THE EFFECTS OF GLUTAMATE AND ADENOSINE RECEPTOR LIGANDS ON NEURONAL AND BONE CELL CULTURES.

By

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A thesis submitted for the degree of Master in Science
in the Faculty of Science, University of Glasgow.

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December 2002.

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Contents.	Page
List of diagrams, figures, tables and photographs.	**6**
Declaration.	**8**
Acknowledgements.	**8**
Abbreviations.	**9**
Abstracts.	**12**
1. Introduction.	**15**
1.1. Glutamate.	**15**
1.1.1. The NMDA receptor.	*15*
1.1.2. Kynurenines.	*15*
1.2. Ischaemia.	**16**
1.2.1. The excitotoxic cascade.	*16*
1.3. Adenosine.	**19**
1.3.1. Adenosine A_{2A} receptors.	*19*
1.3.2. Adenosine A_{2A} receptors and neuroprotection.	*20*
1.3.3. Adenosine A_{2A} receptors and apoptosis.	*23*
1.4. The basic multicellular unit and bone remodelling.	**23**
1.4.1. Bone remodelling.	*23*
1.5. Disorders of bone remodelling.	**24**
1.6. Mechanisms of cell death.	**25**
1.6.1. Necrosis.	*24*
1.6.2. Apoptosis.	*25*
1.7. Mechanisms of excitotoxic cell death.	**25**
1.6.1. Mechanisms of quinolinic acid induced cell death.	*26*
1.7.2. Mechanisms of glutamate induced apoptosis.	*27*
1.7.3. Caspases.	*27*
1.7.4. Caspase-3 induction and apoptosis in osteoblasts.	*29*
1.8. The osteoblast.	**29**
1.8.1. Osteoblast proliferation.	*30*
1.8.2. Osteoblast differentiation.	*31*
1.8.3. Alkaline phosphatase.	*31*
1.8.4. Osteoblastic cell death.	*32*
1.8.5. Postmenopausal osteoporosis and osteoblastic apoptosis.	*33*
1.9. Glutamate and bone cell development.	**34**
1.9.1. The role of glutamate in bone cell viability.	*34*

1.10. Cell culture systems for the assessment of cytotoxicity. **35**
1.10.1. Primary cell cultures. *36*
1.10.2. Cell lines. *37*
1.10.3. MC3T3-E1 cells. *38*
1.10.4. Adenosine A_{2A} receptors and MC3T3-E1 cell development. *39*

1.11. Aims. **40**

2. Methods for the effects of glutamate and adenosine receptor ligands on neuronal cultures.

2.1. Animals. **41**
2.1.1. Animal source. *41*
2.1.2. Animal selection. *41*

2.2. Microsurgery for the dissection of cortical tissue. **41**

2.3. Mechanical and enzymatic disruption of tissue. **42**
2.3.1. Inactivation of trypsin. *43*

2.4. Cell seeding and plating. **43**
2.4.1. Coating of wells. *43*
2.4.2. Cell density. *43*

2.5. Cell characterization. **44**
2.5.1. Cell markers. *44*
2.5.2. The avidin-biotin-peroxidase complex (ABC) method. *45*
2.5.3. Immunocytochemistry protocol. *45*
2.5.4. Procedure for fixing. *47*
2.5.5. Procedure for immunostaining. *47*

2.6. Cell treatment **49**

2.7. Assessment of cell death **49**

2.8. Statistical analysis **50**

3. Methods for the effects of glutamate and adenosine receptor ligands on bone cell cultures.

3.1. Cell culture. **51**
3.1.1. Freezing down of MC3T3-E1 cells. *51*
3.1.2. Subculturing of cells. *52*
3.1.3. Resurrection of frozen cells. *52*

3.2. Mitogenic activity assay. **53**

3.3. Assay for alkaline phosphatase activity. **54**

3.4. Cell death assessment. 56
3.4.1. Necrosis. 56
3.4.2. Apoptosis. 56
3.4.3. Immunocytochemical method for caspase-3 expression. 56

3.5. Drugs. 58

3.6. Statistical analysis. 59

4. Results for the effects of glutamate and adenosine receptor ligands on neuronal cultures.

4.1. Development of cortical and hippocampal neurons in culture. 60
4.1.1. Cell characterization. 60

4.2. Effect of NMDA on cortical neuronal cultures. 63
4.2.1. Effect of AP5 on cell death induced by NMDA. 63

4.3. Effect of kynurenines on cortical neuronal cultures. 63

4.4. Effect of CGS 21680 on cortical neuronal cultures. 70

4.5. Effect of ZM 241385 on cortical neuronal cultures. 70

4.6. Effect of NMDA on hippocampal neuronal cultures. 73

5. Results for the effects of glutamate and adenosine receptor ligands on bone cell cultures.

5.1. Characterization of MC3T3-E1 cells. 74

5.2. Effect of NMDA on MC3T3-E1 cell development. 74
5.2.1. Effect of NMDA on MC3T3-E1 cell proliferation. 74
5.2.1.1. 24 hour exposure. 74
5.2.1.2. 72 hour exposure. 78
5.2.2. Effect of NMDA on MC3T3-E1 cell differentiation. 78

5.3. Effect of QA on MC3T3-E1 cell development. 78
5.3.1. Effect of QA on MC3T3-E1 cell proliferation. 78
5.3.1.1. 24 hour exposure. 78
5.3.1.2. 72 hour exposure. 82
5.3.2. Effect of QA on MC3T3-E1 cell differentiation. 82

5.4. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell development. 82

5.4.1.Effect adenosine A_{2A} receptors on MC3T3-E1 cell proliferation. 82

5.4.2. Effect adenosine A_{2A} receptors on MC3T3-E1 cell differentiation. *86*

5.5. Effect of nirosative and oxidative stress on MC3T3-E1 cell viability. **86**

5.6. Effect of serum withdrawal on MC3T3-E1 cell apoptosis. **89**

6. Discussion of the effects of glutamate and adenosine receptor ligands on neuronal cell cultures.

6.1. Discussion of hippocampal vs. cortical neuronal cultures as *in vitro* models of excitotoxicity. **91**

6.2. Discussion of cell viability assays for quantification of excitotoxic cell death. **93**

6.3. Discussion of effects of agents on cortical neuronal cultures. **95**
6.3.1. Effect of AP5 on cortical neuronal cultures. *95*
6.3.2. Effect of kynurenines on cortical neuronal cultures. *95*
6.3.3. Effect of CGS 21680 on cortical neuronal cultures. *96*
6.3.4. Effect of ZM 241385 cortical neuronal cultures. *97*

6.4. Discussion of the signal transduction mechanism whereby the adenosine A_{2A} receptor elicits neuroprotection. **98**

6.5. Discussion of the mechanism of cell death induced by glutamate receptor ligands. **99**

7. Discussion of the effects of glutamate and adenosine receptor ligands on bone cell cultures.

7.1. Differentiation of MC3T3-E1 cells. **101**

7.2. Effect of NMDA on MC3T3-E1 cell development. **101**

7.2.1. Effect of NMDA on MC3T3-E1 cell proliferation. *112*

7.2.2. Effect of NMDA on MC3T3-E1 cell differentiation. *102*

7.3. Effect of QA on MC3T3-E1 cell development. **102**
7.3.1. Effect of QA on MC3T3-E1 cell proliferation. *102*
7.3.2. Effect of QA on MC3T3-E1 cell differentiation. *102*

7.4. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell development. **103**

7.4.1. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell proliferation. *103*

7.4.2. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell differentiation. *104*

7.5. Effect of nitrosative and oxidative stress on MC3T3-E1 cell viability. **105**

7.5.1. Effect of nitrosative stress on MC3T3-E1 cell viability. *105*

7.5.2. Effect of oxidative stress on MC3T3-E1 cell viability. *106*

7.6. Effect of serum withdrawal on MC3T3-E1 cell viability. **107**

8. General discussion. **108**

8.1. Discussion of the use of a cell line vs. the use of a primary culture for examining the effects of glutamate on cell development. **108**

8.2. Osteoblasts appear not to be vulnerable to excitotoxicity. **108**

8.3. Further work. **110**

8.4. Conclusions. **112**

9. References. **113**

List of diagrams, tables, figures and photographs.

Effects of glutamate and adenosine receptor ligands on neuronal cell cultures.

Table 1. Potential mechanisms by which A_{2A} receptors could modulate neuronal viability in cortical neuronal cultures. **22**

Figure 1. Simplified overview of pathophysiological mechanisms in the ischaemic brain. **17**

Figure 2a. Photomicrograph of immunostained neurons at 14 days *in vitro*. **61**

Figure 2b. Photomicrograph of anti-glial fibrillary acidic protein (GFAP) immunostained hippocampal cultures. **62**

Figure 3. Effect of CGS 21680 and AP5 on cortical neuronal cultures. 64

Figure 4. Effect of ZM 241385 and AP5 on cortical neuronal cultures. 65

Figure 5a. Photomicrograph of untreated cortical neuronal cultures at 14 DIV. 66

Figure 5b. Photomicrograph of effect of 30 μM NMDA on cortical neuronal cultures. 67

Figure 6. Photomicrograph of effect of 100 μM NMDA on cortical neuronal cultures. 68

Figure 7. Effect of kynurenines on cortical neuronal cultures. 69

Figure 8. Effect of NMDA on hippocampal neuronal cultures as assessed by trypan blue exclusion. 71

Figure 9. Effect of NMDA on hippocampal neuronal cultures as assessed by MTS reduction. 72

Effects of glutamate and adenosine receptor ligands on bone cell cultures.

Figure 10. Effect of NMDA on MC3T3-E1 cell viability. 75

Figure 11. Effect of NMDA and H_2O_2 on MC3T3-E1 cell viability. 76

Figure 12. Effect of NMDA on MC3T3-E1 cell differentiation. 77

Figure 13. Effect of quinolinic acid on MC3T3-E1 cell viability. 79

Figure 14. Effect of quinolinic acid and H_2O_2 on MC3T3-E1 cell viability. 80

Figure 15. Effect of quinolinic acid on MC3T3-E1 cell differentiation. 81

Figure 16. Effect of CGS 21680 and ZM 241385 on MC3T3-E1 cell viability. 83

Figure 17. Effect of CGS 21680 and ZM 241385 on MC3T3-E1 cell differentiation. 84

Figure 18. Effect of H_2O_2 on MC3T3-E1 cell viability. 85

Figure 19. Effect of CGS 21680 and H_2O_2 on MC3T3-E1 cell viability. 87

Figure 20. Effect of ZM 241385 and H_2O_2 on MC3T3-E1 cell viability. 88

Figure 21. Effect of serum withdrawal on caspase-3 expression in MC3T3-E1 cells. 90

Figure 22. Summary of the mechanisms whereby adenosine and adenosine A_{2A} receptor ligands could modulate cell damage during an ischaemic insult. 100

Declaration.

I hereby declare that this thesis is my own composition and has not been submitted for consideration for any other degree in this or any other university.

Chris Gudgeon.

Acknowledgements.

First, I would like to thank Professor Stone and Dr. Smith for their valuable supervision and allowing me to undertake this project. I also thank the members of Professor Stone's laboratory, namely Andrew Mallon, Ali Shahraki, Dr. Duncan Macgregor, Dr. Caroline Forrest, Dr. Cathy Clark, Rosalind Macmillan and Jean Smith as well as various other members of the Division of Neuroscience and Biomedical Systems, too numerous to mention here for helpful tips, suggestions and pieces of advice without which the thesis would not have been possible.

Abbreviations.

α-MEM	--alpha minimum essential medium
AA	--ascorbic acid
ABC	--avidin-biotin complex
ALP	--alkaline phosphatase
AP-1	--activator protein-1
Apaf-1	--apoptotic protease activating factor-1
AP5	--2-amino-5-phosphonopentanoic acid
ATP	--adenosine 5'-triphosphate
AMPA	--α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMP buffer	--2-amino-2-methyl-1-propanol buffer
Bcl	--B-cell lymphoma
β-GP	--beta glycerol phosphate
BMU	--basic multicellular unit
CADO	--chloroadenosine
CGS 15943	--[9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo (1,5-c) quinazolin-5-imine]
CGS 21680	--2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamidoadenosine hydrochloride
CHA	--cyclohexyladenosine
C-JUN	--cellular counterpart of v-Jun oncogene (from the Japanese for 17 (ju-nana)) originally discovered in Avian Sarcoma Virus 17.
CNS	--central nervous system
CPA	--cyclopentyladenosine
DAB	--di-azo-aminobenzene
DEPC	--diethylpyrocarbonate
DIV	--days in vitro
DMEM	--Dulbecco's modified Eagle's medium
FADH	--flavin adenine dinucleotide hydrogenase
FCS	--foetal calf serum
GFAP	--glial fibrillary acidic protein
GS	--goat serum
H_2O_2	--hydrogen peroxide
HENECA	--2-hexenyl-5-N-ethyl carboxamido adenosine
IFN-γ	--interferon gamma
Jnk-3	--c-Jun-N terminal kinase
MC3T3-E1	--murine calvariae 3T3-E1
MTS	--(3-(4,5-dimethylthiazol-2-yl)-5-(-3-carboxymethoxyphenyl)-2-(-4-sulfophenyl)-2H-tetrazolium inner salt
MEM	--minimum essential medium
Mg^{2+}	--magnesium ion
NAD	--nicotine adenine dinucleotide
NADPH	--nicotine adenine dinucleotide phosphate hydrogenase
NECA	--2-hexenyl-5-N-ethyl carboxamido adenosine
NF	--neurofilament
NGF	--nerve growth factor
NMDA	--N-methyl-D-aspartate
PB	--phosphate buffer
PBS	--phosphate buffered saline

PCD	--programmed cell death
PDL	--poly-D-lysine
P/S	--penicillin-streptomycin
PKA	--protein kinase A
PKC	--protein kinase C
PLC	--phospholipase C
PTX	--pertussis toxin
QA	--quinolinic acid
SCII 58261	--5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-c]-1,2,4-triazolo [1,5-c] pyrimidine
SNAP	-- S-nitroso-N-acetylpenicillamine
TNF-α	--tumour necrosis factor alpha
Trk	--tyrosine kinase
TUNEL	-- terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling
ZM 241385	--4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol

Abstract.

Effect of glutamate and adenosine A_{2A} receptor agonists and antagonists on neuronal cell cultures.

In vivo data have shown that both adenosine A_{2A} receptor agonists and antagonists are protective against neuronal damage produced by ischaemia. This project aimed to elucidate the direct role of the A_{2A} receptor in neuronal viability using cortical neuronal cultures so that specific experimental questions in the whole animal can be formulated.

Cell death was assessed by trypan blue uptake. In cortical neuronal cultures, N-methyl-D-aspartate ((NMDA) 0, 10, 30 & 100 μM)) induced concentration-dependent cell death. This was attenuated by the NMDA selective antagonist, DL-2-amino-5-phosphonopentanoic acid (AP5) to control levels ($p<0.01$, $n=13$).

0.02 μM 2-p-(2-carboxyethy)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680) reduced the level of cell death induced by 10 μM and 30 μM NMDA to below basal (control) levels. 0.05 μM CGS 21680 showed a tendency to increase neuronal death. However, as the concentration of the compound was increased to 0.5 μM and then 5 μM there was a trend of decreased cell death. This was to the extent that at 5 μM, CGS 21680 reduced cell death induced by 30 μM NMDA to control levels.

0.05 μM and 5 μM 4-(2-[7-amino-2-{2-furyl}{1,2,4} triazolo {2,3-a}{1,3,5}triazin-5-yl-amino]ethyl) phenol (ZM 241385) reduced cell death induced by 100 μM NMDA to near control levels ($p<0.01$, $n=4$).

Quinolinic acid (300, 1000 & 3000 μM) induced a trend of increasing cell death in cortical neuronal cultures, an action which kynurenic acid (1000 μM) had no effect on.

In hippocampal neuronal cultures, N-methyl-D-aspartate (NMDA) also induced concentration-dependent cell death as assessed by trypan blue uptake. However, as assessed by (3-(4,5-dimethylthiazol-2-yl)-5-(-3-carboxymethoxyphenyl)-2-(-4-sulfophenyl)-2H-tetrazolium salt; (MTS) reduction, 1mM NMDA caused an increase in neuronal viability ($p<0.001$, $n=4$) due to superoxide accumulation.

The data suggest that the adenosine A_{2A} receptor activation is neuroprotective when cell damage is mild. However, activation of the adenosine A_{2A} receptor would be neurotoxic when cell damage is more severe due to the pathological accumulation of adenosine enhancing glutamate release. Adenosine A_{2A} receptor ligands might be of value in the treatment of neurodegenerative disorders.

Effect of glutamate and adenosine A_{2A} receptors on bone cell cultures.

There is increasing evidence that glutamate signalling occurs in bone. Excitotoxicity, therefore might underlie the increased osteoblastic cell death that occurs during disorders of bone remodelling such as osteoporosis. This project sought to examine if adenosine A_{2A} receptors could modulate this form of cell death in MC3T3-E1 cells, an osteoblastic cell line.

Cells were characterised by assaying for alkaline phosphatase to ensure they differentiated in accordance with the literature. MC3T3-E1 cells cultured in media containing ascorbate and β-glycerol phosphate showed an almost 2-fold higher alkaline phosphatase activity than cells cultured in media without these supplements ($p<0.01$).

Cell viability was assessed by Alamar Blue reduction. Exposure of MC3T3-E1 cells to 100 μM and 300 μM NMDA caused a time- and dose-dependent increase in their viability ($p<0.05$, $n=3$). However, a prolonged exposure (72 hr) of MC3T3-E1 cells to NMDA did not induce an increase in their viability.

NMDA (100, 300 & 1000 μM) increased alkaline phosphatase activity in MC3T3-E1 cells, an effect which AP5 tended to reduce.

Quinolinic acid (QA 300, 1000 & 3000 μM) induced a time and dose dependent increase in MC3T3-E1 cell viability ($p<0.01$, $n=3$). However, a prolonged exposure (72 hr) of MC3T3-E1 cells to 3000 μM QA did not induce an increase in their viability.

QA (300 & 1000) increased alkaline phosphatase activity in MC3T3-E1 cells, an effect which AP5 tended to reduce. However, 3000 μM QA did not increase alkaline phosphatase activity.

CGS 21680 alone elicited an increase in MC3T3-E1 cell viability. ZM 241385 alone elicited an increase in MC3T3-E1 cell viability ($p<0.05$, $n=6$). CGS 21680 induced a greater increase in cell viability when it was combined with ZM 241385 than it did alone.

CGS 21680 increased alkaline phosphatase activity in MC3T3-E1 cells, an effect which ZM 241385 trended to reduce.

Hydrogen peroxide (1000 μM) dramatically reduced cell viability in MC3T3-E1 cells ($p<0.001$, $n=6$). However, 10 and 100 μM hydrogen peroxide were without effect. NMDA, QA, CGS 21680 and ZM 241385 had no effect on the response to hydrogen peroxide. However, 100 μM H_2O_2 significantly reduced the proliferative effect of the CGS 21680 and ZM 241385 ($p<0.05$, $n=6$).

Serum withdrawal increased the number of MC3T3-E1 cells positively immunostaining for caspase-3 ($p<0.05$). Cells positively staining for caspase-3 also displayed distinct chromatin condensation and cell shrinkage. CGS 21680 & ZM 241385, the adenosine P_1 receptor agonist cyclopentyladenosine and quinolinic acid had no effect on the expression of caspase-3.

In summary, the data confirm that glutamate acts as a trophic factor in bone cell development via the NMDA receptor (Taylor & Skerry, 2001) and that these cells do not succumb to excitotoxicity, at least on a short exposure to excitotoxins. Adenosine A_{2A} receptors have a trophic effect on MC3T3-E1 cell development. Further research into the molecular pharmacology of glutamate and adenosine receptors in bone cells is required. However, it is suggested that NMDA and adenosine A_{2A} as well as the kynurenine pathway could offer a therapeutic target in disorders of bone cell remodelling such as osteoporosis.

1. Introduction.

1.1. Glutamate.

Glutamic acid is the most abundant transmitter in the mammalian central nervous system, accounting for up to 50% of all rapid excitatory synapses in the CNS (Stone & Burton, 1988; Nakanishi et al., 1998). It mediates its diverse physiological effects via two main classes of receptor, ionotropic and metabotropic. Ionotropic glutamate receptors have been defined by their prototypic agonists, namely the N-methyl-D-aspartate (NMDA), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and the kainate receptors. Metabotropic glutamate receptors (of which there are 9) are G-protein coupled receptors (Nakanishi et al., 1998). In recent years, it has been revealed that signalling by glutamate also occurs in peripheral tissues, one of which is bone (Genever & Skerry, 2001).

1.1.1. The NMDA receptor.

This receptor, like other ligand-gated ion channels is thought to be a heteroligomeric protein (Yakamura & Shimoji, 1999). It is composed of an NR1 subunit and at least one isoform of the NR2 subunit and possibly NR3 subunits. The receptor has a high unitary conductance, is permeable to Na^+ & Ca^{2+} (influx) and K^+ (efflux) and exhibits a voltage dependent block by Mg^{2+} under resting conditions (Yakamura & Shimoji, 1999). This latter feature endows the receptor with a state-dependent activation mechanism allowing it to underlie plastic phenomena such as long term potentiation in the CNS.

1.1.2. Kynurenines.

Glutamate is not the only endogenous ligand for the NMDA receptor. Another is quinolinic acid (QA), an intermediate along the kynurenine pathway (Stone, 1993; 2001) which metabolises over 90 % of dietary tryptophan which is not used in protein synthesis to generate the essential cofactors nicotinic acid and NAD.

QA has a preference for NR2A & 2B containing NMDA receptors (Carvalho et al., 1996). Although not as potent a neuroexcitant as NMDA, it is almost as potent a neurotoxin due to its effects on glutamate release and uptake (Tavares et al., 2002), mitochondrial function and generation of reactive oxygen species. This molecule is not the only member of the metabolic pathway active at glutamate receptors. Kynurenic acid was found to behave as a broad spectrum glutamate antagonist soon after the excitatory properties of QA were discovered in the early 1980s. It has since been established that the compound has an especially high affinity, in the low micromolar range, as antagonist at the strychnine-resistant glycine co-agonist site on the NMDA receptor (Stone, 1993). This feature has led to kynurenate being the lead compound for use in a range of CNS disorders (Stone, 1993).

1.2. Ischaemia.

Ischaemic stroke occurs due to a transient or permanent reduction in cerebral blood flow that is restricted to the territory of a major brain artery (Dirnagl et al., 1999). The reduction in flow is, in most cases caused by the occlusion of a cerebral artery either by an embolus or by local thrombosis. With an incidence around 250-400 in 100000 and a mortality of rate of around 30 %, stroke remains the third leading cause of death in industrialized countries (Dirnagl et al., 1999). In the USA alone, four million survivors are coping with its debilitating consequences (Dirnagl et al., 1999). Despite numerous encouraging preclinical data, there has only been one successful clinical trial to date so an effective treatment for the condition is still necessary (De Keyser et al., 1999).

1.2.1. The excitotoxic cascade.

Glutamate needs to be kept at resting levels of < 1 μM to mediate the crucial physiological functions of neuronal growth and development and synaptic plasticity. However brain tissue has a relatively high consumption of oxygen and glucose and depends almost entirely on oxidative phosphorylation for energy production (Krnjevic, 1999).

