it was noticed "the vacuolated form is a precursor stage of apoptosis with many similarities to programmed cell death occurring in the developing brain". In their own work, Cheung et al. (1998) showed that micromolar L-glutamate induces extensive apoptosis in an apoptotic-necrotic continuum of insult-dependent, excitotoxic injury in cultured cortical neurones. From all of these arguments, it is clear that excitotoxicity should not be seen as either strictly apoptotic or strictly necrotic, but rather as an intermediate or hybrid form of cell death, lying along a structural continuum, with apoptosis and necrosis at the extremes. The neuronal swelling that occurs early following massive glutamate-induced influx of sodium and chloride ions and water heralds cell lysis and death by necrosis, while the subsequent neurotoxicity occurring several hours after the insult results from caspase-dependent and -independent apoptosis. It is therefore reasonable to say that in glutamate-induced excitotoxicity, neurones die early by necrosis and those neurones that have survived this early death later succumb to neurodegeneration from apoptosis (see Hou & MacManus, 2002).

5.10. Roles of adenosine A_1 and A_{2A} receptors

Using A_1 and A_{2A} adenosine receptor agonists and antagonists, the ability of adenosine and its receptors to modulate basal viability and oxidative damage in MC3T3-E1 cultures and also to modulate oxidative and excitotoxic damage in CGN cultures was examined.

5.10.1. Modulation of viability and oxidative damage in osteoblasts

Application of adenosine (10μM - 1mM) at 10 div resulted in no significant effect on basal viability of the MC3T3-E1 cultures, but when these cultures were exposed at 4 div, a slight trophic effect was obtained with adenosine at 100μM. However, both the A_1 receptor agonist CPA (100nM) and antagonist DPCPX (50nM, 100nM) significantly enhanced basal viability when applied at 10 div for 1 h, an effect that was also seen with the A_{2A} receptor agonist CGS21680 (100nM). The effects of the A_{2A} antagonist ZM241385 were biphasic, with 50μM showing a tendency to be toxic but 100μM enhancing viability when applied for 1 h. Shimegi (1996) reported that ATP and adenosine acted as mitogens in the MC3T3-E1 osteoblast cells, although the effects of adenosine were to a lesser extent; two years later, the same author showed that the mechanisms of adenosine mitogenicity involved two pathways at least, one mediated by xanthine-sensitive receptor and PTX-sensitive G protein and the other through an unknown xanthine- and PTX-insensitive process (Shimegi, 1998). However, consistent with our findings, P1 agonists adenosine and AMP had no effect on intracellular calcium levels in primary osteoblast cultures at 8 div, whereas the nucleotides (P2 agonists) ATP, UDP and ADP triggered increases in intracellular calcium, demonstrating that P2 agonists may be more important in modulating osteoblast responses during differentiation (Orriss et al., 2006). Effects of adenosine have been verified in other tissues. Sexl and co-workers (1995) did observe increased proliferation in human umbilical vein endothelial cells following A_{2A} and A_1 receptor stimulation, even though A_1 receptor activation was much less potent. Subsequent studies by the same group confirmed that the stimulation of proliferation by A_{2A} receptor activation was mediated by MAP kinase (Sexl et al., 1997). In glial cells, activation of the A_{2A} adenosine receptor inhibits nitric oxide production (Brodie et al., 1998). According to Rathbone et al. (1999), it is possible that, generally speaking, low adenosine concentrations induce cellular proliferation whereas high concentrations induce apoptosis in a variety of cell types.

In relation to cytoprotection, adenosine itself was unable to modify damage by H_2O_2 (500μM), but its more stable analogue, 2-chloroadenosine, offered significant, though partial, protection, indicating that adenosine is possibly being too rapidly degraded intracellularly to be able to produce any observable response or protection in these cultures. It would therefore be interesting to test whether inhibitors of enzymes such as adenosine deaminase (which degrades

adenosine), could stabilise adenosine and therefore enhance its neuroprotection. For example, in the post-ischaemic heart, adenosine deaminase inhibition prevents free radical-mediated injury (Xia et al., 1996), possibly by increasing levels of adenosine which could serve as a potent antioxidant. Moreover, adenosine is known to be produced by ROS-generating systems, attenuating the deleterious consequences of ROS through A_1 receptor activation (Almeida et al., 2003). Extracellular adenosine levels could also be increased by inhibiting adenosine kinase (which phosphorylates adenosine to AMP), blocking its uptake, or facilitating its effects by allosteric modulation (de Mendonca et al., 2000).

When tested for their own ability to protect against the same damage by H_2O_2 (500μM), none of the selective ligands elicited any improvement at 50nM, but at 100nM, the A_1 and A_{2A} agonists, CPA and CGS21680, respectively, each offered partial protection, whereas both antagonists (DPCPX and ZM241385, respectively) were still unable to modify the oxidative damage, suggesting that, while high levels of adenosine receptor agonists may be required to produce observable protective effects against oxidant injury in MC3T3-E1 cells, the antagonists have little effect in protecting these cells against oxidative damage, although they may help to improve basal viability. Even though their roles in protection against oxidant injury have been studied in other tissue types, this study seems to be the first to report adenosine receptor roles in protection against H_2O_2-induced damage, specifically in MC3T3-E1 osteoblast cells. Similar to our observation, the activation of A_1 and A_{2A} receptors in immortalized human proximal tubule (HK-2) cells was seen to attenuate H_2O_2-induced injury, involving distinct signalling pathways that are dependent on gene transcription and new protein synthesis. Chronic treatments with a non-selective adenosine receptor agonist abolished the protection by adenosine while chronic treatments with a non-selective adenosine receptor antagonist increased the endogenous tolerance of HK-2 cells to hydrogen peroxide (Lee & Emala, 2002). This could be explained, at least, in part, by the already reported hint that long-term (e.g., 24 h) treatments with adenosine receptor antagonists can have effects that resemble the acute (e.g., 1 h) effects of adenosine receptor agonists, and vice-versa (Jacobson et al., 1996).

5.10.2. Protection against oxidative and excitotoxic damage in neurones

While A_1 receptor activation or A_{2A} receptor blockade has been variously reported to be neuroprotective in neuronal cultures, A_1 receptor blockade or A_{2A} receptor activation is known to induce damage. The direct roles of adenosine receptors in the context of neuroprotection were therefore verified in this study *in vitro* by testing the abilities of the A_1 receptor agonist CPA and the A_{2A} receptor antagonist ZM241385 to elicit protection against a very neurotoxic concentration (30μM) of H_2O_2, as well as examining the abilities of the A_1 receptor antagonist DPCPX and the A_{2A} receptor agonist CGS21680 to exacerbate damage by a moderately neurotoxic concentration (10μM) of H_2O_2. Based on previous experiments, the ligands were each tested at 100nM for 1 h and cultures were pretreated with antagonists for 15 min. The possible effect of the broad-spectrum adenosine receptor agonist, 2-chloroadenosine, on peroxide damage was also investigated, the result of which showed that it lacked any ability to modulate the damage, despite the fact that adenosine has been previously reported to promote neuronal recovery from ROS-induced lesion in hippocampal slices (Almeida et al., 2003), thus suggesting that the protective effects of adenosine or its analogues may not be uniform across brain regions and could also depend on the experimental models used. Both CPA and ZM241385 protected partially against damage induced by 30μM of H_2O_2, thus confirming that either A_1 receptor activation or A_{2A} blockade is protective against oxidative damage in neurones, including the CGNs. In contrast, however, exacerbation of peroxide damage was not found with DPCPX or CGS21680, suggesting that in CGNs, at least, blockade of A_1 receptors or the activation of A_{2A} receptors may not sufficiently exacerbate oxidative damage in the absence of other potentiating factors or cellular events. Interestingly, a similar result was obtained when these adenosine receptor ligands were tested for their ability to modify glutamate-induced excitotoxicity: CPA offered complete protection, ZM241385 offered almost complete protection, DPCPX tended to exacerbate neuronal damage (effect was not significant), and CGS21680 had no effect. It is known that the adenosine concentration in the extracellular fluid (ECF) is normally in the low micromolar range or less, but this can increase dramatically in conditions like hypoxia, hypoglycaemia or ischaemia, during which endogenous adenosine then becomes sufficient to reduce glutamate-induced currents mediated via the NMDA receptor, thus eliciting a degree of neuroprotection, although this really depends on the degree of ischaemia and the individual dynamics of the adenosine system