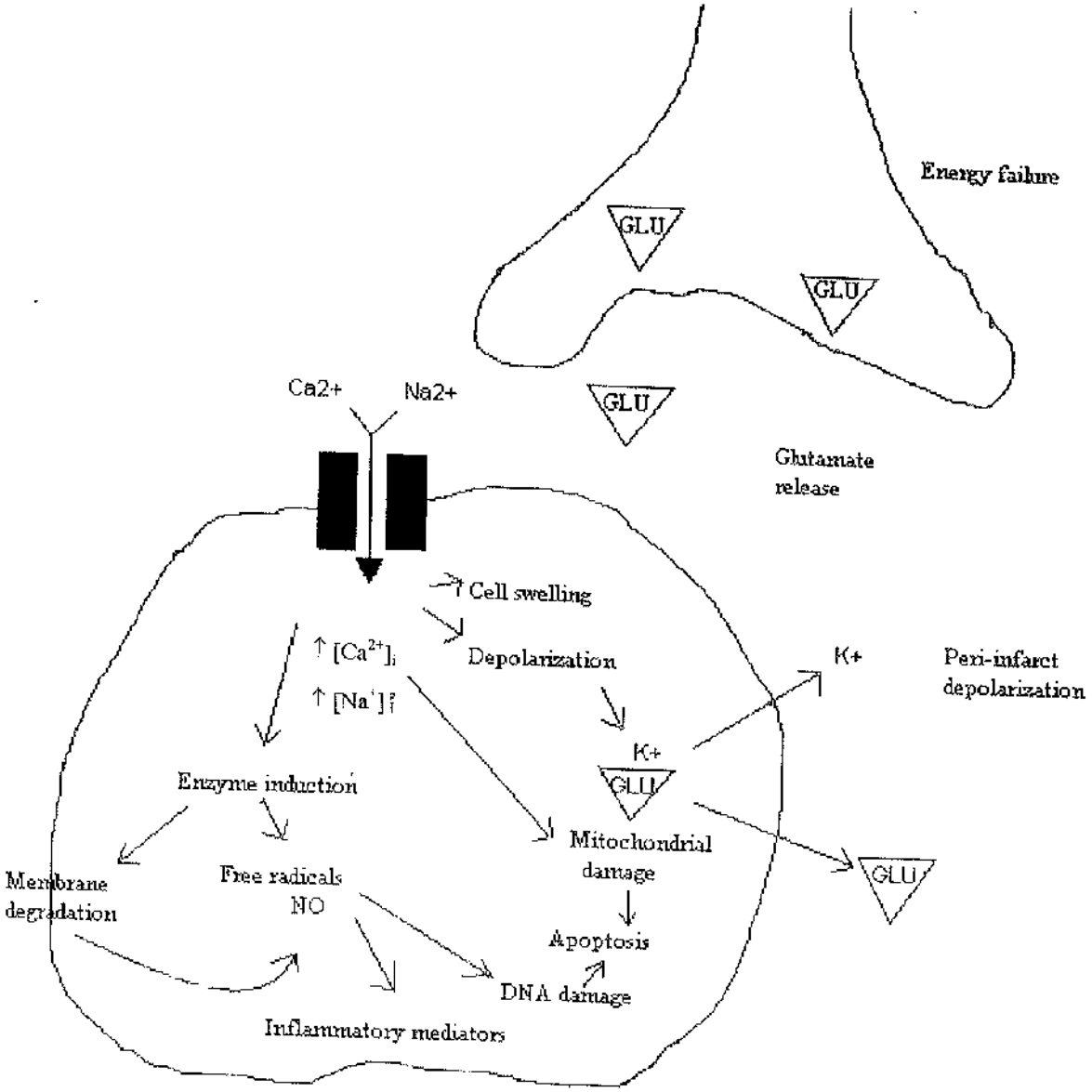

Figure 1. Simplified overview of pathophysiological mechanisms in the ischaemic brain. Energy failure leads to the depolarisation of neurons. Activation of specific glutamate receptors dramatically increases intracellular Ca^{2+}, Na^{+} and Cl^{-} levels while K^{+} is released into the extracellular space. Diffusion of glutamate (GLU) and K^{+} into the extracellular space can propagate a series of waves of depolarisation (peri-infarct depolarizations). Water moves into to the intracellular space via osmotic gradients and cells swell. The universal intracellular messenger Ca^{2+} overactivates numerous enzyme systems (proteases, lipases, endonucleases etc.). Free radicals are generated which damages membranes, mitochondria and DNA, in turn triggering caspase-mediated cell death (apoptosis). NO=nitric oxide. Modified from Dirnagl et al., (1999).

Thus, focal impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose and impairs the energetics required to maintain ionic gradients. With energy depletion, membrane potential is lost and neurones and glia depolarize. As a result somatodendritic and presynaptic voltage dependent Ca^{2+} channels activate, spilling glutamate into the extracellular space (Small et al., 1997). At the same time, the energy dependent processes, such as presynaptic reuptake of excitatory amino acids are impeded, further increasing the accumulation of glutamate into the synaptic cleft.

Activation of AMPA/kainate receptors (Weiss and Sensi, 2000) allows Ca^{2+} entry through these receptors as well as depolarizing the cell enough to remove the Mg^{2+} block of the NMDA receptor (Kumamoto, 1996). Entry via this receptor-channel complex is the main source of cytoplasmic Ca^{2+} entry but metabotropic glutamate receptors also play their part via phospholipase C and Ins(1,4,5) P_3 signalling in inducing intracellular Ca^{2+} overload (Sattler and Tymianski, 2000).

Other ions such as Na^+ and Cl^- enter the neurones via channels for monovalent ions (e.g. the AMPA receptor channel). Water then follows passively as the influx of Na^+ and Cl^- is much larger than the efflux of K^+. The increase in the ubiquitous second messenger, Ca^{2+} (Sattler and Tymianski, 2000) is thought to initiate a series of cytoplasmic and nuclear events that impact the development of tissue damage profoundly. These include activation of proteolytic enzymes that degrade cytoskeletal proteins, for example actin and spectrin (Furukawa et al., 1997) as well as extracellular matrix proteins such as laminin (Chen and Strickland, 1997).

Activation of phospholipase A_2 and cyclooxygenase generates free radical species that overwhelm endogenous scavenging mechanisms, producing lipid peroxidation and membrane damage. Nitric oxide synthesised by the Ca^{2+} dependent enzyme, neuronal nitric oxide synthase (NOS) reacts with a superoxide anion to form the highly reactive species, peroxynitrite that induces tissue damage. In contrast, increased production of nitric oxide by endothelial cells can protect the tissue by improving the microcirculation (Iadecola, 1997).

Mitochondria, which are an important source of reactive oxygen species are impaired by free radical mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport, H^+ extrusion and ATP production (Dugan and Choi, 1994). The mitochondrial membrane becomes leaky, partly owing to the formation of a mitochondrial permeability transition pore, which promotes mitochondrial swelling, the cessation of ATP production and an oxygen free radical burst. Cytochrome C is released from mitochondria and provides a trigger for cell death.

1.3. Adenosine.

Catabolic activity of several major metabolic pathways leads to the production of adenosine (Fredholm, 1997). Extracellularly this purine is widely present in mammalian organisms, especially in the CNS, heart and blood vessels. At rest extracellular ecto-5'-nucleotidases provide another significant source by converting released adenine nucleotides to adenosine (Von Lubitz, 1999). However, during metabolic stress induced by elevated neuronal electrical activity, hypoxia or ischemia the bulk of adenosine originates from the intense degradation of ATP. During ischemia the concentration of the purine increases 30-100 times above the resting level of about 100 nM (Von Lubitz, 1999).

1.3.1. Adenosine A_{2A} receptors.

Adenosine acts on 4 G-protein coupled receptors, namely A_1, A_{2A}, A_{2B} and A_3 receptors. The A_{2A} receptor is the high affinity A_2 receptor and couples mostly to G_s but also to G_i/G_o and $G_{12/13}$ (Moreau & Huber, 1999; Cunha, 2001). A number of selective ligands are available to explore the function of the receptor. The majority of A_{2A} selective agonists are 2-substituted adenosine derivatives or analogs (see Muller, 2000 for a recent review). The rank order of potency of these compounds is 2-hexenyl-5-N-ethyl carboxamido adenosine (NECA) > 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamidoadenosine hydrochloride (CGS 21680) > chloroadenosine (CADO) > cyclohexyladenosine (CHA), with CGS 21680 being the most selective.

However caution should be exercised with the use of CGS 21680 as although it is often referred to as being "A_{2A} selective", it only shows moderate affinity (19 nM at rat, 27 nM at human A_{2A} receptors) and is quite potent at rat, and particularly at human, A_3 receptors (Muller, 2000). Therefore CGS 21680 is only 30-fold selective for A_{2A} receptors in rat and virtually non-selective in humans. Other potent A_{2A} agonists that have been developed are non-selective since they also exhibit high affinity for A_3 receptors as well and some of them are also very potent at A_1 receptors. So far no satisfactory adenosine A_{2A} receptor selective agonist is available (Muller, 2000).

The most commonly used A_{2A} antagonists are [9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo (1,5-c) quinazolin-5-imine] (CGS 15943), 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol
ZM 241385 and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo [4,3-c]-1,2,4-triazolo [1,5-c] pyrimidine (SCH 58261) (Moreau & Huber, 1999) and a study by Ongini and colleagues (1999) sought to evaluate whether these antagonists were "A_{2A} selective" and which one was the most useful in this regard. CGS 15943 had micromolar affinity for the receptor, however, its A_{2A} vs. A_1 selectivity was negligible, thus caution should be exercised with the use of this agent. ZM 241385 and in particular SCH 58261 showed good A_{2A} selectivity so this latter compound should be the preferred antagonist.

1.3.2. Adenosine A_{2A} receptors and neuroprotection.

The adenosine A_{2A} receptor has a range of neuromodulatory effects which can influence neuronal viability (Table 1, p.23). On the detrimental side it is known that the adenosine A_{2A} receptor inhibits A_1 receptors (Cunha, 2001). Furthermore, 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). Antagonism of this action most probably underlies the neuroprotective effect of A_{2A} receptor blockade which has been demonstrated *in vivo*. In the striatum, it has been shown that the A_{2A} receptor can inhibit evoked GABA release (Kirk & Richardson, 1995).

However, this effect may only be a feature of receptors from this brain region which have been proposed as being atypical due to their enhanced G_s coupling (Sebastiao & Ribeiro, 1996).

It is clear that the neuroprotection paradoxically also seen with the adenosine A_{2A} receptor activation *in vivo* can be attributed to effects on cerebral blood flow, glucose utilization, platelet aggregation and superoxide generation from neutrophils (see e.g. Jones et al., 1998a). However, it is known that the adenosine A_{2A} receptor can enhance evoked GABA release from hippocampal synaptosomes (Cunha & Ribeiro, 2000) which could lead to a general attenuation of neuronal excitability and so limit cell death during an ischaemic insult.

Futhermore, it is now becoming clear that the adenosine A_{2A} receptor has a wide range of intracellular effectors which can influence neuronal excitability and viability. For example, in the striatum it has been shown that this receptor can couple to G_i and in this brain region CGS 21680 inhibits the conductance of NMDA receptor channels, by a mechanism that involves the PLC, IP_3, calmodulin and calmodulin II kinase pathway (Wirkner et al., 2000). The adenosine A_{2A} receptor can also inhibit N-type calcium channels in PC12 cells (Park et al., 1997). Furthermore, Haynes (2000) showed that the receptor causes ATP-sensitive potassium channel (K_{ATP}) and large conductance potassium activated calcium channel (BK_{Ca}) activation in the rat epididymis so if the same effector activation occurs in the CNS, it could exert a hyperpolarizing effect on neurons and so limit their cell death during an excitotoxic insult. Furthermore, in PC12 cells it is known that adenosine A_{2A} receptors have a close synergistic relationship with growth factors, especially NGF (Arslan et al., 1997; Arslan & Fredholm, 2000). Also, microglial NGF is enhanced by adenosine A_{2A} receptors (Heese et al., 1997) and this relationship might occur in other brain cells as well. Finally, it has recently been revealed that adenosine A_{2A} receptors can directly activate Trk neurotrophin receptors in the absence of neurotrophins (Lee et al., 2002).

Table 1. Potential mechanisms by which A_{2A} receptors could modulate neuronal viability in cortical neuronal cultures.

Beneficial.	Reference.
↓ NMDA conductance via PLC, IP_3, calmodulin and CamKII activation	Wirkner et al., 2000.
↓ N-type Ca^{2+} channel conductance via PKA activation	Park et al., 1997.
↑ K_{ATP} + BK_{Ca} conductance via PKA and G activation	Haynes et al., 2000.
↑ neurotrophin activity	Arslan et al., 1997; Arslan & Fredholm, 2000; Heese et al., 1997; Lee, Ragapogal & Chao, 2002
Detrimental.	
↑ K^+ stimulated Glu release from neurons via PKC modulation of P-type Ca^{2+} channels	Marchi et al., 2002; Cunha, 2001.
↓ GABA release via PKA & PKC activation	Kirk and Richardson, 1995; Cunha, 2001.
↓ A_1 receptor activity via PKC activation	Cunha, 2001.

1.3.3. Adenosine A_{2A} receptors and apoptosis.

It is generally accepted that at low concentrations adenosine induces cellular proliferation whereas high concentrations induce apoptosis of a variety of cell types (Rathbone et al., 1999). Some recent studies examining the role of the adenosine A_{2A} receptor in apoptosis of blood cell types have given conflicting results with adenosine A_{2A} receptor activation observed to enhance (Barbieri, 1998; Apasov; 2000) apoptosis. However, Huang and colleagues (2001) showed that the adenosine A_{2A} receptor was capable of protecting against apoptosis in PC 12 cells. Very recently, it has been demonstrated that A_{2A} receptors are capable of protecting against apoptosis induced by growth factor withdrawal in hippocampal neurons by direct activation of Trk B receptors in the absence of neurotrophins (Lee & Chao, 2001).

1.4. The basic multicellular unit and bone remodelling.

For centuries the skeleton has been recognised as a means of locomotion, protection of vital organs and provision of a readily available store of calcium and phosphorus. However, it is only comparatively recently has it been appreciated that bone is a dynamic tissue that is constantly remodelling itself, a process carried out by two main types of cells: osteoclasts and osteoblasts. The former erode cavities in bone and the latter then synthesize new bone matrix, all to the single purpose of bone renewal. Remodelling or turnover is carried out by juxtaposed osteoclasts and osteoclasts comprising temporary anatomical structures known as basic multicellular units (BMUs) (Weinstein & Manolagas, 2000).

1.4.1. Bone remodelling.

Bone turnover starts by conversion of an inactive skeletal surface to a remodeling site, a process known as activation. This involves proliferation of new blood vessels needed to bring recruited resorbing cells to the remodelling site and retraction of the flat, pavement-like cells that cover inactive surfaces to expose the mineralized bone surface. The recruited cells become multinucleated osteoclasts, which attach to the newly exposed bone surface with a ring of contractile proteins, sealing off a subosteoblastic resorption compartment.

The osteoclasts then secrete lysosomal enzymes, hydrogen ions and collagenase from their basement membrane to form a resorption bay or cavity (Parfitt et al., 1981). After the cavity is formed, the osteoclasts detach from bone and move away to a new resorption site. When the osteoclasts have moved away, osteoblasts are brought in to reconstitute the previously resorbed cavity with new bone, a process known as coupling. During the 6 to 9 month lifespan of a BMU, the shorter lived osteoclasts and osteoblasts require continual replacement while maintaining the same discrete spatial and temporal relationships to each other as the BMU digs through the bone. By this means, the adult skeleton, containing approximately 35 million bone structural units or BSUs (one from each previous BMU) is almost completely regenerated every 10 years (Corral et al., 1998). In healthy human adults, 3 to 4 million BMUs are initiated each year and approximately 1 million are operating at any one time.

1.5. Disorders of bone remodelling.

One of the most prevalent disorders of bone remodelling is postmenopausal osteoporosis. The condition is characterized by low bone mass and microarchitectural deterioration of the skeleton leading to an increased risk of fracture after minimal trauma. It is the most common form of osteoporosis and the most common of all systemic osteopathies. Annually, in the U.S. alone, the loss of bone in the disorder results in approximately 300,000 hip fractures and $9 billion in direct medical costs (Bason, 1996). These fractures result from the accelerated loss of bone that occurs in women after natural or surgically induced menopause.

1.6. Mechanisms of cell death.

1.6.1. Necrosis.

Traditionally, cell death has been classified, based on morphological criteria as being either necrotic or apoptotic (Wyllie et al., 1980). Necrosis is in general, a form of cell death that rapidly occurs in response to severe insults such as anoxia and cell trauma and is associated with alterations in calcium and sodium ion homeostasis. The main morphological changes in cells undergoing necrosis include cellular and organellar swelling, especially the mitochondria.

Subsequently there is a dissolution of organelles, rupture of the plasma membrane with leakage of cellular contents in to the extracellular space and random DNA degradation following histone proteolysis. This form of cell death usually affects large groups of adjacent cells and is associated with an inflammatory reaction.

1.6.2. Apoptosis.

In contrast, apoptosis is a delayed form of cell death resulting from less severe insults and is associated with activation of a "genetic program". Cells undergoing this form of cell death exhibit shrinkage of the cytoplasm and condensation of nuclear material into "clumps" (Banasiak, 2000).

As the process continues the nucleus undergoes fragmentation and the endoplasmic reticulum fuses with the plasma membrane forming vesicles and convoluting its surface. In the final stages of apoptosis, there is cellular fragmentation forming membrane-bound apoptotic bodies that contain intact cytoplasmic organelles and nuclear fragments.

1.7. Mechanisms of excitotoxic cell death.

It is now becoming clear that a binary scheme for classifying neuronal cell death is severely flawed as excitotoxic cell death often appears as neither apoptotic or necrotic. Thus, in cerebellar granule cells, Dessi et al. (1993) concluded that excitotoxicity is non-apoptotic as protein synthesis inhibitors and an endonuclease inhibitor (aurintricarboxylic acid) could not protect against cell death in these cells. Also, an interesting study by Chihab et al. (1998) showed that glutamate could not induce cell death in neurons less than 6 days old but at 13 days the cells underwent necrosis and not apoptosis. In contrast, Kure et al. (1991) concluded that excitotoxicity is an active suicide process as endonuclease and mRNA synthesis inhibitors prevented cell death. A study by Leist and co-workers (1997) demonstrated, using cerebellar granule cells, that nitric oxide donors elicit apoptosis by caspase activation.

Although it is often assumed that cells die by necrosis when they are exposed to severe insults and by apoptosis when they are subject to mild trauma (Cheung et al., 1998; Banasiak et al., 2000), excitotoxicity can be an exception to this. For example 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. This theory is also backed up by *in vivo* studies mainly performed by Martin and colleagues (1998). These authors observed that in newborn rats, excitotoxic activation of NMDA and non-NMDA glutamate receptors causes neuronal death with phenotypes ranging from apoptotic to necrotic. They observed "structurally different forms of dying cells: a classic apoptotic form, similar to the endocytic –autophagic type and a classic necrotic form".

Also, when these authors examined the progression of excitotoxin-induced, neuronal apoptosis in the newborn brain, they observed that "the vacuolated form is a precursor stage of apoptosis with many similarities to programmed cell death occurring in the developing brain".

1.7.1. Mechanisms of quinolinic acid induced cell death.

It is becoming increasingly evident that pathological concentrations of QA encountered in the brain and cerebrospinal fluid in certain circumstances can cause neuronal damage as well. Although QA is not as strong a neuroexcitant as NMDA, it is almost as potent a neurotoxin as it can enhance glutamate release from neurons. Furthermore, QA can also inhibit uptake of the transmitter into glia (Tavares, 2002) as well as having the capacity to cause mitochondrial dysfunction, lipid peroxidation and generate free radical damage (Stone, 2001). *In vitro* studies have shown that micromolar concentrations of QA are toxic when exposed to cells for several hours (Kim & Choi, 1987; Khaspekov et al., 1990; Galarraga et al., 1990; Keilhoff & Erdo, 1991; Chiarugi et al., 2001).

Furthermore, corticostriatal cell cultures exposed to submicromolar concentrations of QA for several weeks are susceptible to excitotoxicity (Whetsell & Schwarcz, 1989).

Thus, excitotoxic cell death is not identical in every neuronal type as one would not expect it to be due to the high diversity in the expression, localization and function of glutamate receptor subtypes and second messenger systems. Furthermore, it seems that excitotoxicity should not be seen as either strictly apoptotic or strictly necrotic but exists as an intermediate or hybrid form of cell death, lying along a structural continuum with apoptosis and necrosis at the extremes.

1.7.2. Mechanisms of glutamate-induced apoptosis.

Various mechanisms and biochemical events have been implicated in glutamate-induced apoptotic changes. More recently, it has been shown that mitochondria are central in glutamate-induced cell death (Montal, 1998). For example, inhibition of calcineurin, (a calcium-calmodulin regulated phosphatase) prevents the collapse of mitochondrial membrane potential and apoptotic cell death (Ankarcrona et al., 1996; Wang et al., 1999).

Calcineurin dephosphorylates Bcl-xl/Bcl-2 associated death promoter (BAD) a pro-apoptotic gene product which is then translocated to the mitochondria to dimerize with B-cell lymphoma/leukaemia-xl (Bcl-xl), a process that initiates apoptosis (Wang et al., 1996, 1999). It is this dimerization that induces the release of cytochrome c from mitochondria and along with apoptotic protease activating factor-1 (Apaf-1) and pro-proteases, proteases are formed and lead the way to cell death by apoptosis (Neame et al., 1998).

1.7.3. Caspases.

Whereas BAD can initiate apoptosis, it is well recognized that the effector molecules are proteases, serine and cysteine proteases, termed caspases. However, recently, there has been extensive interest in the processes of cell death and controversy continues to surround the definitions that distinguish apoptosis from other forms of cell death.

Because of this confusion in interpretation of these results, the definition of apoptosis has changed. It is defined as the process of cell death associated with caspase activation or caspase-mediated cell death and presumes that caspases represent its final common mechanistic pathway (Cohen, 1997).

Caspases are cysteine proteinases with two aspartate cleavage sites, one of which excises the prodomain and the other that cleaves the large domain from the small domain. Among the group of 14 caspases identified to date (2003), caspase-3 has been recognised as a central player in mediating apoptosis and is the most widely studied.

Caspases are synthesised and exist mostly in the cytoplasm of viable cells as an inactive pro-enzyme. Activation of caspase-zymogens is an early event in the process of apoptosis. In response to the release of cytochrome C from the mitochondria combining with Apaf-1 and pro-caspases (giving the apoptosome) pro-caspases are processed by enzymatic cleavage to generate active enzymes.

The p32 pro-enzyme of the protease first undergoes cleavage at the C-terminal side of Asp^{28} to remove the amino terminal pro-domain (Desagher & Martinou, 2000). Additional cleavage at the C-terminal side of Asp^{175} results in the generation of the p17 and p12 fragments. The X-ray crystal structure of caspase-3 shows the formation of a tetramer composed of two small and two large subunits. The tetramer has been suggested to be the catalytically active form.

Both *in vivo* and *in vitro* data suggest glutamate induces caspase-3 induction in neurons (Du et al., 1997; Gottron et al., 1997; Tenneti et al., 1998; Tenneti & Lipton, 2000; Hirashima et al., 1999; Thomas & Mayle, 2000; Han et al., 2002; Lok et al., 2002; Zhang et al., 2002). With regard to the cell death process induced by quinolinic acid, Chiarugi and colleagues (2001) demonstrated that caspase inhibitors did not prevent quinolinic acid-induced cell death in rat cortical cultures so these proteases might not play a role in mediating cell death induced by this molecule.

The transcription factor AP-1 and the cellular counterpart of v-Jun (c-Jun) activation by its N-terminal kinase Jnk, especially Jnk-3 which is selectively expressed in the CNS are also critical as a Jnk-3 knock out protects mice from excitotoxic apoptosis (Tabuchi et al., 1996; Yang et al., 1997a, 1997b). Glutamate-induced apoptosis also can activate another pathway, that of p53, a tumour suppressor gene, that can lead to cell death by activating a Bax-related pathway in neurons (Uberti et al., 1998).

Hence, glutamate can lead to apoptosis by activating a number of processes that possibly converge into caspase activation. While it is not yet clear as to what are the gene products that constitute all the targets for caspases, it is clear now, from the preceding discussion, that caspases play a crucial role in activating cell death by apoptosis during excitotoxicity.

1.7.4. Caspase-3 induction and apoptosis in osteoblasts.

Only a few studies have addressed the role of caspase-3 induction in apoptosis in MC3T3-E1 cells, a cell line, derived from newborn murine calvariae which displays osteoblast-like characteristics after repeated passages and which was used in the present project (Quarles et al., 1992). Chae and colleagues (2000; 2001) showed that staurosporine and hypoxia both cause activation of the protease in these cells.

1.8. The osteoblast.

As mentioned earlier, osteoblasts are the skeletal cells that synthesize and regulate the deposition and mineralization of the extracellular matrix of bone. The precursors of these cells are pluripotent mesenchymal stem cells, present in both red and yellow marrow (Aubin & Liu, 1996). Based on a number of histological and morphological criteria, *in vivo* bone formation progresses through distinct developmental stages.
These stages are:- 1) commitment of mesenchymally derived cells to the osteoblast lineage 2) mitogenic growth of osteoblast precursors 3) expression of the differentiated osteoblast phenotype and ultimately formation of mineralized extracellular matrix by postmitotic terminally differentiated osteoblasts.

1.8.1. Osteoblast proliferation.

The balance between cell populations in an organism is controlled by the rates of proliferation, differentiation and programmed cell death or apoptosis (Quarles, 1992). Bone formation during bone development and remodelling necessitates stringent control of osteoblast proliferation and differentiation (Stein et al., 1996). *In vivo*, proliferation of osteoprogenitor cells occurs for formation of the embryonic skeleton and expansion of the bone stem cell population. Differentiated osteocytes and osteoprogenitor cells are then rendered quiescent, the osteoprogenitor cells reinitiate a limited extent of proliferation on demand during skeletal remodelling or fracture repair.

An analogous requirement for proliferation (regulatory mechanisms) is observed in primary cultures of normal diploid osteoblasts (Aubin & Liu, 1996). Initially isolation of osteoblasts from *in vivo* environments to *in vitro* culture results in proliferation of cells. The *exponential* growth supports expansion of the osteoblast cell population.

Post-mitotic proliferation subsequently supports focal multilayering of bone forming cells and biosynthesis of the type 1 collagen matrix extracellular which establishes a bone tissue-like organization (Stein et al., 1996). A limited extent of *compensatory proliferation* occurs in mature, mineralizing bone nodules in association with apoptosis and upregulated collagenase activity.