(Stone & Addae, 2002). As part of its mechanism of action, the adenosine A_1 receptor is able to inhibit glutamate release into the synaptic cleft from the presynaptic neurone and can also cause hyperpolarization of the postsynaptic membrane through the ATP-sensitive potassium channels (K_{ATP}) as well as direct inhibition of certain kinds of Ca^{2+} channels (Wu & Saggau, 1994; Ribeiro, 1995). All these processes contribute to the net result of neuroprotection by decreasing Ca^{2+} entry, which is a key step in excitotoxic damage. A_1 adenosine receptor upregulation and activation has been shown to attenuate neuroinflammation and demyelination in a model of multiple sclerosis (Tsutsui et al., 2004). However, despite their impressive protective ability, there is an increasing level of concern with the prospect of using A_1 receptor agonists as neuroprotective agents, owing to the discovery that activation of the NMDA receptor reduces neuronal sensitivity to adenosine (Bartrup & Stone, 1988; 1990; Nikbakht & Stone, 2001; Shahraki & Stone, 2003). In order to circumvent this potential problem, antagonists at the A_{2A} receptors, such as SCH58261 and ZM241385, are now of particular interest with a growing literature indicating an ability to protect neurones against damage caused by a range of toxins, including glutamate and NMDA receptor agonists (Jones et al., 1998a; 1998b), and in situations involving oxidative stress such as cerebral hypoxia and ischaemia (Stone, 2002). This has led to the development of A_{2A} receptor antagonists for use in neurodegenerative disorder clinical trials (Phillis & Goshgarian, 2001). Indeed, it has been demonstrated that antagonists at adenosine A_{2A} receptors are neuroprotective in animal models of ischaemia (Sheardown & Knutsen, 1996; Monopoli et al., 1998; Ongini, et al., 1997) and excitotoxicity (Jones et al., 1998a; 1998b; Stone et al., 2001). The use of knockout models has also enabled confirmation that the effects of A_{2A} receptor antagonists are due to the blockade of receptors, rather than to an unrecognized or non-specific action of the compounds (Stone, 2005). An A_{2A} receptor antagonist, KW-6002 (istradefylline), has shown potential in a recently completed phase II clinical trial as a novel treatment for Parkinson's disease and has now entered phase III trials (Hauser et al., 2003; Weiss et al., 2003), and others such as V2006, which was derived from the antimalarial drug mefloquine and is well tolerated in high doses, are in development (Weiss et al., 2003; Matasi et al., 2005; Peng et al., 2004). Because the A_{2A} receptors are low-affinity receptors for adenosine as opposed to the high-affinity A_1 receptors, blockade of A_{2A} receptors is therefore not likely to impair the beneficial effects of A_1 receptor activation by endogenous adenosine. Activation of A_{2A} receptors- and even A3

receptors (von Lubitz, 1999)- could inhibit A_1 receptor activation. Using A_{2A} receptor antagonists, this inhibitory effect of the A_{2A} receptor on A_1 receptor activation could therefore be removed, resulting in additional neuroprotective effect furnished by the latter. It is now well known that the mechanism of damage by activation of A_{2A} receptors involves their enhancement of glutamate release, which can facilitate oxidative stress in the cell. A_{2A} receptors can also mediate inhibition of NMDA receptor function, although A_{2A} antagonists may be unable to directly inhibit NMDA effects (Popoli et al., 2003). In conditions like hypoxia, hypoglycaemia, and ischaemia, the balance between adenosine A_{2A} receptor-mediated direct inhibition of NMDA receptor function and their stimulation of glutamate release could be critical to neuroprotection. When elevation of extracellular levels of adenosine is within the range 1 - 10μM, it will begin to activate the potentially protective adenosine A_1 receptor population (von Lubitz et al., 1988; von Lubitz et al., 1989; MacGregor & Stone, 1993; MacGregor et al., 1993). However, should the levels of adenosine rise further to around 20μM or above, the activation of adenosine A_{2A} receptors may begin to predominate and since these receptors increase the release of glutamate and some other transmitters, these high adenosine levels could result in an exacerbation of neuronal damage. The protection by A_1 receptor activation or A_{2A} receptor blockade against oxidative and excitotoxic damage in this study suggests that the mechanism of adenosine protection against glutamate-induced excitotoxicity could involve substantial antioxidant action, thus providing further evidence that oxidative stress is a critical downstream effector of glutamate-induced excitotoxicity and that adenosine has significant antioxidant properties, possibly through facilitation of A_1 receptors. Despite the impressive potential of A_{2A} receptor antagonists as neuroprotective agents, a study by Blum et al. (2003) has cautioned that their therapeutic use, especially in Huntington's disease, could exhibit undesirable biphasic neuroprotective-neurotoxic effects because blockade of A_{2A} receptors has differential effects on striatal cell death *in vivo*, depending on its ability to modulate presynaptic over postsynaptic receptor activity. With regard to this, Sebastiao & Ribeiro (1996) have suggested that this effect may only be specific to receptors from this brain region that are already proposed as being atypical, owing to their enhanced G_s coupling. Nevertheless, blockade of A_{2A} adenosine receptors has been shown to prevent basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes (Brambilla et al., 2003).