In MC3T3-E1 cells, the initial phase of osteoblast development is characterised by active replication of undifferentiated cells. In this regard, during days 1-9 *in vitro* subconfluent cultures display rapid increases in cell number and high rates of DNA synthesis. However by day 10, growth rates reach a plateau and DNA synthesis becomes negligible (Quarles et al., 1992).

1.8.2. Osteoblast differentiation.

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 postproliferative bone phenotypic genes (Stein et al., 1996).

The mature osteoblast phenotype is characterised by the ability of the cells to synthesize collagen type 1 and a wide variety of noncollagenous proteins such as osteocalcin, bone salioprotein, osteopontin, proteoglycans and hormone as well as growth factor receptors (Aubin & Liu, 1996). Expression of the bone/liver/kidney (also called tissue nonspecific) isoform of alkaline phosphatase is positively correlated with bone formation. It is accepted that 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).

1.8.3. Alkaline phosphatase

Alkaline phosphatase (ALP) is a ubiquitous, membrane bound tetrameric enzyme attached to glycosyl-phosphatidyl-inositol moieties on the outer cell surface and is commonly used as a marker of osteogenic development *in vitro* and in the clinic (Henthorn, 1996). It was first postulated by Robison (1923) 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 is now known to be alkaline phosphatase.

Although the precise physiological role of alkaline phosphatase is still unknown, it has been hypothesized to be involved in the mineralization process. Its expression is partly transcriptionally regulated but there is little evidence for translational and post translational control (Henthorn, 1996).

In MC3T3-E1 cells, the expression of alkaline phosphatase is time, but not cell density, dependent and evident only at the onset of growth arrest at about day 9 and is enhanced 2-fold by ascorbate and β-glycerol phosphate (Quarles et al., 1992).

Selective inhibition of ALP does not affect cell growth but decreases osteocalcin production, mineralized nodule formation and accumulation of matrix calcium phosphate (Suguwara et al., 2002). It is known that a variety of agents that have a trophic influence on MC3T3-E1 development increase the expression of alkaline phosphatase as measured by the extent to which the enzyme can release free nitrophenol from p-nitrophenylphosphate (e.g.Yada et al., 1994; Suzuki et al., 1998; Genever & Skerry, 2001). Thus this enzyme definitely plays a role in mineralization by MC3T3-E1 cells.

1.8.4. Osteoblastic cell death.

Although cell renewal and cell death are opposing processes, they are inexorably linked in bone, as both are essential for bone formation. After osteoclasts finally stop resorbing bone, they die by apoptosis and are quickly removed by phagocytes during the early portion of the reversal phase. However, during the longer lifespan of the osteoblasts more changes and choices arise.

A variety of mediators in the bone microenvironment can influence osteoblastic apoptosis. Thus, MC3T3-E1 cells undergo apoptosis in response to serum withdrawal or addition of tumour necrosis factor alpha (TNF-α) as indicated by TUNEL labeling and DNA fragmentation (Jilka et al., 1998). The potential role of nitrosative stress in the cell death mediated by TNF-α has been investigated.

Induction of nitric oxide synthase activity caused osteoblastic apoptosis and this was enhanced by pretreatment with TNF-α (Damoulis & Hauschka, 1997). Furthermore, both these agents promoted macrophage mediated cytotoxicity of MC3T3-E1 cells in co-cultures with macrophages (Damoulis & Hauschka, 1997).

The role of oxidative stress in MC3T3-E1 development has also been assessed. Hydrogen peroxide (H_2O_2) and xanthine oxidase are capable of inducing significant cell death in these cells as well as inhibiting their differentiation (Mody et al., 2001).

1.8.5. Postmenopausal osteoporosis and osteoblastic apoptosis.

The oestrogen loss that occurs during menopause causes an increase in interleukin-1, interleukin-6 and TNF-α production which mediate an increase in osteoblast and osteoclast numbers and consequentially an increased frequency of their activation (Jilka, 1998). As well as changes occurring in the number of bone cells, erosion of deeper than normal cavities also takes place. This increased remodeling 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.

Also, if resorption penetrates through a trabecular structure, the substrate for the coupled bone formation is lost forever. In this way oestrogen deficiency removes some cancellous elements completely.

Concurrent loss of cortical bone occurs by enlargement and coalescence of subendocortical spaces, a process resulting from deeper penetration of endocortical osteoclasts. This deeper erosion is explained by a loss of oestrogen action in promoting osteoclastic apoptosis (Hughes et al., 1996; Kameda et al., 1997; Raisz, 1996). Loss of this effect prolongs the lifespan of osteoclasts and increases their numbers 2 to 3-fold, thus accounting for the perforation of tabeculae and grinding away of endocortical margins.

As ovariectomy increases the turnover of murine vertebrae osteoblasts 10-fold and osteocytes 4-fold (Weinstein & Manolagas, 2000), this indicates that 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. In fact, it has been suggested that up to as many as 65% of osteoblasts that originally assemble at the remodelling site die by apoptosis before refilling the erosion cavity (Jilka et al., 1998).

1.9. Glutamate and bone cell development.

Over the past few years it has become evident that glutamate signalling may occur in bone. On the whole, this occurs in the same manner as in the central nervous system (reviewed by Skerry & Taylor, 2001). Thus, osteoblasts express the whole range of glutamate receptors which display the same electrophysiological characteristics as their neuronal counterparts. They also express glutamate transporters as well as the exocytotic machinery needed for complete glutamate release.

Not surprisingly, the precise function of glutamate receptors in bone is still being evaluated. However, it is known that bone cells need to undergo changes in mass in response to exercise for example. Glutamatergic transmission could play a vital role in these skeletal adaptations, not only as a trophic factor but also perhaps by induction of a long term potentiation-like process (Skerry & Taylor, 2001).

1.9.1. The role of glutamate in bone cell viability.

There are, however, some important differences between glutamate signalling in bone and central nervous system glutamatergic function. So far excitotoxic effects have not been demonstrated in bone cells and indeed all the evidence suggests that this neurotransmitter acts as a trophic factor in osteoblast development even at concentrations which are neurotoxic in the CNS (Genever & Skerry, 2001). Glutamate release parallels alkaline phosphatase activity (Bhangu et al., 2001), suggesting that as MC3T3-E1 cells differentiate they express more of a glutamate phenotype. Also, attenuation of glutamate release by riluzole is able to inhibit alkaline phosphatase activity and at higher concentrations can induce apoptosis in MC3T3-E1 cells. Therefore, glutamate also has a trophic role in MC3T3-E1 cell development. Furthermore, glutamate at a concentration of 50 μM – 1mM increases the survival rates of human primary osteoblasts exposed to TNF-α & IFN-γ (Genever & Skerry, 2001).

Also, unlike in the CNS, glutamate release is negatively regulated by voltage-dependent calcium entry as a continued depolarization with 30-60 mM KCl inhibits glutamate release (Genever & Skerry, 2001). However, in the CNS, glutamate can elicit neuronal damage partly via free radical release. In addition, quinolinic acid, a glutamatergic ligand and speculative endogenous central neurotoxin, at concentrations which are by itself not injurious to neurons, can be excitotoxic to hippocampal neurons *in vivo* in the presence of donors of nitrosative and oxidative stress (Behan & Stone, 2002). As mentioned earlier (p. 32) nitric oxide enhances TNF-α induced cytotoxicity in MC3T3-E1 cells (Damoulis & Hauschka, 1997) and both these molecules increase macrophage mediated death of these cells. Therefore, it is possible that in the presence of oxidative and nitrosative stress these mechanisms occur in osteoblasts as well and glutamatergic ligands such as quinolinic acid could have a toxic action on MC3T3-E1 cells.

1.10. Cell culture systems for the assessment of cytotoxicity.

In vivo data have shown that both adenosine A_{2A} receptor agonists and antagonists are neuroprotective. It is known that activation of A_2 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. Furthermore, adenosine A_{2A} receptor activation depresses cerebral glucose utilization in the cortex (Nehlig et al., 1994), which may be advantageous to neuronal viability by decreasing the requirement for depleted nutrients during ischaemia. Furthermore, activation of adenosine A_{2A} receptors decreases superoxide anion production in neutrophils (Cronstein et al., 1985). If the same action occurs in neurons, it is possible that adenosine A_{2A} receptor activation could reduce free radical associated cell damage which occurs in ischaemia and excitotoxicity (Lafon-Cazal et al., 1993; Reynolds & Hastings, 1995; Patel et al., 1996). Therefore activation of adenosine A_{2A} receptors could exert neuroprotection *in vivo* by peripheral mechanisms of action. However, it is possible that the adenosine A_{2A} receptor also exerts a direct neuroprotective effect, because, as detailed in Table 1 (p.22) the receptor has a wide range of neuromodulatory effects which could be beneficial to neuronal survival during an excitotoxic insult.

Therefore, in order to eliminate the role of central vs. peripheral mechanisms of A_{2A} receptor activation in the neuroprotection demonstrated *in vivo* and to establish the direct neuromodulatory role of the A_{2A} receptor on neuronal viability it is necessary to have a system that consists entirely of this cell type.

As stated by 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". In other words, an *in vitro* model of excitotoxicity is needed.

There are two main systems which could provide an *in vitro* model of excitotoxicity. These are primary cultures or the use of a cell line. According to Harry et al. (1998), primary cultures are "cells harvested directly from the organism, dissociated in to single cells before seeding into the culture vessel, and maintained *in vitro* for periods exceeding 24 hours". Cell lines, however are "cultures that have been serially transplanted or subcultured through a number of generations and can be propagated for an extended period of time". Whether a primary culture or cell line should be used as the *in vitro* system depends upon a number of factors. Although neuronal cell lines are 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). Although some cell lines (e.g. phaechromocytoma) are useful for examining peripheral neuronal cell death, central neuronal cell lines are expensive, difficult to set up and maintain.

1.10.1. Primary cell cultures.

For these reasons, primary cell cultures are the preferred system for use in excitotoxic studies. Also, it is generally accepted that they more closely represent the *in vivo* situation (Buchhalter & Dichter, 1996). The most frequently used primary cell cultures for this purpose are cortical, cerebellar granule and hippocampal cultures. Which tissue should be cultured depends really upon the investigator's personal preference as there seems little to tell between receptor expression for the "classic" neurotransmitters between these brain areas.

The cortex and cerebellum are much larger than the hippocampus and so it is possible to plate many more testable cultures when these brain regions are cultured (Cambray-Deakin, 1995). However, as the hippocampus is often used for electrophysiological studies, some laboratories prefer to use hippocampal cultures too to see if a certain electrophysiological property extends to neurotoxicity (Buchhalter & Dichter, 1992).

1.10.2. Cell lines.

Continuous cell lines are transformed cells derived from tumourigenic tissue which have a life span of approximately 50 divisions. Cell lines of limited life span often undergo crisis after which their growth potential changes and their lifespan becomes unlimited. The major attributes of clonal cell lines cell are homogeneity and the ease of which a large quantity of cells can be grown. Features of established cell lines include 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, 1997). In addition, once the phenotype of the cell line is established, it does not change. However, it has been observed quite often that the cells can "drift" with regard to physiological responsiveness with increased passages (Harry et al., 1998).

Information about regulation of osteogenesis is incomplete, partly because of the lack of reliable *in vitro* models to study the developmental sequence associated with replication of osteoblast precursor and differentiation. There are a number of cell lines available for the investigation of bone formation *in vitro*. Malignant osteoblast (transformed) cell lines, although of uniform phenotype, have unrepressed replicative activity and fail to display the normal coupling of differentiation and growth arrest (Pardee et al., 1978). The simultaneous expression of partially differentiated and replicative function severely limit such transformed cell lines as models to study the process of osteoblast development. Studies in primary osteoblast cultures support the concept of sequential expression of the osteoblast phenotype, but interpretation of these data is limited by the presence, in isolated calvarial and primary cell cultures, of various cell populations at various developmental stages, as well as de-differentiated cell subpopulations.

Also, subcultivation of primary cultures results in loss of osteoblast specific characteristics (Aronow et al., 1990). An understanding of the osteoblast developmental sequence has been greatly facilitated by an immortalized cell culture system that permits independent investigation of osteoprogenitors, osteoblast differentiation and mature osteoblast function. The MC3T3-E1 cell line, derived from newborn murine calvariae displays osteoblast-like characteristics after repeated passages (Quarles et al., 1992).

1.10.3. MC3T3-E1 cells.

Sudo et al. (1983) demonstrated that this cell line expresses parameters of the osteoblast phenotype, including type 1 collagen synthesis, alkaline phosphatase and nodular extracellular matrix mineralization resembling woven bone. Quarles et al. (1992) furthered these observations by revealing that in culture these cells show a temporal sequence of development characterized by distinct proliferative and differentiated stages.

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. They also have an inability to effectively assimilate newly synthesised collagen into extracellular matrix and the absence of mineralization (Stein et al., 1996). Downregulation of replication and expression of differentiated osteoblast functions characterize the next developmental stage which occurs 10 days after plating. 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. A final phase of MC3T3-E1 maturation begins about day 20 and is defined by matrix calcification associated with progressive increases in extracellular matrix accumulation and alkaline phosphatase activity (Stein et al., 1996).

The effects of ascorbate and β-glycerol phosphate (BGP) on the differentiation of these cells have also been examined (Quarles et al., 1992). In the absence of these agents MC3T3-E1 cells attain growth arrest and express low levels of alkaline phosphatase at day 10. However, at this stage, they fail to express a fully differentiated osteoblast phenotype as evidenced by the inability to form and mineralize extracellular matrix. Ascorbate facilitated the expression of osteoblast phenotype in these cells, without affecting the timing of maturation. Addition of ascorbate to the culture medium significantly increases cellular alkaline phosphatase activity in differentiated cells, whereas the time course of osteoblast phenotype development as defined by cessation of mitogenic growth and the onset of alkaline phosphatase expression were unaffected by ascorbate. These cells represent immature osteoblasts that undergo a temporal program consistent with osteoblast differentiation in stages analogous to *in vivo* bone formation.

1.10.4. Adenosine A_{2A} receptors and MC3T3-E1 development.

Another mediator of bone remodelling in the osseous microenvironment alongside growth factors, cytokines and glutamate is adenosine. The purine has a mitogenic effect on MC3T3-E1 cells (Shimegi, 1996). With regard to the role of receptor subtypes for this purine in MC3T3-E1 cells, Shimegi (1998) showed that adenosine could by itself enhance MC3T3-E1 proliferation and interestingly also enhanced platelet derived growth factor induced mitogenecity in them. Cyclopentylxanthine and pertussis toxin (PTX) partly antagonized this proliferative effect confirming that A_1 receptors partly mediated this action. However, as some of this mitogenicity was not xanthine and PTX sensitive, it is clear that other adenosine receptors played a part in eliciting this effect.

1.11. Aims.

In vivo studies have demonstrated that both adenosine A_{2A} receptor agonists and antagonists are neuroprotective (Jones et al., 1998 a,b). It is certain that some of the neuroprotection exhibited by the agonists is peripherally mediated (Jones et al., 1998 a,b) but the antagonists mediate their protection via a central mechanism.

However, *in vitro* studies are needed to clarify what the actual role of the adenosine A_{2A} receptor is in modulation of neuronal viability, in order to refine specific experimental questions in the whole animal (Harry et al., 1998) as when ischaemia is mild the use of an A_{2A} receptor antagonist might block valuable protective A_{2A} receptor-mediated peripheral effects. Cortical neuronal cultures are an established *in vitro* model for examining the effects of neuroprotective agents. Thus, the primary aim of the project is to elucidate the direct role of the adenosine A_{2A} receptor in neuronal viability using this model. Furthermore, disorders of bone remodelling, such as postmenopausal osteoporosis and periodontal disease, are still in need of an effective treatment. As there is increasing evidence that glutamate signalling occurs in bone, it is not inconceivable that excitotoxicity might underlie the excess osteoblastic cell death that occurs in these conditions. Adenosine A_{2A} receptors might, therefore, be able to modulate osteoblastic as well as central excitotoxicity. Thus, a second aim of this project was to elucidate the role of the adenosine A_{2A} receptor in osteoblastic cell viability, using MC3T3-E1 cells, an established *in vitro* model of osteogenesis.

2. Methods for the effects of glutamate and adenosine receptor ligands on neuronal cell cultures.

2.1. Animals.

2.1.1. Animal source.

Female rats (Wistar) were obtained from a commercial source (Harlan, U.K.). They were maintained by standard hygienic procedures and the behaviour and weight (450-550 g) of animals were monitored routinely to exclude them from disease.

2.1.2. Animal selection.

In excitotoxic studies using primary neuronal cultures, the rat has become the favoured species over the mouse probably because the microdissection involved in removing the tissue can be more easily performed on this species. Although some papers report using embryonic tissue from rats at a gestation period as early as 15 days, in the present study, it was found the brain tissue from embryos of this age was too fragile to culture successfully. Therefore, dams of a gestation period of between 17-19 days were used.

2.2. Microsurgery for the dissection of cortical tissue.

The following steps were all performed under sterile conditions. In a sterile laminar flowhood, 50 ml of MEM (Gibco) was divided between a 140 mm diameter and a 35 mm diameter Petri dish (Falcon, U.K.). 5 ml of a 25 % solution of urethane intraperitoneally injected was used to sacrifice the pregnant animal. 70 % alcohol was sprayed over the abdominal region to minimize accumulation of fur during the dissection. A ventral incision was made about 1 cm below the sternum to the pubic symphysis. The gravid uterine horns were excised and the embryonic sacs detached where they attached to the placenta and placed in the lid of the 140 mm diameter Petri dish in one ml of MEM. Embryos were carefully removed from their sacs using forceps.

Embryos were then decapitated using scissors and the heads placed in a 35 mm diameter Petri dish containing 2 ml of MEM. Cortical tissue was isolated as follows. The brain was gently squeezed from the cranium with forceps after making a small longitudinal incision in the middle of the skull. Using a stereobinocular dissecting microscope (previously swabbed with ethanol), the cortex or hippocampus was removed following the procedure of Hansson & Ronnback (1989), the meninges carefully removed using fine tip forceps and the tissue placed in a 35 mm diameter Petri dish containing MEM. The procedure was repeated using as many embryos, regardless of their sex as necessary to collect enough tissue. Typically, this was about 10 embryos when the dam was at gestation day 17 and about 6 when at gestation day 19.

2.3. Mechanical and enzymatic digestion of tissue.

A standard razor blade was used to mechanically disrupt the tissue until smaller pieces of it could not be obtained. As much of the tissue was then triturated using a wide tip plastic pipette and placed in a 15 ml centrifuge tube. The tissue was then washed twice in MEM by simply triturating the medium-tissue mixture with a fine tip pastette and allowing the tissue to settle. The tissue was enzymatically digested with 0.4 % trypsin in MEM for 30 min at 37°C. This concentration and time of exposure of the enzyme was considered optimal after experience showed that 5 % trypsin for 40 minutes resulted in complete cell lysis. This was evident as when plating the tissue after a 40 min trypsin treatment, cell debris and no viable neuronal network was observed even after several days *in vitro*. However, 0.25 % trypsin for 30 min resulted in insufficient digestion of connective tissue, which resulted in cell clumps and again, a lack of a viable neuronal network after several days *in vitro*. 0.4 % trypsin was prepared by adding a 1 ml aliquot of 2.5 % trypsin in phosphate buffered saline (PBS) (Gibco) to 5 ml MEM.

2.3.1. Inactivation of trypsin.

The enzymatic digestion was ended by adding 5 ml of foetal calf serum (FCS) to the centrifuge tube. To remove the trypsin and the FCS, the cells were resuspended in MEM using a fine tip pastette followed by centrifuging for 3-4 min at 500-700 rpm (50 g). This procedure was repeated twice.

2.4. Cell seeding and plating.

2.4.1. Coating of wells.

Laminin (Cambridge Bioscience, U.K.) and poly-D-lysine (PDL) (Sigma, U.K.) together represent a good substrate for neurite outgrowth in primary culture (see Buchhalter & Dichter, 1992; Paulsson, 1992). This substrate combination was used therefore as the coating for the eight well chamber slides. Solutions of the reagents were prepared as follows:- 4 mls of diethylpyrocarbonate (DEPC) treated distilled water was added to 5 mg of thawed (PDL). 128 µl of this PDL solution and 240 µl of thawed laminin (1 mg/ml.) were added to 40 ml of DEPC treated water in a Falcon tube to give final concentrations of 4 µg/ml for PDL and 6 µg/ml for laminin. The solution was applied to the plates the night before use and any remaining liquid aspirated off with a fine tip pastette on the morning of the dissection and the plates left to air dry for at least an hour prior to cell seeding.

2.4.2. Cell density.

Cells were seeded in DMEM (supplemented with 1% FCS and 100 IU/ml of penicillin and 100 µg/ml streptomycin (P/S) (Gibco)). The cell pellet was resuspended in 5 ml of culture media (preheated to 37°C). The density of viable neurons in this solution was determined by aliquotting 1 ml of cell suspension and incubating this with 100 µl of 4 % Trypan Blue solution (Sigma) at 37°C for 10 minutes.

Two cell counts (minimum 100 cells/count) were performed using a haemocytometer at 400X magnification using a standard light phase microscope (Nikon), in order to calculate the percentage of viable neurons, defined as those excluding trypan blue. The volume of the resuspension solution was then adjusted to yield a seeding density of 200,000 to 500,000 cells/ml and the cells were then seeded using a mixing needle (Henley, U.K.) by the addition of 2 drops well. This gave a seeding density of 32,000 cells/well when the seeding density was 200,000/ml. and a seeding density of 80,000 cells/well when the seeding density was 500,000 cells/ml.

This seeding density of 200,000 to 500,000 cells/ml. was chosen after experience showed that when cells were seeded at a density below 200,000/ml they did not form a monolayer with extensive neuritic connections and arborizations at the time of their use in experiments (12-16 days *in vitro*). However, when seeded above a density of 500,000/ml, cells had a tendency to detach before their use in experiments. Cells were examined to see if they had adhered at 24 & 48 hr after seeding. If this was the case, DMEM was replaced with Neurobasal (containing with 5 ml each of B27 supplement and penicillin/streptomycin antibiotics, Gibco) medium. This was changed every 2-3 days thereafter. This serum free medium was chosen, as the maintenance medium as it has been shown that it virtually eliminates glial growth in primary neuronal cultures and is better in enhancing neuronal growth compared with serum containing media (Buchhalter & Dichter, 1992; Brewer, 1995).

2.5. Cell characterization.

2.5.1. Cell markers.

Despite the claims that Neurobasal medium eliminates glial growth (Brewer, 1995), it is essential that primary neuronal cultures are characterized as regards cell type. Immunocytochemistry offers a reliable and specific method for this (Barnstable, 1992). Rabbit anti-neurofilament 200 (NF, Sigma) which recognises the 200 kD intermediate filament specific for neurons was chosen as the neural marker. Glial fibrillary acidic protein (GFAP, Sigma) was selected as the marker for astrocytes, as it is accepted that it is an unequivocal marker for this cell type (Juurlink & Hertz, 1992).

Although it is possible to use certain antibodies, namely Ran-2, A2B5 and LB1 (Marriott et al., 1996) to distinguish between type-1 and type-2 astrocytes, it is generally appreciated that it is sufficient to label astrocytes with GFAP to distinguish them from neurons in primary cell culture (Juurlink & Hertz, 1992). Integrin αM (Chemicon) was used for microglia detection (Milligan et al., 1991) and O4 (Chemicon) used for oligodendrocytes (Collarini, 1996).

2.5.2. The avidin-biotin-peroxidase complex (ABC) method.

The avidin-biotin system was first applied to immunocytochemistry for fluorescence microscopy in 1977 (Heggeness & Ash, 1977). The reaction between avidin and biotin by non-covalent bonds is very rapid, sensitive and stable and is one of the strongest bonds known in nature.

Biotin is conjugated to the secondary antibody by an indirect method and the antigen signals amplified. When the avidin-biotin-peroxidase complex (ABC) is added, a second amplification of antigen signals is completed. Therefore, the ABC method ensures maximal sensitivity in immunocytochemistry since an antibody can be coated with 150 biotin residues and each of them is able to conjugate with each ABC, ensuring a very high marker/antigen ratio (Coggi et al., 1986). Peroxidase functions as a marker which catalyzes the chemical reaction of hydrogen peroxide and thereby allows staining visualization in the presence of di-azo-aminobenzene (DAB). In view of this sensitivity, the ABC method was chosen to examine the expression of markers for the various brain cell types in this study.

2.5.3. Immunocytochemistry protocol.

All reagents used. These were prepared on day of use except phosphate buffer and phosphate buffered saline which were stored for up to 3 months at 4°C.

Phosphate buffer (PB; 0.2 M).

14.48g sodium dihydrogen phosphate ($NaH_2PO_4.2H_20$) & 40.78g disodium hydrogen phosphate (Na_2HPO_4) in 2 L distilled water.