In our *in vitro* study, no neuroprotective or neurodetrimental effect was found with A_{2A} receptor activation, but *in vivo* data have shown that both adenosine A_{2A} receptor agonists and antagonists are neuroprotective. The neuroprotection that is paradoxically seen with adenosine A_{2A} receptor activation *in vivo* could be attributed to effects on cerebral blood flow, glucose utilization, platelet aggregation and superoxide generation from neutrophils. Activation of A_{2A} receptors increases cerebral blood flow and inhibits platelet aggregation (Stella et al., 1996; Hourani, 1996). These effects would increase blood and nutrient supply to any ischaemically compromised area of the brain. Adenosine A_{2A} receptor activation also depresses cerebral glucose utilization in the cortex (Nehlig et al., 1994), which may be of help to neuronal viability by reducing the requirement for depleted nutrients during ischaemia. Activation of adenosine A_{2A} receptors decreases superoxide anion production in neutrophils (Cronstein et al., 1985) and it is possible that adenosine could reduce free radical-associated cell damage that occurs in ischaemia and excitotoxicity (Lafon-Cazal et al., 1993; Reynolds & Hastings, 1995; Patel et al., 1996). Adenosine A_{2A} receptors may therefore exert neuroprotection *in vivo* via peripheral mechanisms of action. Centrally, it is known that, adenosine A_{2A} receptor can enhance evoked GABA release from hippocampal synaptosomes (Cunha & Ribeiro, 2000a) and this could result in a general reduction of neuronal excitability and hence limit cell death during an ischaemic insult. In addition, it is also now clear that adenosine A_{2A} receptor has a wide range of intracellular effectors, which can influence neuronal excitability and viability, thus exerting a direct neuroprotective effect. In the striatum, this receptor couples to G_s and in this brain region, CGS21680 inhibits the conductance of NMDA receptor channels by a mechanism that involves the PLC / IP_3 / calmodulin and calmodulin kinase II pathway (Wirkner et al., 2000). The adenosine A_{2A} receptor can also inhibit N-type calcium channels in PC12 cells (Park et al., 1998). It causes the activation of ATP-sensitive potassium channels (K_{ATP}) and large conductance calcium-activated potassium channels (BK_{Ca}) in the rat epididymis, meaning that, if the same effector activation occurs in the CNS, it could exert a hyperpolarizing effect on neurones and so limit their death during an excitotoxic insult (Haynes, 2000). In PC12 cells, adenosine A_{2A} receptors have a close synergistic relationship with growth factors, especially NGF (Arslan et al., 1997; Arslan & Fredholm, 2000). Microglial NGF is enhanced by adenosine A_{2A} receptors (Heese et al., 1997) and this relationship may be found in other cells also. Furthermore, it has been discovered that

adenosine A_{2A} receptors can directly activate Trk neurotrophin receptors in the absence of neurotrophins, thus protecting against apoptosis induced by growth factor withdrawal in hippocampal neurones (Lee & Chao, 2001; Lee et al., 2002). While there is evidence for the stimulation of expression for the adenosine A_{2A} receptor gene by hypoxia in PC12 cells (Kobayashi & Millhorn, 1999), chronic hypoxia reduces adenosine A_{2A} receptor-mediated inhibition of calcium current in rat PC12 cells via downregulation of protein kinase A (Kobayashi et al., 1998).

In relation to the involvement of non-neuronal cells in the neuroprotective effects of purines, Ciccarelli et al. (2001) has argued that there is an involvement of astrocytes in purine-mediated reparative processes in the brain. AIT-082 is neuroprotective against kainate-induced neuronal injury in rats. It is a hypoxanthine derivative that stimulates *in vitro* neurite outgrowth and the production of adenosine and neurotrophins from astrocytes (Di Iorio et al., 2001). Ferreira & Paes-de-Carvalho (2001) showed that adenosine inhibits glutamate toxicity in retinal neurones through a long-term activation of A_{2A} receptors and elevation of intracellular cyclic AMP levels.

5.11. Areas of further research

It is certain from the results of this study that many more areas of investigations would be worth pursuing in future. Of late, there has arisen a lot of active interest in signalling pathways involving a multiplicity of intra- and inter-cellular factors and mediators. Studies of these intriguing cascades of signalling networks now provide experimental evidence helping to fine-tune explanations of complex phenomena, including mechanisms of cell proliferation, cell damage and death, and cytoprotection. Most of these attempts invariably involve the powerful tools of cell and molecular biology. Investigating at the molecular level the various signalling pathways that could be involved in the observed differential effects of glutamate and other agents on osteoblastic and neuronal viability may therefore further tremendously the interesting pharmacological endeavours described in this study. Possible answers may lie in differences in the pattern of genetic regulation of intracellular mediators or events, either at the transcriptional, translational, or post-translational level.

6. CONCLUSIONS

The following conclusions may be drawn from the results presented in this thesis:

1. The alamar blue reduction assay for the routine determination of cell viability and the quantification of effects of toxicants is a quick and simple method that is accurate and gives reproducible results.
2. The MC3T3-E1 osteoblast-like cell line is a suitable model for osteogenic development *in vitro*.
3. Osteoblasts may represent a less fastidious cell type than neuronal cells in their requirements for survival in culture.
4. β-glycerol phosphate acts synergistically with ascorbate to increase osteoblastic proliferation and differentiation and these supplements are capable of increasing alkaline phophatase (ALP) activity up to four-fold.
5. The increased differentiation of osteoblasts in the presence of ascorbate and β-glycerol phosphate sensitizes the cells to oxidative damage.
6. Glutamate or NMDA has trophic rather than toxic effects on osteoblasts, even at concentrations that are known to be profoundly neurotoxic. Glutamate toxicity may therefore not extend to bone tissue, although in the presence of inducers of oxidative or nitrosative stress, toxicity may result.
7. Glutamate toxicity in cerebellar granule neurones occurs mainly through overstimulation of the NMDA receptor and involves substantial activation of both apoptotic and necrotic death pathways.
8. Neuronal cultures are more sensitive to oxidative stress resulting from a variety of sources including hydrogen peroxide, xanthine/xanthine oxidase and kynurenines (e.g., 3-hydroxykynurenine) than osteoblast cultures. The same is true for nitrosative stress. It is therefore possible that, while neurones depend critically on oxidative phosphorylation for the generation of energy (ATP), the osteoblasts may preferentially rely on the glycolytic pathway.
9. Hydrogen peroxide-induced oxidative damage to osteoblasts and neurones occurs through both apoptosis and necrosis.
10. Scavengers of the hydroxyl radical such as mannitol may precipitate paradoxical toxicity under certain conditions, possibly related to a disturbance of cellular ionic homeostasis.
11. Under certain experimental conditions, the superoxide-generating mixture of xanthine and xanthine oxidase may generate predominantly hydrogen peroxide.

12. The mitochondrial poisons potassium cyanide and 3-nitropropionic acid elicit neurotoxicity via activation of the NMDA receptor.
13. Adenosine and its receptors participate in protection against damage and death in osteoblasts and neurones, though possibly via different mechanisms in these cell types. While both A_1 and A_{2A} agonists were protective against peroxide-induced damage in osteoblasts and antagonists lacked any protective effect, it was A_1 agonists and A_{2A} antagonists that showed protection against the same damage in neurones, while A_1 antagonists and A_{2A} agonists failed to exacerbate damage. Following treatment of CGNs with glutamate, the same pattern of protection by adenosine receptors against peroxide damage in these cultures was observed.

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