0.1 M PB was obtained by diluting an equal volume of 0.2 M PB with distilled water.

Phosphate buffer solution (PBS (2X)).

36g sodium chloride (NaCl) & 200 ml PB & 1800 mls distilled H_2O.

4 % formaldehyde.

0.75 ml 36 % formaldehyde soln. + 6 ml 0.1M PB.

0.2 % triton in PBS (X1).

8 µl triton X-100 in 4 ml PBS (X1). This was achieved by slowly pipetting up the triton X-100, ensuring that the volume in the pipette was liquid only and free of air bubbles. When adding the triton X-100 mixture to PBS, care was taken to ensure that the total volume of liquid from the pipette tip was added by using a sharp and forceful push to expel the triton X-100 so that it formed a snake like formation on being introduced into the PBS. It was ensured that the triton was fully dissolved in the PBS prior to its use by heating in a water bath for at least 10 minutes and vortexing the mixture 3 times.

1 % hydrogen peroxide in methanol.

133 µl 30% hydrogen peroxide in 4 ml methanol.

20 % blocking serum (BS).

1.16 ml goat serum (GS, Diagnostics Scotland) and 4.64 ml PBS.

Primary antibody (1:100 diln.).

12 µl stock primary antibody + 1188 µl BS.

Secondary antibody (1:200 diln.).

600 µl GS and 15 µl stock secondary antibody + 2.385 ml PBS.

ABC solution.

One drop A (avidin BH) and one drop B (biotinylated horse radish peroxidase) added to 2.5 ml PBS (left for 30 min at room temperature before use).

DAB solution (Vector, USA).

To 5 ml distilled water, 2 drops of buffer stock solution were added. The entire solution was then mixed well. 4 drops of DAB stock solution were then added and the entire solution mixed well again. 2 drops of hydrogen peroxide solution were then added and the entire solution mixed well. Finally, 2 drops of nickel stock solution were added and the entire solution mixed well.

2.5.4. Procedure for fixing.

1. Fix sister cultures at same time of treatment with excitotoxins by rinsing with PB and then incubating them with 4% formaldehyde solution for 20-30 min at room temperature.

2. Rinse with PBS 3 times. Cells can be left in PBS for a few days before proceeding to immunostaining if necessary.

Working solutions for the primary antibodies mentioned above were prepared and tested over a range of concentrations. It was ascertained that NF and GFAP could be used at 1:500 in blocking serum. However, O4 and integrin αM were used at a dilution of 1:100.

2.5.5. Procedure for immunostaining.

Unless otherwise stated, the volume of reagent added to each well was 200 µl/well.

1. Rinse once with PBS.

2. Add 0.2 % triton X-100 in PBS (to enhance membrane permeability) for 10 min.

3.Wash 3 times with PBS.

4. Add 1 % H_2O_2 in methanol (to block endogenous peroxidase) for 10 min.

5. Wash 3 times with PBS.

6. Incubate with 20% blocking serum for 60 min. at room temperature (to block non-antigenic sites).

7. Remove blocking serum and incubate specimen with primary antibody (100 µl/well) overnight at room temperature. Omit the primary antibody from 2 wells (control) to ensure the secondary antibody is reacting specifically with the primary antibody.

8. Wash 3 times with PBS.

9. Incubate cells for 60 minutes with 1:200 biotinylated goat anti-rabbit second antibody (200 µl/well) (make up ABC solution 30 min. before this step ends).

10. Wash 3 times with PBS.

11. Perform ABC incubation by adding 100 µl of ABC solution to wells. Leave for 30 min.

12. Wash once with PBS.

13. Visualize immunoreactivity in each well in turn by adding 100 µl DAB solution. Examine regularly for maximal staining intensity using light phase microscopy.

14. Wash 3 times with PBS and store in PBS.

15. Dehydrate in alcohol and Histoclear and then fix using Histofix.

At least 100 cells at a times 400 magnification were counted in 4 separate fields/well. A field was designated an area of the well in which the cell population did not overlap with a previously examined area. 3 different wells over 3 different culture preparations were examined using a phase contrast microscope (Nikon).

2.6. Cell treatment.

Cortical neuronal cultures prepared from embryonic tissue are not susceptible to NMDA mediated excitotoxicity until about 12 days *in vitro* due to a number of factors. These factors are lack of receptor expression (Frandsen & Schousboe, 1990; Drian et al., 1991, Lustig et al., 1992; Weller et al., 1993; Akaneya et al., 1994; Griffiths et al., 1997; Cho et al., 2000; Hewett et al., 2000; Rudolph et al., 2000 & Skaper et al. 2001), lack of maturation of 2nd messenger systems (Keilhoff & Erdo, 1991; Cai & Erdo, 1992), expression of neuroD (Katayama et al., 1997) and decreased oxidation state of the NMDA receptor (Sinor et al., 1997).

Experiments using NMDA as the excitotoxin were therefore performed on viable cell cultures between 12-16 days *in vitro*. However, when quinolinic acid was used to induce cell death, cultures older than 17 DIV were used as they become susceptible to this excitotoxin at this time point *in vitro* (Kim & Choi, 1987).

At the time in the project (June, 2001) when the effects of the adenosine A_{2A} receptor ligands on cortical neuronal viability were being looked at, only one report had examined the protective effect of any of the adenosine A_{2A} receptor ligands *in vitro*. Ferreira et al. (2001) demonstrated that it was necessary to incubate retinal neurons for 24 hr with CGS 21680 before addition of 1 mM glutamate for the compound to exert neuroprotection via a metabolic effect. Therefore, it was decided initially to use this same incubation time period for the A_{2A} ligands before addition of excitotoxins. After addition of the A_{2A} ligands for 24 hr, excitotoxins were added for a further 48 hr before final cell death assessment.

2.7. Assessment of cell death.

Trypan blue uptake (Tennant, 1964) offers a quick and reliable method for measuring necrosis as cells succumbing to this form of cell death lose their membrane integrity, allowing uptake of the dye. Its use is established for measuring excitotoxic cell death (Mattson et al., 1995).

These authors also reported that neurons adjacent to astrocytes are more resistant to excitotoxicity than neurons not in contact with these cells. This was also noted in the present study, so care was taken not to include neurons within a glial "pocket" in cell counts during assessment of cell death by this method. The cells were initially incubated with 0.4 % Trypan Blue solution (Sigma) for 10-15 min and then fixed in 4 % formaldehyde for 25-30 min. Wells were then visualized under light microscopy at a magnification of times 400 (Nikon). Cells which failed to exclude trypan blue were scored as necrotic. The number of necrotic cells in 4 fields was counted (minimum 100 cells/field) and % necrotic cell death/well calculated as follows:-

$$\% \text{ necrotic cell death/well} = \frac{\%\text{field } 1 + \%\text{field } 2 + \%\text{field } 3 + \%\text{field } 4}{4}$$

2.8. Statistical analysis.

See section 3.6..

3. Methods for investigating the effects of glutamate and adenosine receptor ligands on bone cell cultures.

3.1. Cell culture.

Murine calvaria derived MC3T3-E1 osteoblast-like cells were used in the present study. They were a generous gift from Professor Tim Skerry, Royal Veterinary College, London. A 75 cm^3 flask of the cells (passage 7) was received in alpha-minimum essential medium (α-MEM, Gibco) containing 20 % FCS (Gibco) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (P/S).

3.1.1. Freezing down of MC3T3-E1 cells.

The cells were allowed to obtain full confluence and then frozen at -70°C so that aliquots of the cells would be available for future experiments. The following procedure was followed (aseptic):-

Cell cultures (one flask) were trypsinized by removal of the culture medium and adding just sufficient 0.5 % trypsin in MEM (Gibco, preheated to 37°C) to cover the cells. This was 10 ml. for a 75 cm^3 flask and 5 ml for a 25 cm^3 flask. The flask was then returned to the incubator and checked every 5 min for signs of cell detachment. When it was clear that the majority of the cells had detached, the flask was flooded with MEM (supplemented with 20% FCS) and all the contents pipetted in to a 15 ml centrifuge tube, ensuring all the detached cells were removed. They were then washed by initially centrifuging gently (3 mins at 500g) and resuspended in 5 ml α-MEM (with 20 % FCS and P/S). The washing process was repeated and the cells resuspended in 5 ml MEM. The cells were then counted in the same way that cortical neuron concentration was calculated and then the number adjusted by adding α-MEM (supplemented with 20% FCS + P/S) to give a concentration of 500,000/ml. A third of the cells were then reseeded in 25 ml culture medium (α-MEM with 20% FCS and P/S) in a 75 cm^3 flask and returned to the incubator for later experiments.

The remaining two thirds of the cells were then frozen for later use in case cells were lost due to contamination and they had to be resurrected. The freezing process was achieved by transferring to culture medium containing 10 % DMSO. The DMSO/cells/medium mixture was then dispensed into 1 ml aliquots in ampoules and cooled at 4°C for 30 min. The cells were then slowly frozen by putting in a styrofoam box, first in a –20 °C freezer for 30 min and then in a -80°C freezer for storage.

3.1.2. Subculturing of cells.

Subcultures were obtained every 5-7 days by removing the cells from the flask by trypsinization, washing, resuspension and counting as detailed above. Approximately one third of the cells were reseeded in a 75 ml flask in 25 ml α-MEM with 10 % FCS (changed every 2-3 days) and maintained at 37°C in a humidified atmosphere of 5 % CO_2-95 % air. The rest of the cells were seeded using a mixing needle (1 drop/well) in α-MEM (supp.with 10% FCS and P/S) in 96 well plates. Seeding density varied according to the experimental assay to be performed later (see below). After 24 hours the plated cells were switched to media supplemented with 50 μg/ml ascorbate and 5 mM β-glycerol phosphate and P/S. These supplements were added to the media as they accelerate the proliferation and differentiation of these cells (Harada et al., 1991; Quarles et al., 1992). In particular, it is known that glutamate release parallels alkaline phosphatase expression (a marker of differentiation, Bhangu et al., 2001). Since one of the main aims of the project was to assess the role of this transmitter in osteoblastic cell development and death, these supplements were required so that the cells expressed a maximal glutamatergic phenotype at the time of treatment.

3.1.3. Resurrection of frozen cells.

This was achieved by gently thawing the previously frozen DMSO/cells/medium mixture by heating the end of a tube in a water bath. On thawing, the mixture was immediately pipetted into a 15 ml centrifuge tube.

The storage tube was rinsed once with 5 ml α-MEM (with 20 % FCS and P/S) to ensure all of the cells were passed into the centrifuge tube. The cells were then washed once by initially centrifuging at 500g for 5 mins., aspirating the medium only and then resuspending the pellet in 10 ml medium. The entire cell suspension was then transferred to a 75 cm^3 flask. To ensure that all the cells were transferred to the flask, the centrifuge tube was then flooded with 10 ml α-MEM (with 20 % FCS and P/S). This flooding procedure was repeated and so the cells were seeded in 30 ml. of media. Half the media in the flasks was replaced after 24 hr and then every 2-3 days until subculturing.

3.2. Mitogenic activity assay.

Alamar Blue (Biosource) was used to assess cell viability. The active ingredient in this colorimetric assay is 7-hydroxy-3H-phenoxazin-3-one-10-oxide (resazurin, O'Brien et al., 2000). The dye relies on the principal that the internal environment of the proliferating cell is more reduced than that of non-proliferating cells. As the ratios of nicotine adenine dinucleotide phosphohydrogenase (NADPH)/nicotine adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide hydrogenase (FADH)/ flavin adenine dinucleotide (FAD), flavin mononucleotide hydrogenase FMNH and nicotine adenine dinucleotide hydrogenase (NADH) increase during proliferation, Alamar Blue can be reduced by all of these intermediates which is accompanied by a measurable shift in colour (Nociari et al., 1998).

Furthermore, as this dye can be reduced by cytochromes whereas tetrazolium salts cannot (O'Brien et al., 2000), it offers increased sensitivity over these assays. The optimum incubation time for Alamar Blue when used with MC3T3-E1 cells was determined. This was found to be 4 hr when the cells were plated between 5,000/ml. and 20,000/ml.

The general protocol for measuring cell viability in MC3T3-E1 cells was as follows:-

1. The old media was aspirated and fresh media (200 µl) added to test wells in 96 well plates
2. 20 µl of Alamar Blue was added to each test well.
3. The plate was then returned to the incubator for 4 hr
4. The absorbance (A) was measured at 540 nm (lower wavelength, LW) and then 595 nm (higher wavelength, HW) using a spectrophotometric plate reader (Dynex, USA)
5. The absorbance of 220 µl of media only was then measured at the same wavelengths mentioned in step 4.
6. The absorbance values of media only (step 5) were substracted from the absorbance values of Alamar Blue and media (step 4) at both wavelengths to give the absorbance of the oxidized form of Alamar Blue in media – absorbance of media only. The absorbance of the oxidized form at lower wavelength was called AO_{LW} and absorbance of oxidized form at higher wavelength was called AO_{HW}.
7. The correction factor: R_O. ($R_O = AO_{LW}/AO_{HW}$) was then calculated.
8. The percentage of reduced Alamar Blue, AR was then calculated. $AR = A_{LW}(A_{HW} \times R_O) \times 100)$

To assess the effects of various compounds on MC3T3-E1 proliferation, cells were seeded at a density of 5,000/ml. Medium was changed to ascorbate and β-glycerol phosphate media ("switch") media (with 10 % FCS) after 24 hr. At 6 days *in vitro* an initial cell viability assay according to the manufacturers instructions (above) was performed with Alamar Blue (Biosource). Agents were then added and cell viability assessed again at the desired time point after agent addition. Cell proliferation was expressed as the percentage of the pretreatment level.

3.3. Assay for alkaline phosphatase (ALP) activity.

To assess the effect of the agents on MC3T3-E1 ALP activity, the protocol of Suzuki et al. (1998), who examined the effects of epinephrine on expression of this enzyme in these cells was followed.

Furthermore, as cell lines can show phenotypic drift after multiple passages and it is possible that the phenotype can differ between different laboratories, it was necessary to ensure the cells were characterised as regards their development. This was achieved by ensuring they differentiated in the same way as has previously been reported by assaying for alkaline phosphatase. Cells were plated at a density of 5,000/ml. They were then switched to, and maintained in, ascorbate and β-glycerol phosphate (with 10% FCS and P/S) containing media for 11 days, after which time they were switched to the same media containing just 1% FCS for 24 hr Agents were then added for 48 hr For the initial cell characterization, the cells were fixed at 11 DIV.

ALP activity in 96 well plates (adapted from Boyan (1989) & Lowry (1956)) was assessed as follows. Initially, the cells were fixed in 96 well plates. After washing twice in PBS, 100 µl of 0.05 % Triton (Sigma) was added to each well.

The 96 well plates were then frozen by putting them in a -70°C freezer and then having them thawed by placing in a water bath at 37°C. The mixture was then triturated carefully, ensuring no air bubbles were introduced as experience showed these can give erroneous readings during spectrophotometry. This freeze-thaw-titration process was then repeated.

100 µl ice cold 16 mM p-nitrophenylphosphate (BDH) in 2-amino-2-methyl-1-propanol (AMP) buffer (Sigma) was then added to each well. The plates were then incubated in a water bath at 37°C for 60 min and then read at 410 nm using a plate reader.

ALP catalyses the conversion of p-nitrophenylphosphate (colourless) to free nitrophenol (yellow) which in alkaline solution has strong absorption at a wavelength (410 nm) at which the substrate has little or no absorption (Lowry, 1956).

$$\text{p-nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALP}} \text{p-nitrophenyl} + P_i$$

3.4. Cell death assessment.

3.4.1. Necrosis.

Necrotic cell death in MC3T3-E1 cells was assessed using trypan blue uptake (see neuronal cultures (section **2.7**)).

3.4.2. Apoptosis.

Cells were seeded at a density of 15,000/ml. in a 96 well plate in α-MEM with 20 % FCS and P/S. 24 hr later the medium was switched to ascorbate and β-glycerol phosphate containing media. At 3 days *in vitro*, the cells were washed 3 times with α-MEM with 0.5 % BSA and then exposed to the test compounds in α-MEM with 0.5 % BSA. Caspase-3 activity was measured by immunostaining 18 hr later, a time point when apoptosis occurs in response to serum withdrawal in MC3T3-E1 cells (Jilka et al., 1998) and when expression of the protease is maximal (Chae et al., 2000).

3.4.3. Immunocytochemical method for assessment of caspase-3 expression.

Reagents used (unless stated otherwise, 200 µl of each reagent was used).

4 % Paraformaldehyde in PBS.

0.75 ml 36 % paraformaldehyde solution + 6 ml 0.1 M PBS.

0.2 % Triton in PBS.

8 µl triton in 4 ml PBS.

Blocking buffer.

5 ml PBS + 5 µl Tween 20 + 250 µl goat serum.

Anti-active caspase-3 antibody (Primary antibody, 1: 320 dilution).

Add a 5 µl aliquot of the antibody to 1600 µl blocking buffer = 50 µl added to each well if for 32 wells.

Secondary antibody (1:200 diln.).

600 µl GS & 15 µl stock secondary antibody + 2.385 ml PBS.

ABC (avidin-biotin- peroxidase complex) solution (Vector, U.K.).

One drop A (avidin BH) & one drop B (biotinylated horse radish peroxidase) added to 2.5 ml PBS (left for 30 min at room temperature.)

DAB (di-azo-aminobenzene) solution (Vector, U.K.).

To 5 ml distilled water, 2 drops of buffer stock solution were added. The entire solution was then mixed well. 4 drops of DAB stock solution were then added and the entire solution mixed well again. 2 drops of hydrogen peroxide solution were then added and the entire solution mixed well. Finally, 2 drops of nickel stock solution were added and the entire solution mixed well.

Unless stated otherwise the volume added to each well was 200 µl.

1. Fix cells by incubating in 4 % paraformaldehyde at room temperature for 25-30 min Wash 3 times in PBS and then proceed to immunostaining.

2. Permeabilize fixed cells by incubating in 0.2 % Triton X-100 in PBS for 5 min at room temperature.

3. Wash 3 times in PBS.

4. Drain plus add 100 µl of blocking buffer and incubate for 2 hr at room temperature in a humidified chamber.

5. Wash once with PBS.

6. Add 50 µl of anti-active caspase 3 antibody in blocking buffer and incubate overnight in a humidified chamber at 4°C.

7. The following day, wash twice in PBS for 10 min, twice in PBS/0.1% Tween 20 for 10 min and again twice in PBS for 10 mins. at room temperature.

8. Drain and add 100 µl of the secondary antibody in blocking buffer. Incubate for 2 hr at room temperature in a humidified chamber. Make up ABC soln. 30 mins. before this step ends.

9. Wash twice in PBS for 5 mins., once in PBS/0.1% Tween 20 for 5 min and once again in PBS for 5 min

10. Wash 3 times in PBS.

11. Perform ABC incubation by adding 100 µl of ABC solution to wells. Leave for 30 min.

12. Wash once with PBS.

13. Visualize immunoreactivity in each well in turn by adding 100 µl DAB solution and examining regularly for maximal staining intensity using light phase microscopy.

14. Wash in PBS 3 times and take photos if necessary.

The number of cells positively staining for caspase-3 was quantified by using phase contrast light microscopy in the same way that counts for trypan blue positive stained cells were performed in assessing cell death of cortical neurons as mentioned earlier (section **2.7.**).

3.5. Drugs.

All drugs were obtained from Sigma, Dorset, U.K. except 2-amino-5-phosphonopentanoic acid (AP5) and 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxyamidoadenosine hydrochloride (CGS 21680) (Tocris, Bristol, U.K..). 4-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-a][1,3,5]triazo-5-yl-amino]ethyl)phenol (ZM 241385) was obtained from Zeneca, Macclesfield, U.K.

3.6. Statistical analysis.

Results are expressed as the mean ± S.E.M. (standard error of the mean) of the stated number (n) of wells. When the basal or control expression level is shown graphically, it is the same value as that induced by 0 μM of an agent. The statistical significance of any difference was assessed by either the unpaired t-test or one way analysis of variance (ANOVA) with a post test of Student-Newman-Keuls multiple comparisons test (SNK test). Differences were considered significant when $p < 0.05$ unless stated otherwise.

4. The effects of glutamate and adenosine receptor ligands on neuronal cultures.

4.1. Development of cortical and hippocampal neurons in culture.

As assessed by light phase microscopy, 3 hr after plating, neurons, recognised by their spherical shape, already showed process development, marked by neuritic extension. After 24 hr *in vitro*, cells began to aggregate and differentiate into morphological subtypes. After 72 hr a neuritic network between adjacent cell aggregates started to form. Between 72 hr and 14 days *in vitro*, this neuritic extension continued with a corresponding increase in neuron size and complexity of processes. At the time of experiments (12-16 DIV), viable neurons were distinctly recognisable by having bright oval somata (Kuriyama & Ohkuma, 1990; Mattson et al., 1995) with extensive neuritic arborisation between adjacent cell aggregates. Pyramidal neurons, recognised by their triangular soma, stout apical dendrite and smaller diameter basilar dendrites (Buchhalter & Dichter, 1992) were evident. Non-pyramidal, stellate neurons were also present (Figure 2a, p. 61). The overall morphological development of the cultures was similar to those observed by others (Kuriyama & Ohkuma, 1979; Buchhalter & Dichter, 1992; Mattson et al., 1995).

4.1.1. Cell characterization.

Immunostaining against neurofilament (NF-200) revealed that the cultures consisted of an homogeneous neuronal population of about 90 % of this cell type (Figure 2b, p.62). Staining against GFAP showed that the remainder of the cell population consisted of astrocytes. Most of the non-neuronal cell population (about 8 %) of this cell type were protoplasmic, recognised by having processes which were variable in shape and which closely followed the contours of adjacent cells. There was also some contamination (about 2 %) by fibrous astrocytes, recognised by their smaller size and finger-like processes (Juurlink & Hertz, 1995). Contamination by astrocytes was restricted to "pockets" which tended to appear around the edges of the wells. Staining against integrin αM for microglial detection and O4 for oligodendrocytes showed there was negligible (< 0.1 %) contamination in the cultures from these cell types.

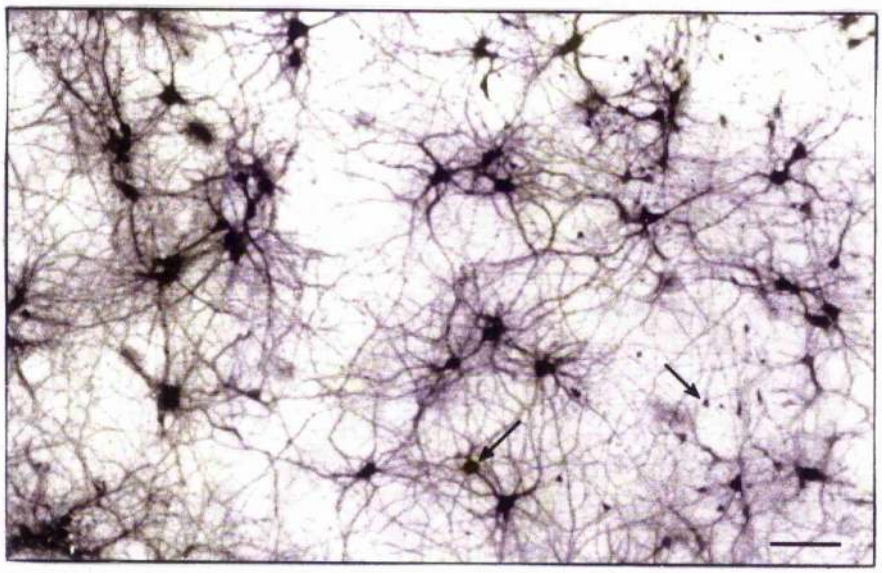

Figure 2a. Anti-neurofilament immunostained hippocampal neuronal cultures. Hippocampal neuronal cultures at 14 DIV were fixed and immunostained against the 200 kD intermediate filament (1:500 dilution). Pyramidal cells (arrow on right), recogniseable by their triangular soma, stout apical dendrite and smaller basilar dendrites can be seen as well as non-pyramidal, stellate neurons (arrow on left). Bar = 40 μM.

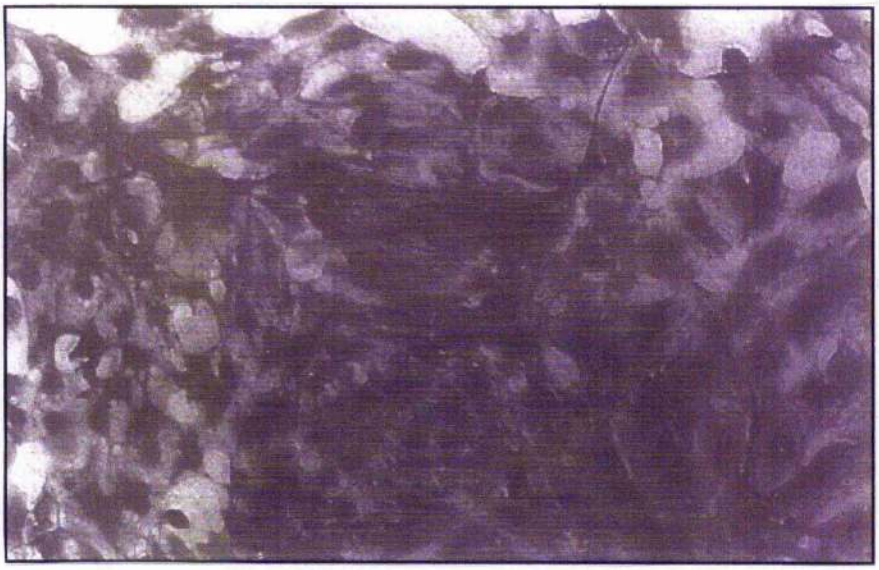

Figure 2b. Anti glial fibrillary acidic protein (GFAP) stained hippocampal cultures. Hippocampal cultures were fixed at 14 DIV and immunostained against GFAP (1:500 dilution). This pocket of glial contamination contains mostly type-1 astrocytes.

4.2. Effect of NMDA on cortical neuron cultures.

Figures 3 and 4 show NMDA induced concentration-dependent cell death in cortical neuronal cultures. After 24 hr, there was no evidence of neuronal damage by NMDA. However, after 48 hr, 30 μM NMDA non-significantly increased neuronal cell death from a basal level of 9±2.5 % (n=13) to 22±5 %. At a concentration of 100 μM, NMDA significantly increased cell death to 31±4 % ($p<0.001$ vs. basal cell death level, n=17, SNK test) Viable neurons with round oval somata and extensive neuritic arborization displayed distinct somatic swelling/vacuolization and neuritic fragmentation on exposure to the excitotoxin (Figures 5b & 6, p. 67 & 68). Their cell bodies also stained positive with trypan blue.

4.2.1. Effect of 2-amino-5-phosphonopentanoic acid (AP5) on cell death induced by NMDA.

Addition of the NMDA selective antagonist, DL-2-aminophosphonopentanoic acid, (AP5) (Evans et al., 1982) afforded protection against NMDA-elicited cell damage Figs. 3 & 4). AP5 non-significantly reduced 30 μM NMDA-induced cell death from 22±5 % to 8±2 % (n=8) but significantly attenuated cell death induced by 100 μM NMDA from 31±4 % to 7.5±2 % ($p<0.01$, n=13, SNK test).

4.3. Effect of kynurenines on cortical neuronal cultures.

Quinolinic acid non-significantly increased cell death in cortical neuronal cultures (Figure 7, n=3). Cell damage induced by quinolinic acid was morphologically similar to that induced by NMDA, in that the cells showed somatic swelling/vacuolisation and neuritic fragmentation. They also took up trypan blue. The broad spectrum glutamate receptor antagonist, kynurenic acid (Stone & Burton, 1988) had no effect on the response to quinolinic acid (n=2).

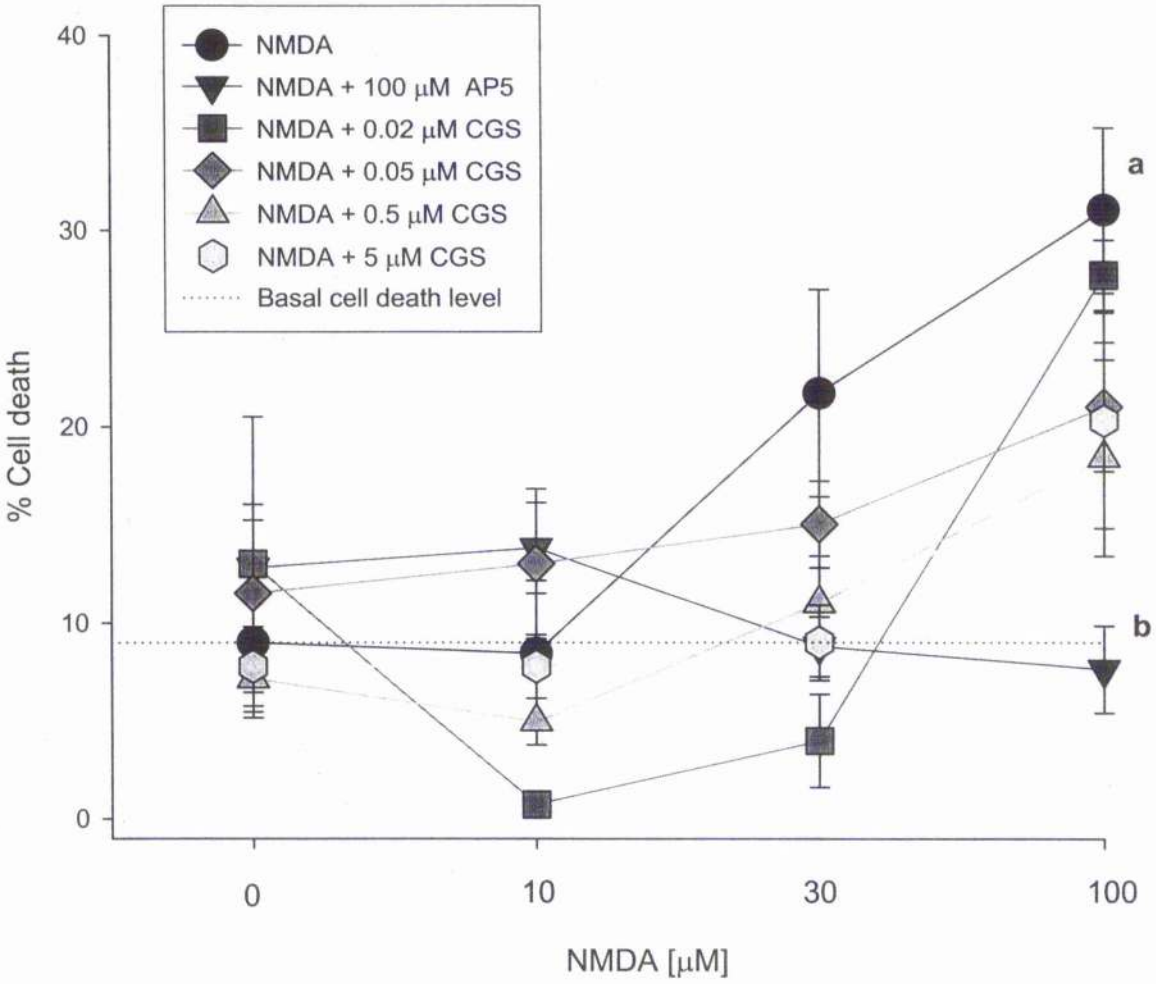

Figure 3. Effect of CGS 21680 (CGS) and AP5 on cortical neuronal cultures. At 12-16 DIV, cells were incubated with CGS 21680 for 24 hr. NMDA was then added and cell death was assessed an additional 48 hr after NMDA addition by trypan blue exclusion (n=4).

a. $p<0.001$ vs. 0 μM & 10 μM NMDA (n=7, SNK test).
b. $p<0.01$ vs. 100 μM NMDA (n=17, SNK test).
$F=7.941$.

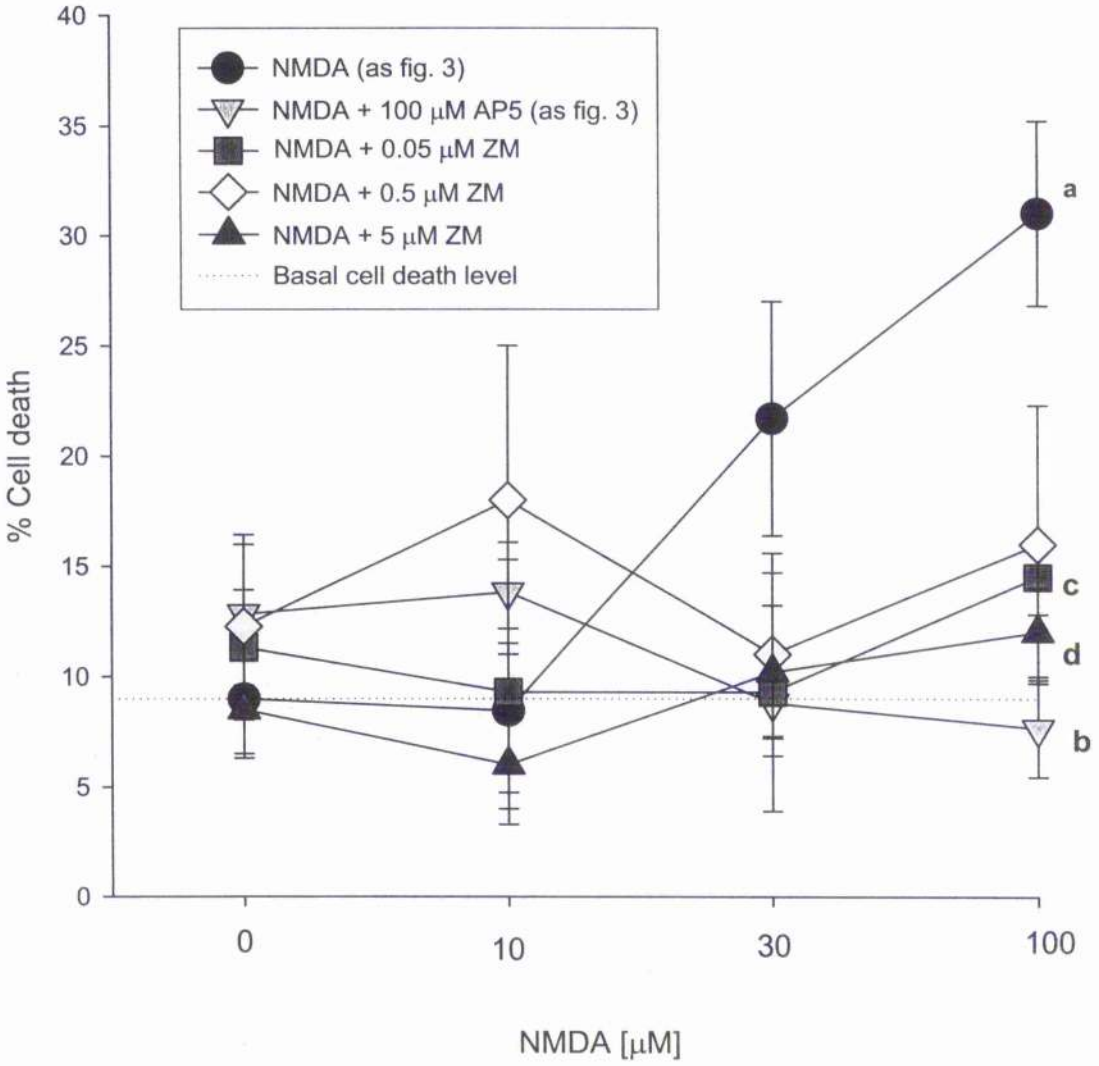

Figure 4. Effect of ZM 241385 (ZM) and AP5 on cortical neuronal cultures. At 12-16 DIV, cells were incubated with ZM 241385 for 24 hr. NMDA was then added and cell death was assessed an additional 48 hr after NMDA addition by trypan blue exclusion (minimum n=4).

a. $p<0.001$ vs. 0 μM & 10 μM NMDA (n=7, SNK test).
b. $p<0.001$ vs. 100 μM NMDA (n=17, SNK test).
c. 0.05 μM ZM: $p<0.001$ vs. 100 μM NMDA (n=13, SNK test).
d. 5 μM ZM: $p<0.01$ vs.100 μM NMDA (n=11,SNK test).
$F=7.724$.

Figure 5a. Untreated cortical neuronal cultures at 14 DIV. Viable neurons at this stage in culture are recognised by having compact, oval somata linked by extensive neuritic arborisation.

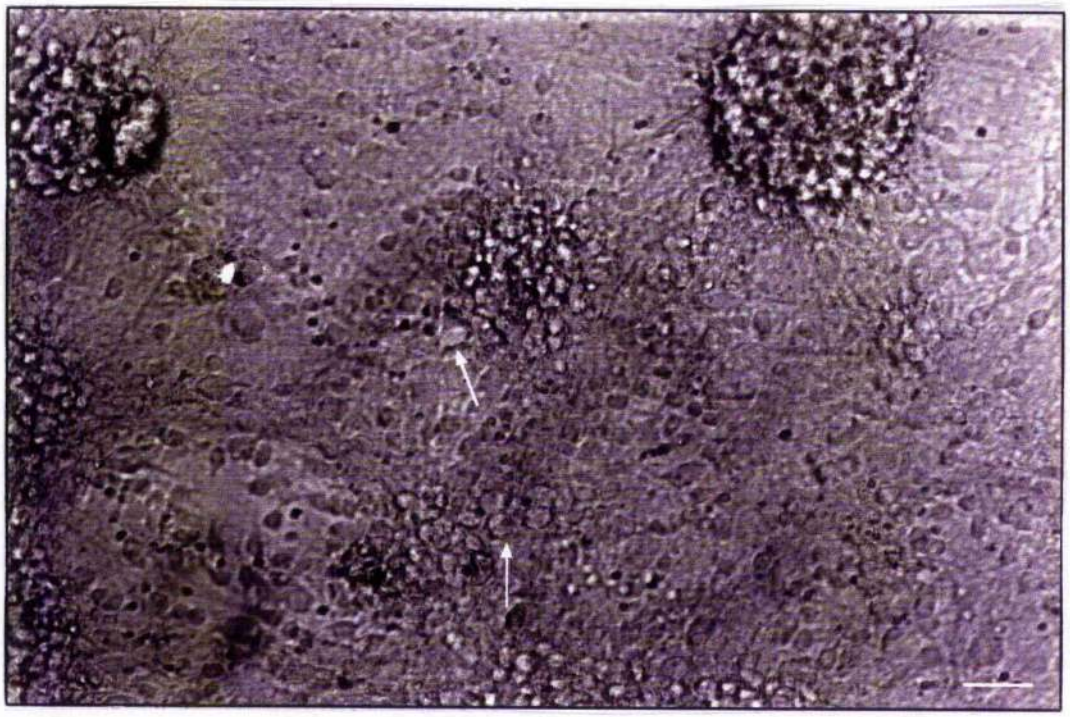

Figure 5b. Effect of 30 μM NMDA on cortical neuronal cultures. Somatic swelling/vacuolisation as a morphological hallmark of excitotoxicity *in vitro* can be seen (arrows). Bar = 40 μM.

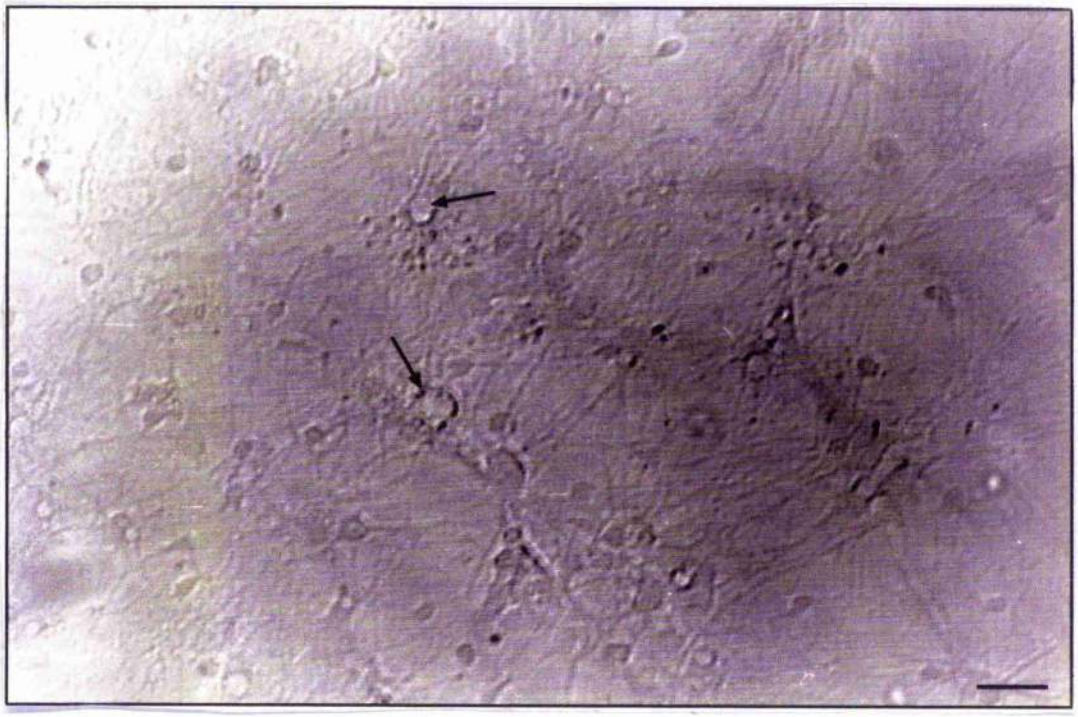

Figure 6. Effect of 100 μM NMDA on cortical neuronal cultures. The morphological hallmarks of excitotoxicity *in vitro,* namely somatic swelling/vacuolisation and neuritic fragmentation are more marked when cells are exposed to this higher concentration of NMDA (arrows). Bar = 40 μM.

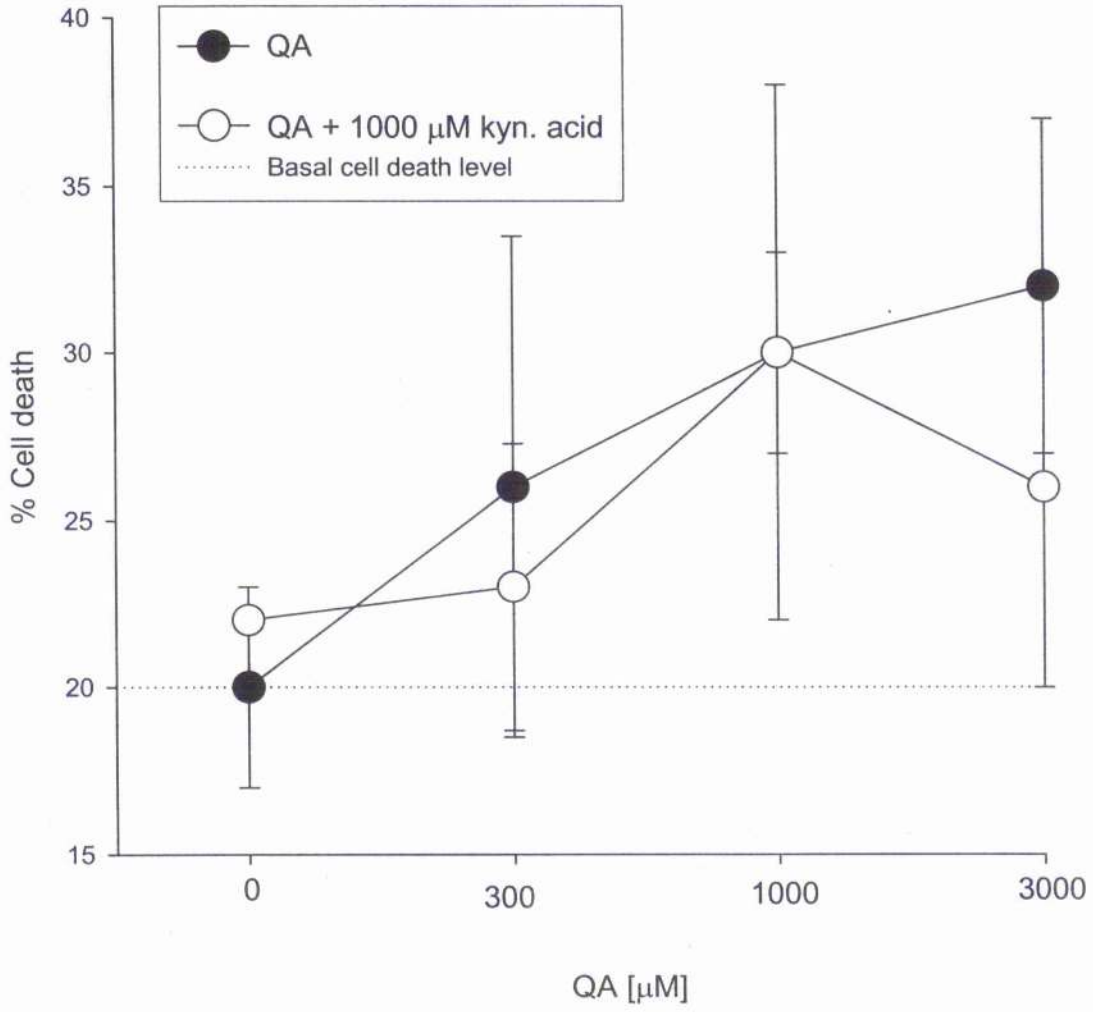

Figure 7. Effect of quinolinic acid (QA) and kynurenic acid (kyn. acid) on cortical neuronal cultures. At 17-21 DIV, cells were incubated with kynurenic acid for 24 hr. QA was then added and cell death was assessed an additional 48 hr after QA addition by trypan blue exclusion (n=3 for QA data and n=2 for QA+kyn. acid data).

4.4. Effect of CGS 21680 on cortical neuronal cultures.

As can be seen in Figure 3, 0.02 μM CGS 21680 had no effect on the basal level of cell death but non-significantly reduced the level of cell death induced by 10 μM NMDA from 8.5±4 % (n=7) to 0.7±0.5 % (n=4) and reduced cell death induced by 30 μM NMDA from 22±5 % to 4±3 % (n=4). However, CGS 21680 did not affect cell death induced by 100 μM NMDA.

At 0.05 μM, CGS 21680 did not attenuate cell death induced by NMDA. As the concentration was increased to 0.5 μM and then 5 μM, there was a non-significant trend that the agent decreases neuronal damage. This was to the extent that at 5 μM, the compound was able to non-significantly reduce cell death evoked by 30 μM NMDA to 8.5±2 % (n=5). However, 5 μM CGS 21680 did not protect against cell death elicited by 100 μM NMDA.

4.5. Effect of ZM 241385 on cortical neuronal cultures.

0.05 μM and 5 μM ZM 241385 had no effect on cell death induced by 10 μM NMDA or 30 μM NMDA (Figure 4). 0.05 μM significantly reduced cell death induced by 100 μM NMDA from 32±4 % to 14±2 % ($p<0.001$, n=11, SNK test) and 5 μM ZM 241385 significantly reduced cell death induced by 100 μM NMDA to 12±2 % ($p<0.01$, n=4, SNK test).

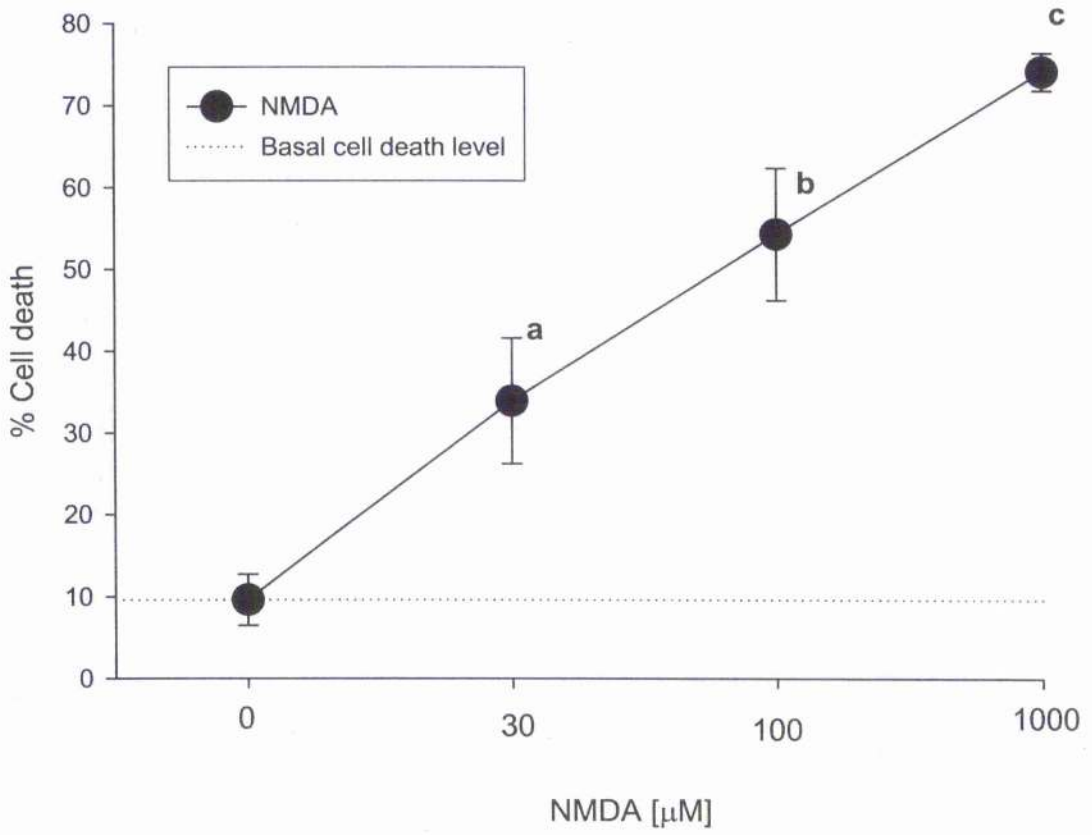

Figure 8. Effect of NMDA on hippocampal neuronal cultures. At 12-16 DIV, cells were exposed to NMDA and cell death assessed by trypan blue uptake an additional 48 hr after NMDA addition (n=4).

a. $p<0.05$ vs. 0 μM NMDA (n=4, SNK test).
b. $p<0.001$ vs. 0 μM & $p<0.05$ vs. 30 μM NMDA (n=4, SNK test).
c. $p<0.001$ vs. 0 μM, $p<0.01$ vs. 30 μM & $p<0.05$ vs. 100 μM NMDA (n=4, SNK test).
$F=21.931$.

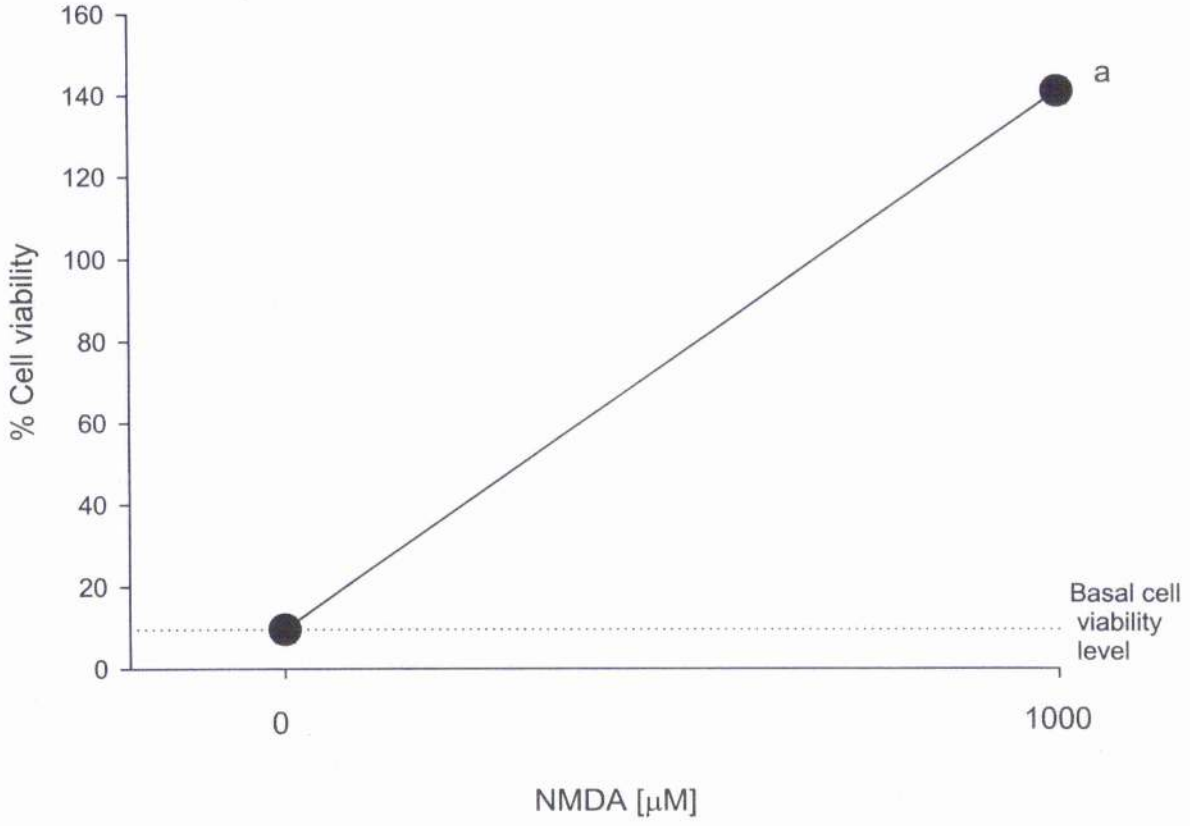

Figure 9. Effect of NMDA on hippocampal neuronal viability as assessed by MTS reduction. Cultures were incubated with NMDA and cell viability assessed an additional 48 hr after NMDA addition (n=3).

a. $p<0.0001$ (two-tailed value) vs. 0 μM.
(unpaired t-test, n=3).

4.6. Effect of NMDA on hippocampal neuronal cultures.

Figure 8 reveals that NMDA induced concentration-dependent cell death in hippocampal cultures as assessed by trypan blue uptake. 30 μM NMDA significantly increased cell death from a basal level of 9.5±3 % to 34±8 % ($p<0.05$, n=4, SNK test). 100 μM of the agent significantly increased cell death to 54±8 % ($p<0.05$ vs. 0 μM, n=4, SNK test). 1mM NMDA significantly increased neuronal death to 74±2 % ($p<0.01$ vs. 0 μM and 30 μM NMDA, n=4). As assessed by light phase microscopy, cell death in these cultures was morphologically identical to cell death in cortical neuronal cultures with NMDA causing distinct somatic swelling/vacuolisation and neuritic fragmentation to hippocampal neurons.

However, as assessed by MTS reduction, 1mM NMDA caused a significant increase in neuronal viability or cell number to 141±3 % ($p<0.0001$ (two-tailed value, n=3, unpaired t-test)) of basal levels after 48 hr (Figure 9).

5. The effects of glutamate and adenosine receptor ligands on bone cell cultures.

5.1. Characterisation of MC3T3-E1 cells.

MC3T3-E1 cells cultured in ascorbate and β-glycerol phosphate supplemented ("switch") media showed a significantly higher ALP activity (0.4±0.02 U/ml, n=8) compared to cells cultured in medium without these supplements ("normal") which showed an ALP activity of 0.26±0.01 U/ml ($p<0.01$, unpaired t-test, n=8). Morphologically, cells maintained in switch medium showed a more cuboidal appearance compared to cells maintained in normal medium which appeared mesenchymal.

5.2. Effect of N-methyl-D-aspartate (NMDA) on MC3T3-E1 cell development.

5.2. Effect of NMDA on MC3T3-E1 cell proliferation.

5.2.1. 24 hr exposure.

None of the concentrations of NMDA had a significant effect on MC3T3-E1 proliferation after 24 hr (Figure 10). However, after 48 hr, 300 μM NMDA significantly increased MC3T3-E1 cell viability from a basal level of 104±2 % to 127±2 % ($p<0.001$, n=4, SNK test). After 72 hrs., 300 μM NMDA had significantly increased viability to 112±2 % ($p<0.05$ vs. basal cell viability level, n=4, SNK test). However, 1000 μM NMDA did not have any significant effect on cell viability levels after 48 or 72 hr and had no effect on morphology of MC3T3-E1 cells as assessed by light phase microscopy.

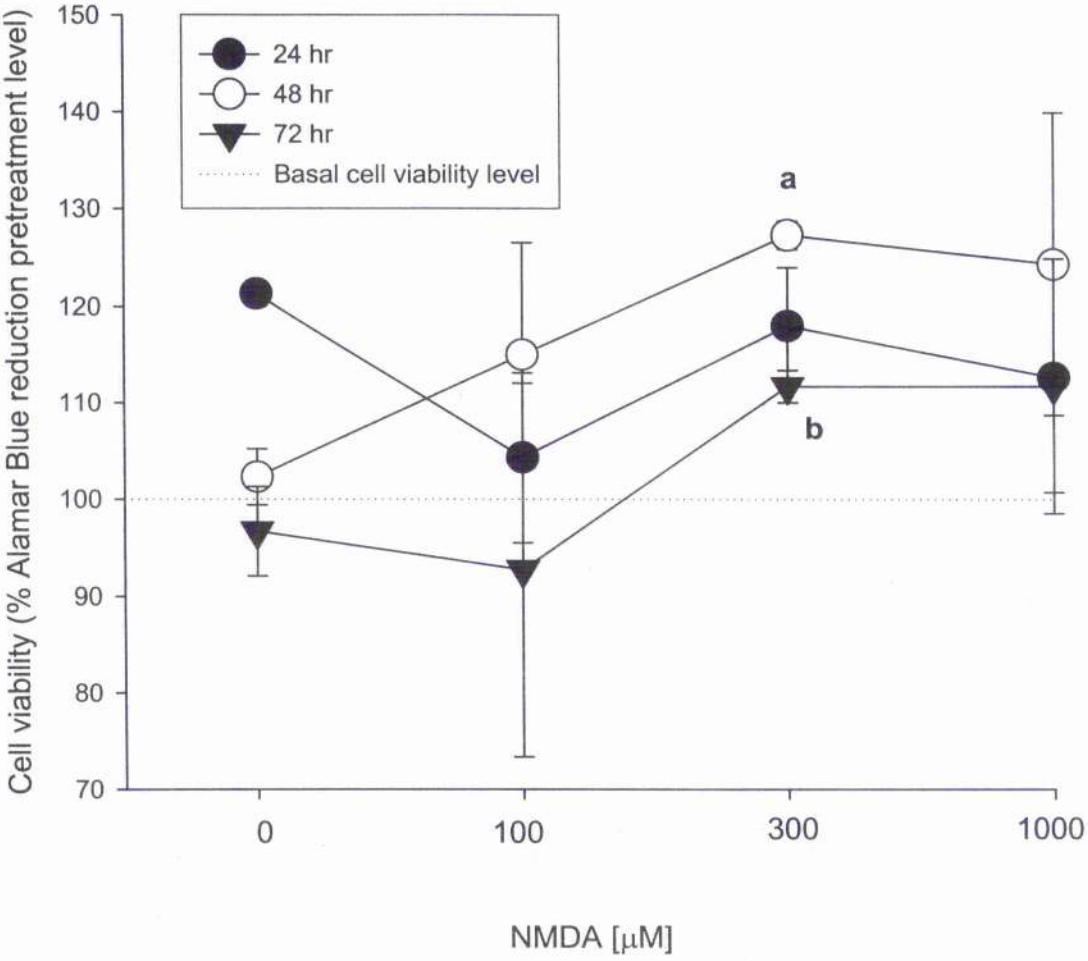

Figure 10. Effect of N-methyl-D-aspartate (NMDA) on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then treated with NMDA. Cell viability was assessed after an additional 24, 48 & 72 hr after NMDA addition (n=4).

a. $p<0.001$ vs. basal cell viability level (n=4, SNK test).
b. 72 hr. $p<0.05$ vs. basal cell viability level (n=4, SNK test).
$F=20.370$.

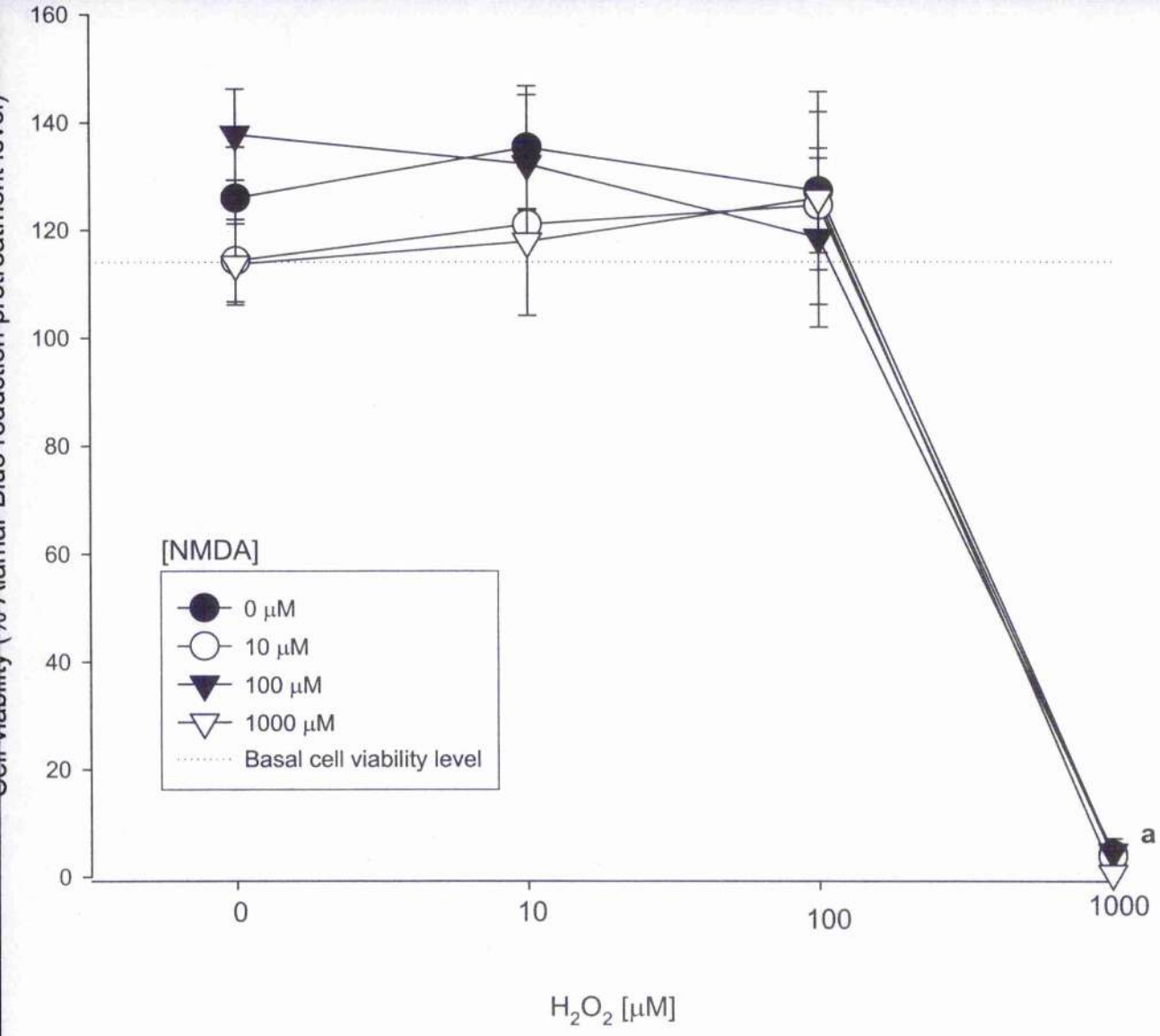

Figure 11. Effect of NMDA and H_2O_2 on MC3T3-E1 cell viability. At 6 DIV, cells were subjected to a pretreatment viability assay and then incubated with the agents. Cell viability was assessed after an additional 72 hr after NMDA & H_2O_2 addition (n=6).

a. $p<0.001$ vs. all other treatments (n=6, SNK test).
$F=144.67$.

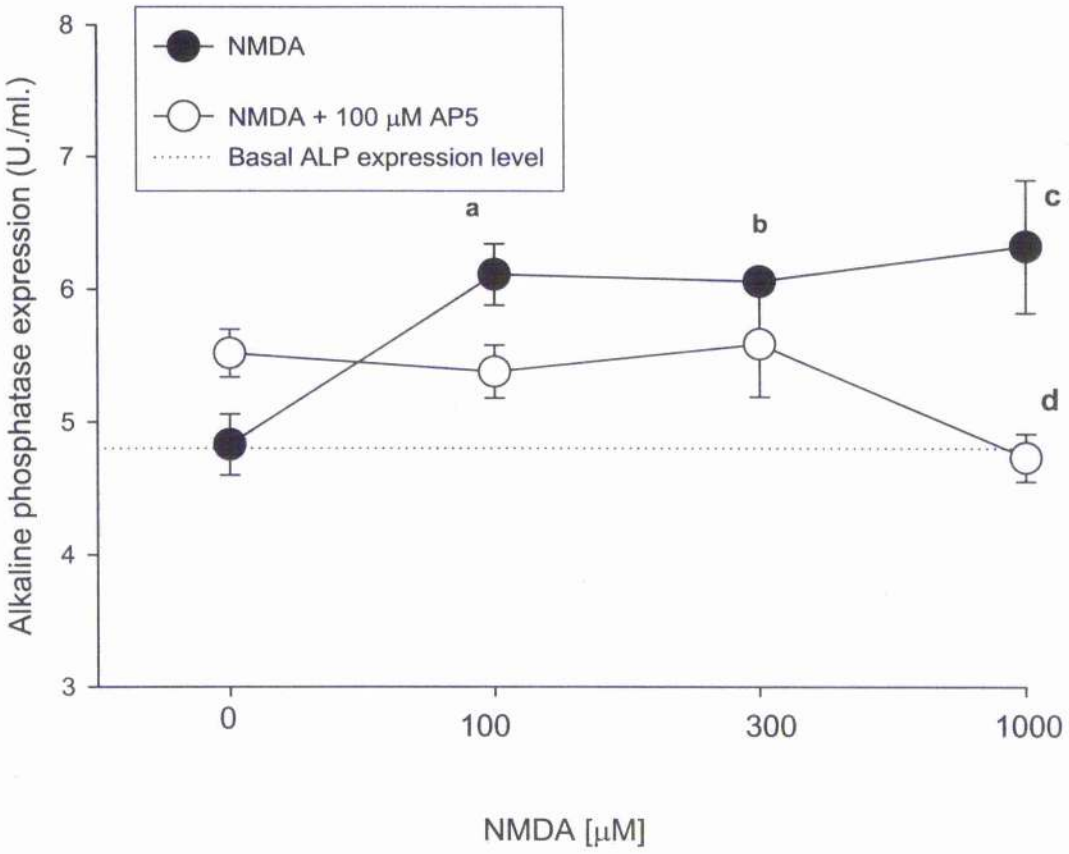

Figure 12. Effect of NMDA on MC3T3-E1 cell differentiation. At 11 DIV, maintenance medium was switched to medium containing 1 % FCS for 24 hr. Agents were then added and cell differentiation was assessed after an additional 48 hr by assaying for alkaline phosphatase (n=4).

a. $p < 0.05$ vs. basal expression level (n=4, SNK test).
b. $p<0.05$ vs. basal expression level (n=4, SNK test).
c. $p<0.05$ vs. basal expression level (n=4, SNK test).
d. $p<0.05$ vs.1000 μM NMDA (n=4, SNK test).
$F=4.348$.

5.2.1.2. 72 hour exposure.

NMDA did not have a significant mitogenic effect when exposed to MC3T3-E1 cells for 72 hr (Figure 11)

5.2.2. Effect of NMDA on MC3T3-E1 cell differentiation.

NMDA at 100 μM significantly increased alkaline phosphatase expression from a basal level of 4.8±0.2 U/ml to 6.1±0.2 U/ml ($p<0.05$, n=4, SNK test) an effect non-significantly reduced by the NMDA selective antagonist, AP5 (100 μM) to 5.4±0.2 U/ml ($p<0.05$, n=4, Figure 12). 300 μM NMDA significantly increased alkaline phosphatase expression to 6±0.1 U/ml ($p<0.05$ vs. basal levels, n=4, SNK test) an effect again non-significantly reduced by AP5. 1000 μM NMDA significantly increased alkaline phosphatase expression to 6.3±0.5 U/ml ($p<0.05$ vs. basal levels, n=4, SNK test), an effect significantly reduced by AP5 to 4.73 U/ml ($p<0.05$, n=4, SNK test). No difference in morphology was observed between untreated and treated cells.

5.3. Effect of QA on MC3T3-E1 cell development.

5.3.1. Effect of QA on MC3T3-E1 cell proliferation.

5.3.1.1. 24 hour exposure.

After 24 hr, quinolinic acid (300 & 1000 μM) had no significant effect on MC3T3-E1 viability (Figure 13). However, 3000 μM QA significantly increased cell viability to 158±7 % of that of basal levels ($p<0.05$, n=4, SNK test). After 48 hr, 3000 μM QA caused a significant increase in cell viability to 167±10 % of basal levels ($p<0.05$, n=4, SNK test). After 72 hr, 300 and 1000 μM quinolinic acid were able to induce a non-significant tendency to increase cell viability. 3000 μM QA significantly increased cell viability to 165±14 % of basal levels ($p<0.05$, n=4, SNK test).

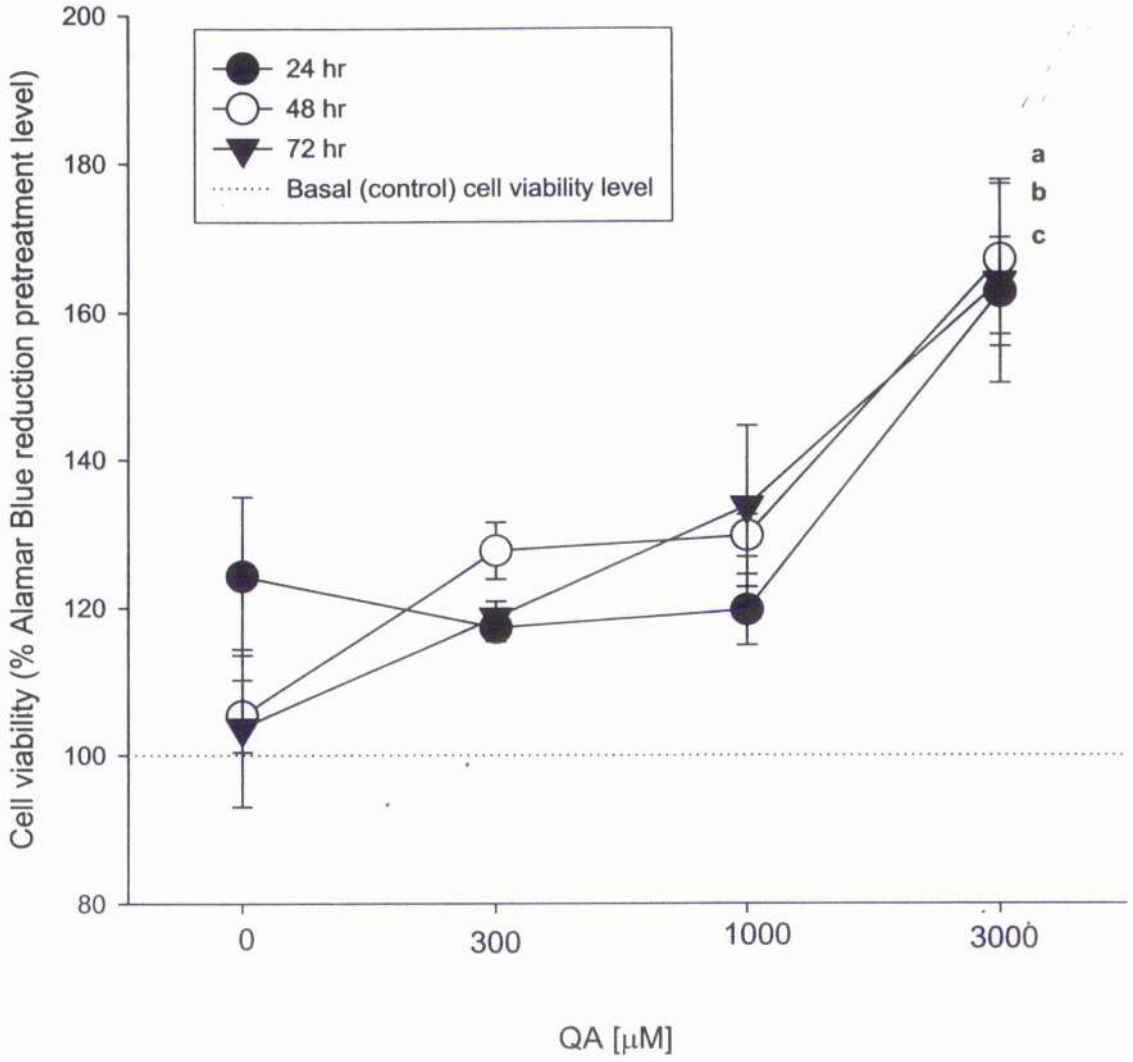

Figure 13. Effect of quinolinic acid (QA) on MC3T3-E1 cell viability. At 6 DIV, cells were subjected to a pretreatment cell viability assay and then exposed to quinolinic acid for 24 hr. Cell viability was assessed after an additional 24, 48 & 72 hr after QA addition (n=3).

a. 24 hr: $p<0.05$ vs. 0 μM, 300 μM and 1000 μM QA (n=3, SNK test).
b. 48 hr: $p<0.05$ vs. 0 μM, 300 μM and 1000 μM QA (n=3, SNK test).
c. 72 hr: $p<0.05$ vs. 0 μM and 300 μM (n=3, SNK test).
$F=9.184$.

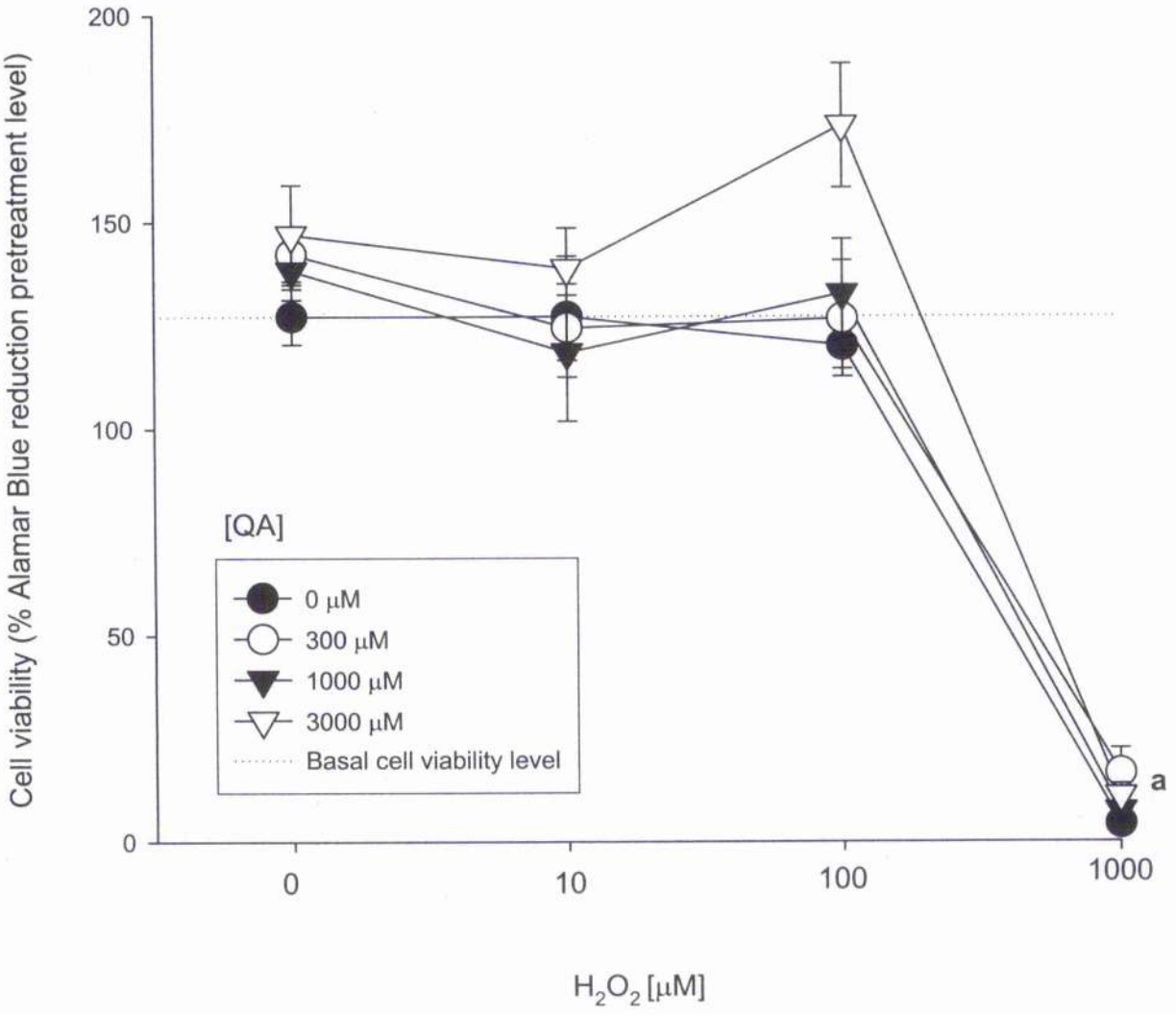

Figure 14. Effect of quinolinic acid (QA) and H_2O_2 on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then exposed to the agents. Cell viability was assessed an additional 72 hr after QA & H_2O_2 addition (n=6).

a. $p<0.001$ vs. all other treatments (n=6, SNK test). $F=143.82$.

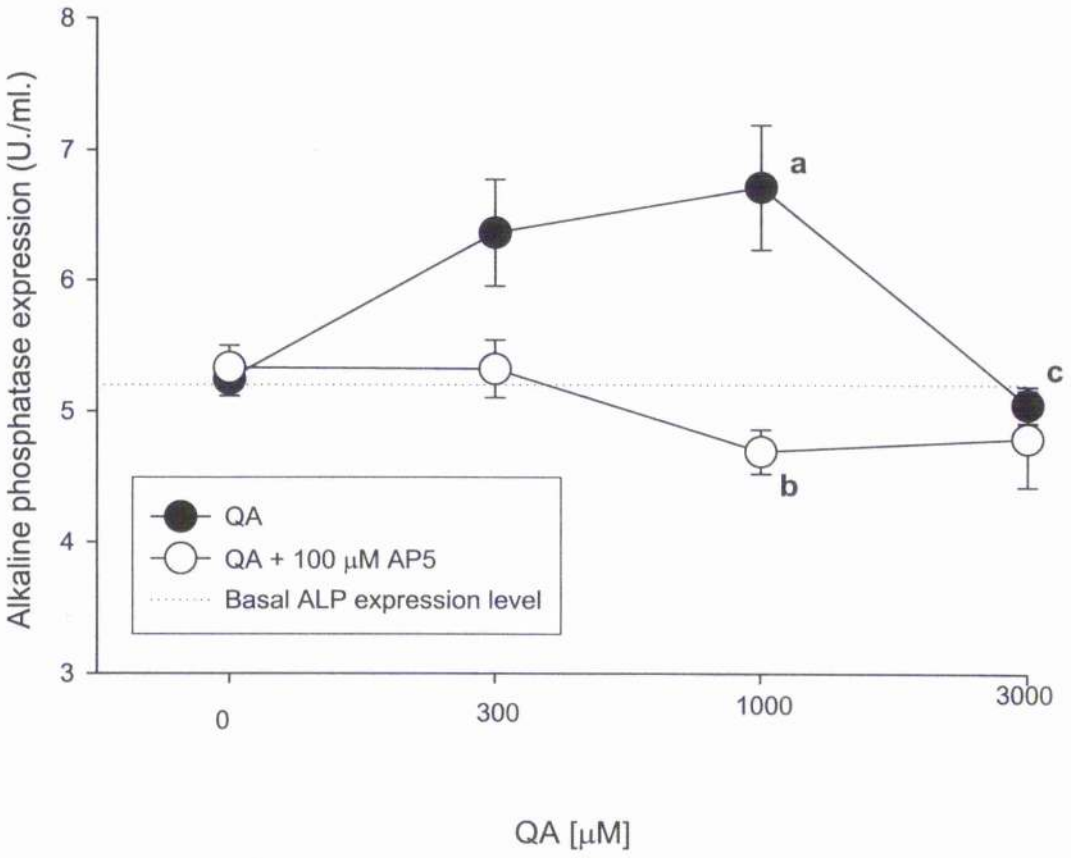

Figure 15. Effect of quinolinic acid (QA) on MC3T3-E1 differentiation. At 11 DIV, cells were switched to maintenance media containing 1% FCS for 24 hr. Agents were then added and differentiation was assessed an additional 48 hr after QA & AP5 addition by assaying for alkaline phosphatase (n=4).

a. $p<0.05$ vs. 0 μM (n=4, SNK test) .
b. $p<0.01$ vs. 1000 μM QA (n=4, SNK test).
c. 3000 μM QA: $p< 0.01$ vs. 1000 μM QA and $p<0.05$ vs. 300 μM QA n=4, SNK test).
F=6.104.

5.3.1.2. 72 hour exposure.

It can be seen in Figure 14, that quinolinic acid did not have a significant mitogenic effect when exposed to MC3T3-E1 cells for 72 hr.

5.3.2. Effect of quinolinic acid on MC3T3-E1 cell differentiation.

At 300 μM, quinolinic acid (QA) non-significantly increased alkaline phosphatase expression, an effect which AP5 (100 μM) non-significantly reduced. 1000 μM QA significantly increased alkaline phosphatase expression to 6.7±0.1 U/ml from a basal expression level of 5.2±0.9 U/ml ($p<0.05$, n=4, SNK test, Figure 15), an effect reduced by AP5 to 4.7±1 U/ml ($p<0.05$, n=4, SNK test). 3000 μM QA had no effect on alkaline phosphatase expression relative to basal levels. In fact, this concentration of QA was able to significantly reduce alkaline phosphatase expression to 5±1 U/ml This was significantly different from 1000 μM QA ($p<0.01$, n=4, SNK test) and 300 μM QA ($p<0.05$, n=4, SNK test), and was not attenuated by AP5. No morphological difference was observed between treated and control cells.

5.4. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell development.

5.4.1. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 proliferation.

CGS 21680 (0.001, 0.01 & 0.1 μM) non-significantly caused a concentration-dependent increase in cell viability (Figure 16).

At concentrations of 0.001 and 0.01 μM, ZM 241385 also non-significantly increased cell viability. At 0.1 μM, ZM 241385 significantly increased cell viability to 191±14 % of basal levels ($p<0.05$, n=6, SNK test).

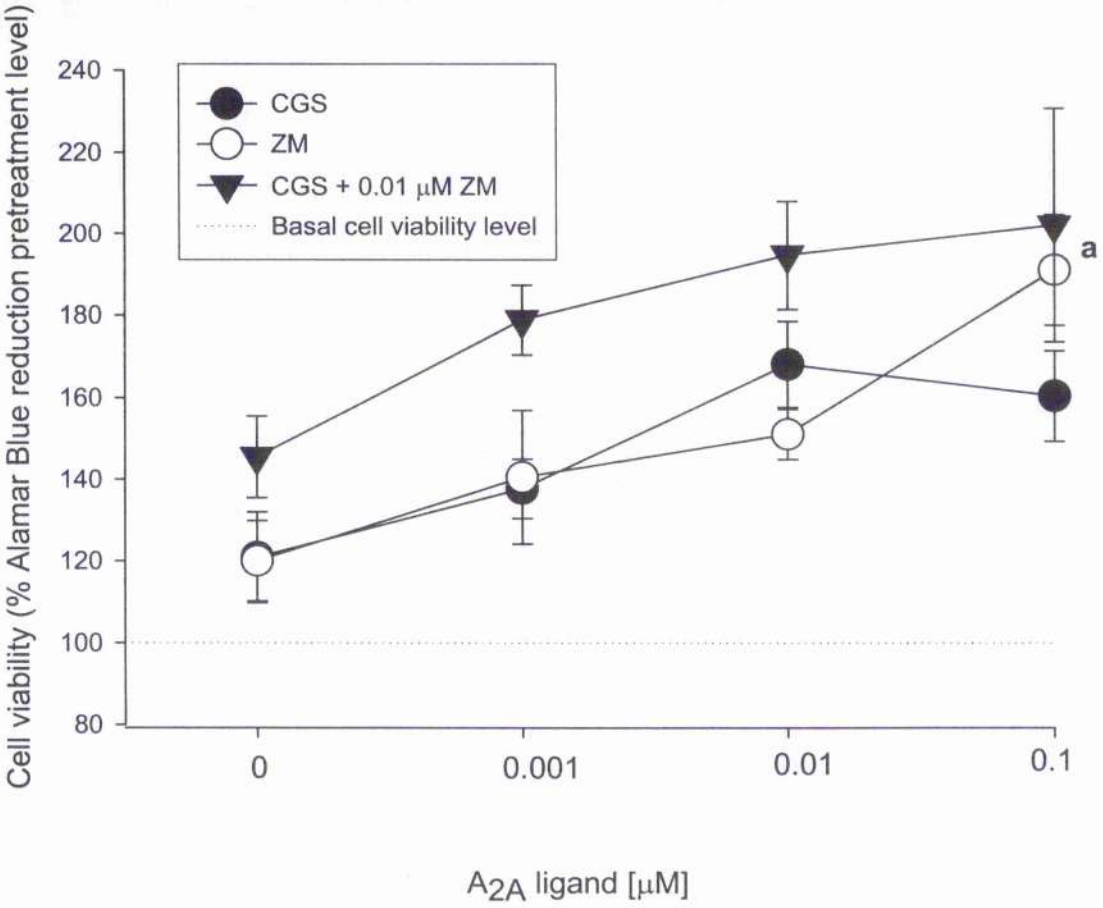

Figure 16. Effect of CGS 21680 (CGS) and ZM 241385 (ZM) on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then exposed to either CGS 21680 alone, ZM 241385 alone or the agonist combined with the antagonist. Cell viability was assessed 72 hr after CGS & ZM addition (minimum n=6).

a. 0.1 μM ZM: $p<0.05$ vs. 0 μM ZM (n=6, SNK test). $F=4.374$.

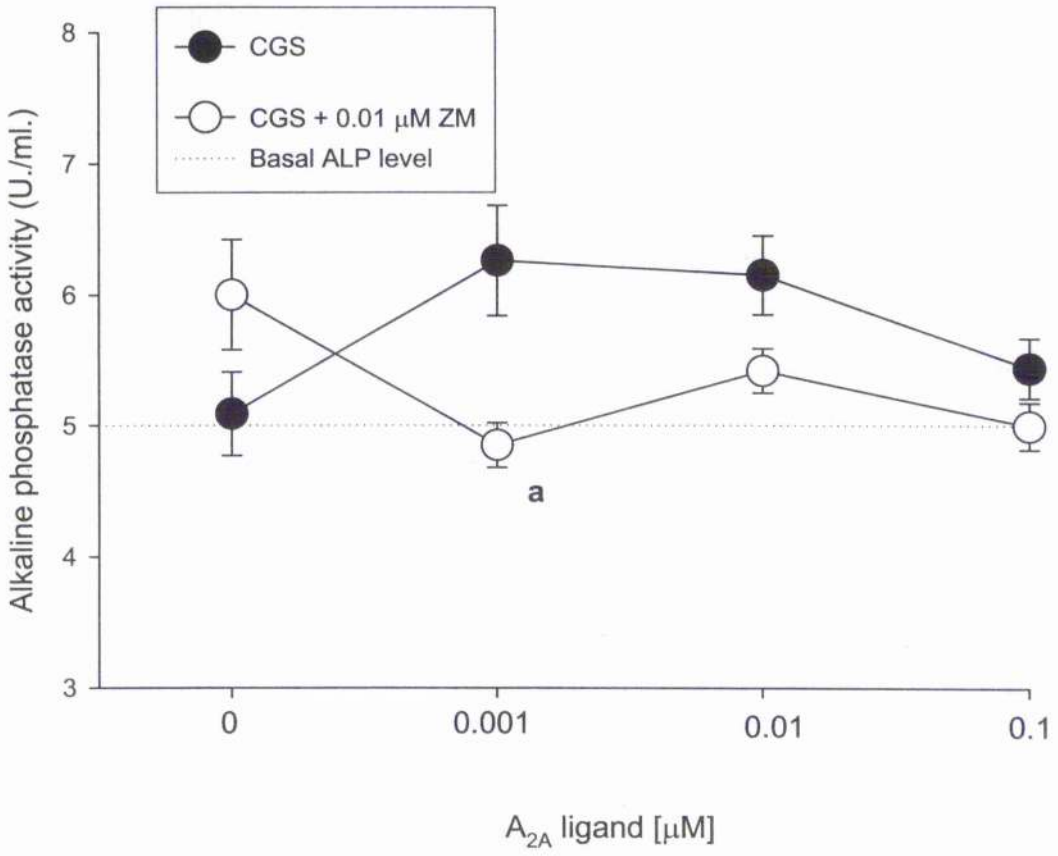

Figure 17. Effect of CGS 21680 (CGS) and ZM 241385 (ZM) on differentiation of MC3T3-E1 cells. Cells were treated as described in methods. At 11 DIV the cells were switched to maintenance media containing 1 % FCS for 24 hr. Agents were then added and cell differentiation assessed by assaying for alkaline phosphatase an additional 48 hr after CGS & ZM addition (n=4).

a. $p<0.05$ vs. 0.001 μM CGS (SNK test, n=4).
F=3.529.

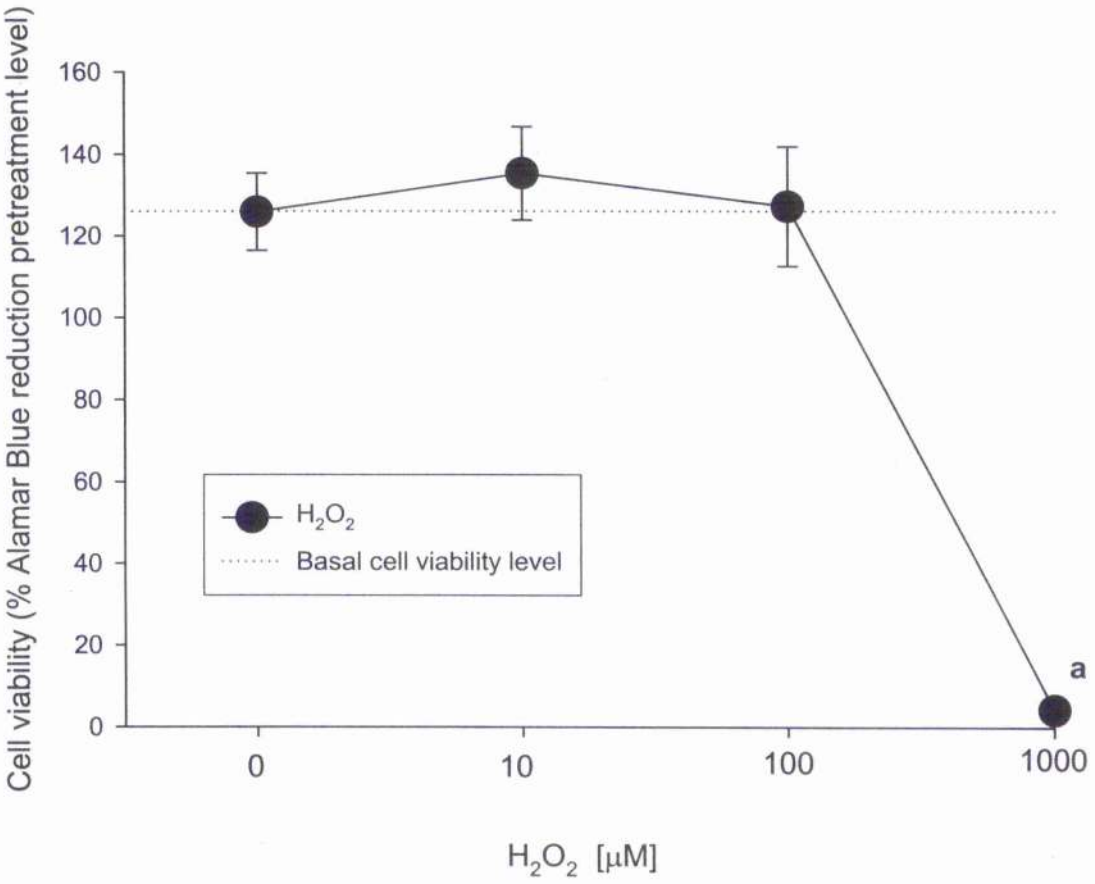

Figure 18. Effect of hydrogen peroxide (H_2O_2) on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then treated with hydrogen peroxide (H_2O_2). Cell viability was assessed an additional 72 hr after H_2O_2 addition (n=6).

a $p < 0.0001$ (two-tailed value) vs. all other treatments (unpaired t-test, n=6). F=89.333.

When combined with ZM 241385, 0.001 μM CGS 21680 non-significantly increased cell viability to 179±8 % of basal levels and 0.01 μM CGS 21680 non-significantly increased cell viability to 195±13 % of basal.

5.4.2. Effect of adenosine A_{2A} receptors on MC3T3-E1 differentiation.

CGS 21680 (0.001 μM) non-significantly increased alkaline phosphatase expression from a basal level of 5±0.3 U/ml to 6.3±0.4 U/ml, an effect significantly reduced by 0.01 μM ZM 241385 to 4.9±0.2 U/ml ($p<0.05$, n=4, SNK test, Figure 17). 0.01 μM CGS 21680 non-significantly increased alkaline phosphatase expression to 6.2±0.3 U/ml. Neither of the agents induced any morphological changes as assessed by light phase microscopy.

5.5. Effect of nitrosative and oxidative stress on MC3T3-E1 cell viability.

S-nitroso-N-acetyl-penicillamine (SNAP) had no effect on MC3T3-E1 cell viability (data not shown) and hydrogen peroxide (H_2O_2) at 10 μM & 100 μM was not significantly injurious to MC3T3-E1 cells (Figure 18). In fact, 10 μM H_2O_2 induced a non-significant increase in cell viability relative to control. However, 1000 μM H_2O_2 dramatically significantly decreased cell viability from a basal level of 130±7 % to 12.7±3 % ($p<0.001$, n=6, SNK test). Morphologically, cell damage induced by H_2O_2 was marked by swelling of the cytoplasm and lysis of the plasma membrane. Cell viability as assessed by trypan blue uptake showed a good correlation with that obtained by Alamar Blue reduction (data not shown).

Neither NMDA (Figure 11), quinolinic acid (Figure 14) or the adenosine A_{2A} receptor ligands had a significant effect on the response to hydrogen peroxide. However, 100 μM H_2O_2 significantly reduced the proliferative effect of 0.1 μM CGS 21680 alone (Figure 19) from 181±11 % to 143±11 % ($p<0.01$, n=6, SNK test) and significantly reduced the proliferative effect of 0.1 μM ZM 241385 from 191±11 % to 143±11 % ($p<0.01$, n=6, SNK test, Figure 20).

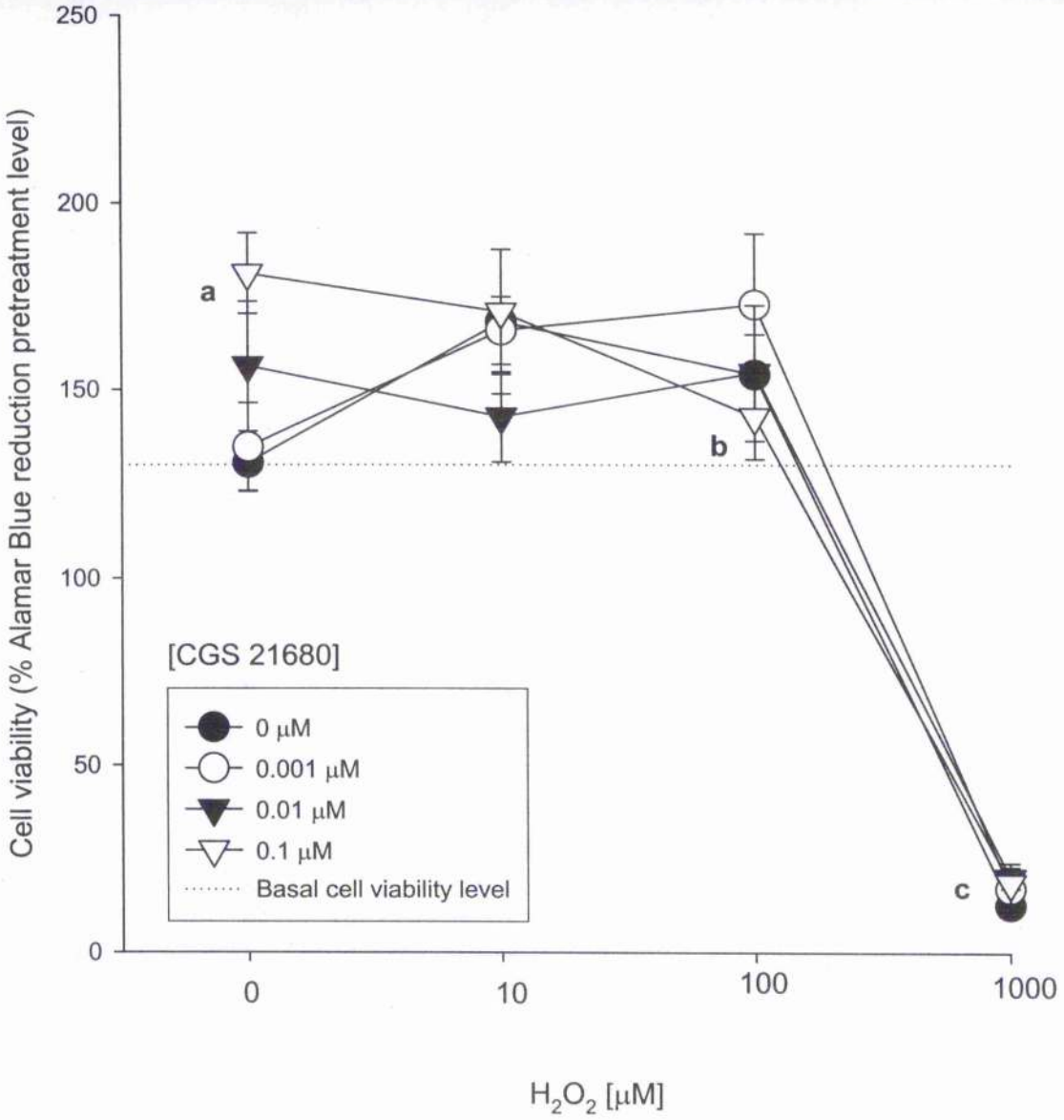

Figure 19. Effect of H_2O_2 and CGS 21680 (CGS) on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then treated with CGS 21680 and various concentrations of hydrogen peroxide (H_2O_2). Cell viability was assessed an additional 72 hr after CGS & H_2O_2 addition (n=6).

a. $p < 0.01$vs. 0 μM CGS/no H_2O_2 (n=6, SNK test).

b. $p<0.01$ vs. 0.1 μM CGS/no H_2O_2 (n=6, SNK test).

c. $p<0.001$ vs. all other treatments (n=6, SNK test).

F=69.1.

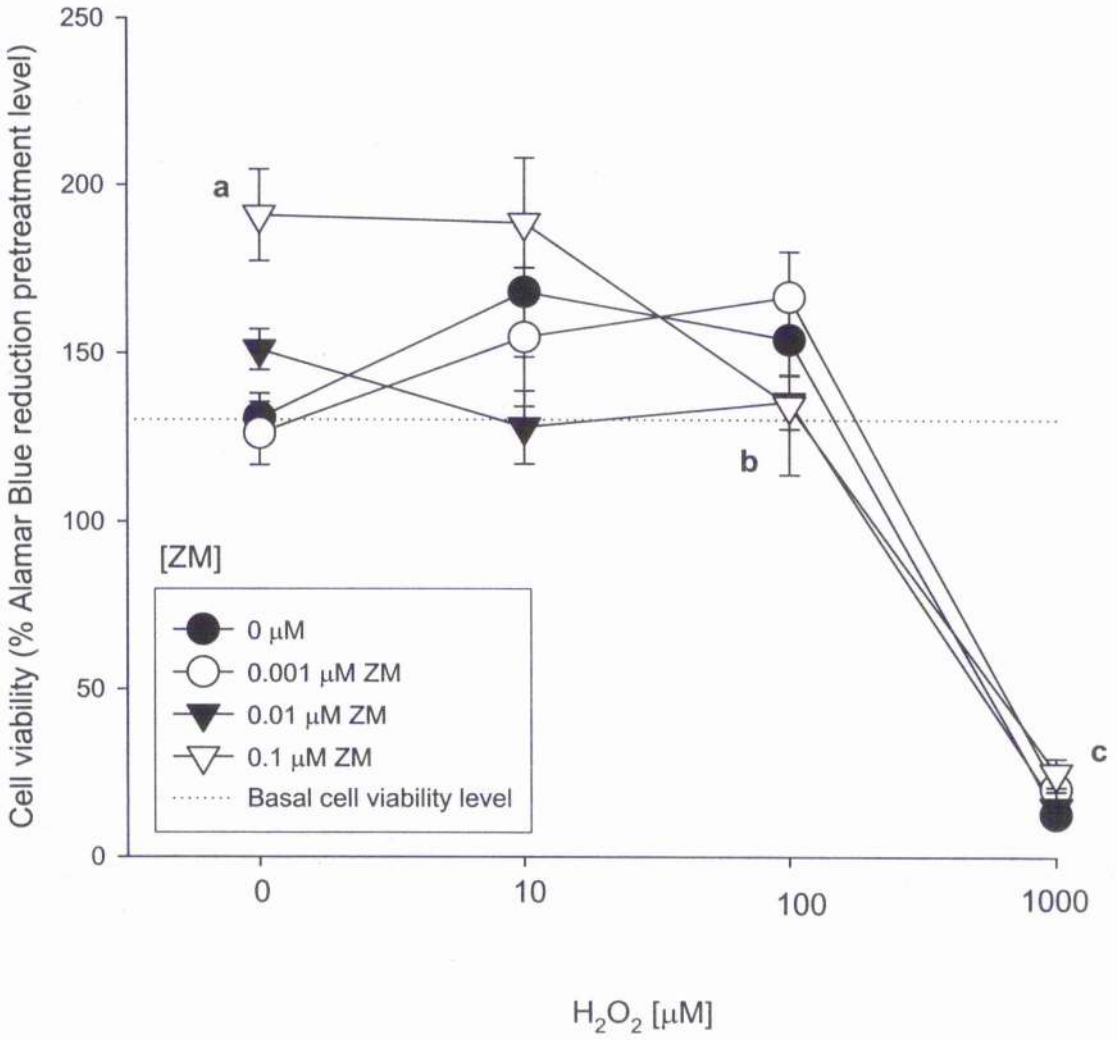

Figure 20. Effect of H_2O_2 and ZM 241385 (ZM) on MC3T3-E1 cell viability. At 6 DIV, cells were subject to a pretreatment viability assay and then exposed to ZM 241385 and various concentrations of hydrogen peroxide (H_2O_2). Cell viability was assessed an additional 72 hr after ZM & H_2O_2 addition (n=6).

a. $p<0.01$ vs. ZM/no H_2O_2 (n=6, SNK test).

b. 0.1 μM ZM+100 μM H_2O_2: $p<0.01$ vs. 0.1 μM ZM/no H_2O_2 (n=6, SNK test).

c. $p<0.001$ vs. all other treatments (n=6, SNK test).
F=32.106.

5.6. Effect of serum withdrawal on MC3T3-E1 cell apoptosis.

Serum withdrawal significantly increased the number of cells positively staining for caspase-3 expression to 10±1 % ($p<0.05$, n=4, SNK test, Figure 21) from a control level of 7±0.4 % (n=4). Cells positively staining for this protease also displayed distinct chromatin condensation and cell shrinkage as assessed by light phase microscopy. The A_{2A} receptor ligands, the selective A_1 receptor agonist cyclopentyladenosine (CPA, 0.02 μM) and quinolinic acid (3000 μM) had no significant effect on expression of caspase-3.

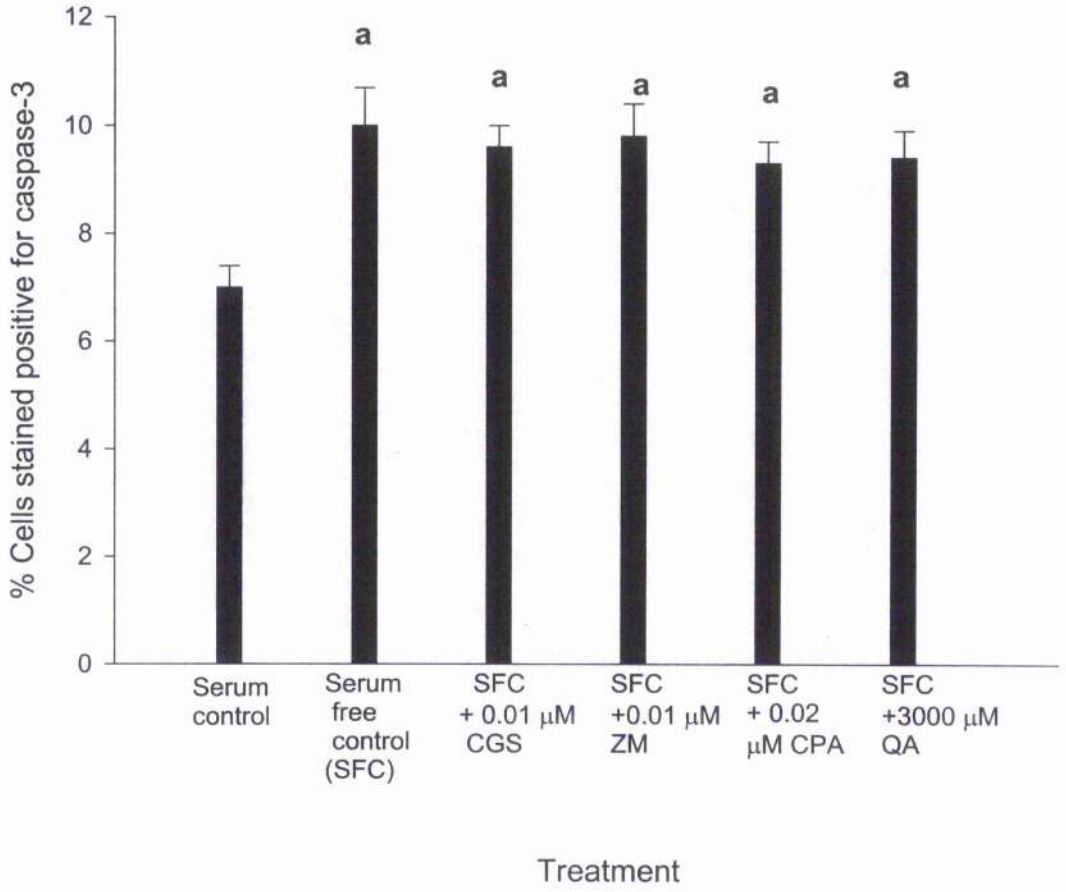

Figure 21. Effect of serum withdrawal on caspase-3 expression in MC3T3-E1 cells. At 3 DIV, cells were washed 3 times in α-MEM containing 0.5 % bovine serum albumin. Caspase-3 activity was assessed by immunostaining 18 hr later as described in methods. CGS 21680 (CGS), ZM 241385 (ZM), cyclopentyladenosine (CPA) & quinolinic acid ((QA), (n=4)).

a. $p<0.05$ vs. serum control (n=4, SNK test).
F=4.594.

6. Discussion of the effects of glutamate and adenosine receptor ligands on neuronal cell cultures.

6.1. Discussion of the use of hippocampal vs. cortical neuronal cultures as *in vitro* models of excitotoxicity.

Hippocampal neuronal cultures were more sensitive to NMDA than cortical neuronal cultures were, as has previously been reported (Rondouin et al., 1988). The number of papers in the literature reporting the use of these neuronal cultures for *in vitro* models of excitotoxicity is about equal and extensive for both cell types. Several laboratories have examined why there is this difference in vulnerability to excitotoxicity and a number of factors appear to influence the susceptibility of a neuron to the phenomenon. These are glutamate receptor expression (Mattson & Kater, 1988), maturation of the receptor complex (Peterson et al., 1989), maturation of 2nd messenger systems (Behrens et al., 1996; Marks et al., 2000) the oxidation state of the receptor (Sinor et al., 1997), the presence of neuroD (Katayama et al., 1997) or calretinin (Lukas & Jones, 1994) as well as the state of development of an inhibitory neurotransmitter system (e.g. GABA) (Cai & Erdo, 1991).

In agreement with the literature, both cortical and hippocampal cultures did not become susceptible to NMDA-mediated excitotoxicity until about 14 days *in vitro*. It is known that the NMDA receptor is developmentally regulated in both neuronal culture types (Frandsen & Schousboe, 1990; Drian et al., 1991, Lustig et al., 1992; Weller et al., 1993; Akaneya et al., 1994; Griffiths et al., 1997; Gonzalez-Zulueta et al., 1998; Cho et al., 2000; Hewett et al., 2000; Rudolph et al., 2000; Sinor et al., 2000; Skaper et al. 2001). However, Peterson et al. (1989) revealed that glutamate binding to the NMDA receptor during development *in vivo* is greater in the hippocampus than it is in the cortex. Furthermore, Olverman et al. (1984) showed that the density of binding sites for [^{3}H]-D-AP5 is greater in rat hippocampal membranes than it is in cortical membranes, suggesting that the number of functional NMDA receptors is higher in the hippocampus than it is in the cortex *in vitro*.
It is likely that this same level of receptor expression occurs in cultures of these brain areas and this would explain the increased vulnerability of hippocampal cultures over cortical cultures to excitotoxicity.

In support of this, Kovacs et al. (2001) found that the expression of functional NMDA receptors was greater *in vivo* in the cortex relative to the striatum. This enhanced expression of NMDA receptors in cortical tissue was preserved *in vitro* and cortical neuronal cultures proved more sensitive to excitotoxicity than striatal neuronal cultures. However, it cannot be excluded that other factors apart from enhanced expression of NMDA receptors contributed to the increased sensitivity of hippocampal neuronal cultures over cortical neuronal cultures observed in the present project.

In the project, cortical neuronal cultures served as an adequate *in vitro* model for investigating neuroprotective mechanisms of action. Cortical neuronal cultures in general were easier to establish than hippocampal cultures. As the cortex is a much larger brain area than the hippocampus, the dissection procedure is a lot quicker and so the likelihood of establishing viable neuronal cultures using the cortex is much greater. Also, when the cortex is cultured, more experimental wells are yielded. Furthermore, experience showed that cortical neuronal cultures could be established when the cells were seeded in 96 well plates using just poly-D-lysine (10 μg/ml) without laminin as the substrate. This necessitated the cells to be seeded at a density of between 400,000 and 500,000 cells/ml which would not be possible using hippocampal tissue. As laminin is an expensive substrate, for economic reasons as well, cortical neurons are more advantageous to use than hippocampal neurons.

Despite the fact that hippocampal neurons are more more susceptible to excitotoxicity than cortical neurons, the advantages of the latter cell type for use in neuronal cell culture as an *in vitro* model of excitotoxicity outweigh the disadvantages. Thus, unless one has a specific reason for using hippocampal neuronal cultures, it is suggested that cortical neuronal cultures are a better choice to investigate the mechanism of action of neuroprotective agents than hippocampal neuronal cultures.

6.2. Discussion of cell viability assays for quantification of excitotoxic cell death.

During the project, a number of assays were used to quantify excitotoxic cell death. Intially, a colorimetric assay, (3-(4,5-dimethylthiazol-2-yl)-5-(-3-carboxymethoxyphenyl)-2-(-4-sulfophenyl)-2H-tetrazolium inner salt; MTS) was used to determine cell viability. Recently, a number of assays based upon the reduction of tetrazolium salts have been promoted in the form of several commercial kits aimed at measuring "cell proliferation". These methods rely on the concept that tetrazolium salts are readily reduced to their respective formazans by metabolically active cells.

The general method for the use of these reagents is that the tetrazolium salt is added to microcultures of cells, incubated until the formazan product is produced (1-4 hrs. in the case of MTS). The contents of the well are then triturated to ensure an even distribution of this soluble formazan and the absorbance read in triplicate in a 96 well plate at 490 nm. According to the manufacturers (Promega), "MTS is bioreduced by cells into a coloured formazan product that is soluble in tissue culture medium". Furthermore it is stated that the "quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture" (Promega technical bulletin No. 245, 1999). Thus, it was hoped that this colorimetric dye would offer a quick, convenient, non-subjective assay to quantify cell death. However, it was soon realized that a number of excitotoxins were not inducing concentration-dependent cell death as assessed by this method in cultures in the present study. Why this is the case is now discussed.

Major emphasis in the literature is put on the role of mitochondrial dehydrogenases and more specifically the role of succinic dehydrogenase and the mitochondrial electron carriers in the respiratory chain subsequent to ubiquinone when discussing the bioreduction of tetrazolium salts (Marshall et al., 1995). As reduction is frequently depicted as the addition of hydrogen, it is fundamentally due to the addition of electrons, the simultaneous transfer of protons is incidental.

Indeed, tetrazolium salts are sometimes referred to as artificial electron acceptors. Furthermore, the reaction whereby tetrazolium salts is reduced occurs via the formation of a free radical intermediate, tetrazolinyl in which only one electron has been transferred to the tetrazole ring (Marshall et al., 1995).

It is widely accepted that NMDA mediated excitotoxicity occurs in part via intracellular accumulation of superoxide free radicals (Lafon-Cazal et al., 1993; Reynolds & Hastings., 1995; Patel et al., 1996), that mitochondrial electron transport is enhanced during NMDA mediated cell death (Sengpiel et al., 1998) and that glutamate itself supports MTT reduction by isolated mitochondria (Liu et al., 1997). Thus it is possible that superoxide generation during cell death by these agents could cause the reduction of tetrazolium salts. Furthermore, it should be noted that the reduction of nitroblue tetrazolium actually forms the basis of a well established indirect assay for superoxide dismutase (Marshall et al., 1995). Also, although it has been observed by other investigators that MTT reduction may occur extramitochondrially (Berridge & Tan, 1993; Liu et al., 1997), superoxide free radical generation by excitotoxins could still affect this (Patel et al., 1996). Indeed, other authors have noted discrepancies between tetrazolium salt assays and other assays of cell viability (Uliasz & Hewett, 2000).

Therefore it is recommended that caution be exercised in the use of tetrazolium salts as indicators of excitotoxic cell death because although cell death may be occurring, cell viability as measured by formazan production may appear to be normal or even enhanced by excitotoxins.

It was therefore decided to use trypan blue exclusion (Tennant, 1964) to quantify cell death. The use of the dye in quantification of excitotoxic cell death is established (see Mattson et al., 1996). Although cheap, the use of this method as a marker of cell death has severe limitations, namely, it requires the use of cell counts which can be very time-consuming and tedious. Furthermore, assessment of cell death using cell counts can be subjective. Also, trypan blue exclusion suffices only as a marker of necrosis and will not quantify apoptotic cell death.

Thus it was attempted to use a spectrophotometric method for trypan blue quantification of cell death (Uliasz & Hewett, 2000) in the hope that this would overcome the limitations of cell counts with the dye. However, this method was also found to be quite tedious as it necessitated cell lysis with sodium dodecyl sulphate and careful tituration of the lysate without introducing air bubbles. It also required an initial cell seeding density of at least 300,000 cells/ml. which could not be obtained with hippocampal neuronal cultures which were the culture type being used at that stage in the project.

Alamar Blue (Biosource) reduction was used to measure bone cell viability and was found to be a quick, reliable and sensitive colorimetric method. This dye was able to quantify hydrogen peroxide-induced injury in these cells which showed a good correlation with cell death quantification assessed by cell counts with trypan blue. Thus, it seems that this colorimetric method is not susceptible to interference by superoxide free radical production and would offer a quick reliable method for measuring excitotoxic cell death as well.

6.3. Discussion of effects of agents on cortical neuronal cultures.

6.3.1. Effect of AP5 on cortical neuronal cultures.

The aim of this project was to establish what the exact role of the adenosine A_{2A} receptor is in neuronal viability. Using a well established *in vitro* model for examining the action of neuroprotective agents i.e. cortical neuronal cultures, AP5 was able to attenuate NMDA elicited cell damage in the neuronal cultures in the present study, suggesting that cortical neuronal cultures were serving as a good *in vitro* model for excitotoxicity.

6.3.2. Effect of kynurenines on cortical neuronal cultures.

Quinolinic acid showed a trend of eliciting cell death in cortical neuronal cultures (Figure 7), an observation compatible with the growing body of evidence that QA is an endogenous neurotoxin as its concentration is known to be elevated in a number a number of neurodegenerative disorders (Stone, 2001).

That kynurenic acid was unable to block the effects of quinolinic acid could be due to the fact that the compound was inducing cell damage primarily via free radical generation, independent of glutamate receptors as has previously been suggested (Rios & Santamaria, 1991; Behan et al., 1999).

6.3.2. Effect of CGS 21680 on cortical neuronal cultures.

So what is the role of the adenosine A_{2A} receptor in modulation of neuronal viability? It is generally accepted in the literature that CGS 21680 is selective for A_{2A} receptors at a concentration of 0.02 μM (Klotz, 1999; Muller, 2000). In the current study at this concentration, CGS 21680 was able to prevent NMDA induced cell death.

This is in agreement with the study of Ferreira et al. (2001) who demonstrated that CGS 21680 was neuroprotective in cultures of avian retinal neurons and the report of Lee & Chao (2001). These latter authors revealed that CGS 21680 (0.001 μM) was able to rescue primary hippocampal neurons from withdrawal of growth factor induced apoptosis.

When the concentration of CGS 21680 was increased to 0.05 μM the compound apparently increases neuronal cell death. However when the concentration of this compound was increased to 0.5 μM and 5 μM there was a trend in that the compound was neuroprotective and at the highest dose used neuroprotection is observed. The adenosine A_{2A} receptor has a wide range of neuromodulatory effects which could be either neuroprotective or neurodetrimental. One of the latter effects is inhibition of neuroprotective adenosine A_1 receptors and glutamate release (Cunha, 2001), an action that would occur at a low concentration of CGS 21680. Thus it is likely that this inhibitory effect was occurring at 0.05 μM and was starting to overcome the neuroprotective neuromodulatory effects of CGS 21680.

Although CGS 21680 is the prototypical A_{2A} selective agonist, at 0.5 μM and especially at 5 μM the compound can act as a direct agonist at adenosine A_1 receptors (Muller, 2000; Klotz, 1999).

Thus CGS 21680 was probably directly activating A_1 receptors at the highest dose used in the project which would have overcome any inhibition of this receptor resulting from A_{2A} receptor activity. Activation of the adenosine A_1 receptor is neuroprotective in cell culture (Mendonca et al., 2000) and so this effect would explain the neuroprotection observed at this highest dose of CGS 21680.

6.3.3. Effect of ZM 241385 on cortical neuronal cultures.

In vivo data have shown that adenosine A_{2A} receptor antagonists are neuroprotective and that this is a centrally mediated effect (Jones et al., 1998a; Behan & Stone, 2002). 0.05 μM is a concentration at which ZM 241385 is selective for adenosine A_{2A} receptors (Moreau & Huber, 1999). At this concentration, the agent protected against 100 μM NMDA-induced cell damage but not against that induced by 30 μM of the excitotoxin. Why this was the case is suggested below.

During the pathology of ischaemia and excitotoxicity, there is a release of adenosine (Latini & Pedata, 2001). The release of the "retaliatory metabolite" may be either neuroprotective or neurodetrimental depending on the degree of ischaemia and cell damage. Thus, when cell damage is mild the extracellular concentration (1-10 μM) of adenosine would only be sufficient to activate adenosine A_1 receptors, an effect which would be protective. However, when cell damage is more severe and the concentration of adenosine rises to above about 20 μM it would activate A_{2A} receptors, an action which would inhibit A_1 receptor activation and then increase glutamate release. This effect could be neurodetrimental.

NMDA is known to induce release of adenosine from the cortex *in vitro* (see Latini & Pedata, 2001). Furthermore, Schousboe et al. (1989) showed that adenosine release can be induced by NMDA in cerebellar granule cell cultures. Therefore, it is likely that this same NMDA induced release of adenosine would occur in cortical neurons *in vitro*. Thus, it is appealing to speculate that the reason ZM 241385 was able to protect against damage induced by the highest concentration of NMDA is because it was releasing the A_{2A} receptor mediated inhibition of A_1 receptors, so allowing adenosine to exert neuroprotection via this receptor.

It would also be antagonising any A_{2A} receptor mediated glutamate release induced by a pathological extracellular accumulation of the purine evoked by NMDA.

However, it should be noted that there is a possibility that the effects of ZM 241385 could be due to effects of this compound on receptor number. For example, Varani & colleagues (1999, 2000) reported that chronic blockade of adenosine A_{2A} receptors caused an upregulation of the receptor and sensitization of the responses to 2-hexenyl-5-N-ethyl carboxamido adenosine (HE-NECA). It is possible that this same receptor adaptation occurred in the neuronal cultures in the present project as the cells were exposed to ZM 241385 for 72 hr which could be long enough for an upregulation of the adenosine A_{2A} receptor to occur so that the antagonist induces the same response as the agonist. Therefore, although it is less likely than the hypothesis detailed above, it cannot be excluded that ZM 241385 was upregulating adenosine A_{2A} receptors and allowing protection by endogenous adenosine through these upregulated receptors.

6.4. Discussion of the signal transduction mechanism by which the adenosine A_{2A} receptor elicits neuroprotection.

Although further experiments would be necessary to elucidate the signal transduction mechanisms responsible for the neuroprotection observed with CGS 21680, it is not unreasonable to speculate on this now. There are a wide range of neuromodulatory mechanisms whereby CGS 21680 could have exerted this neuroprotection (see Table 1 Introduction). However, the two most likely mechanisms are transactivation of neurotrophin receptors via Akt activation (Lee & Chao, 2001) and inhibition of NMDA conductance via CamKII activation (Wirkner et al., 2000) as these effects have been demonstrated in primary neuronal culture.

CGS 21680 was able to reduce cell death induced by 10 μM NMDA but the NMDA-selective antagonist, AP5 was unable to reduce cell damage induced by this concentration of the excitotoxin.

Furthermore, CGS 21680 showed an apparent tendency to be more effective in reducing cell damage elicited by 30 μM NMDA than AP5. Also, ZM 241385 had no effect on cell damage induced by these concentrations of NMDA. It has been shown that adenosine A_{2A} receptor blockade enhances NMDA calcium conductances in striatal neuronal cultures (Popoli et al., 2002). Therefore, had ZM 241385 been blocking any adenosine A_{2A} receptor mediated effects on NMDA channel conductance, one would have expected ZM 241385 to increase cell damage induced by NMDA to above control levels. This did not occur. Thus, it is tentatively suggested that CGS 21680 was transactivating neurotrophin receptors in this A_{2A} receptor mediated- protective effect (Figure 22).

6.5. Discussion of the mechanism of cell death induced by glutamate receptor ligands.

The mechanism of cell death elicited by both quinolinic acid and NMDA appeared to be necrotic as assessed by trypan blue uptake. However, it is now evident that the distinction between apoptosis and necrosis in excitotoxicity on morphological grounds is becoming increasingly blurred. Thus, it cannot be excluded that there was an apoptotic component to excitotoxicity in the present study. Indeed, it has been shown that NMDA (Tenneti et al., 1998; Tenneti & Lipton, 2000) and quinolinic acid (Qin et al., 2000) can induce caspase-3 dependent neuronal cell death. Thus it is possible that this form of cell death was occurring as well as necrosis.

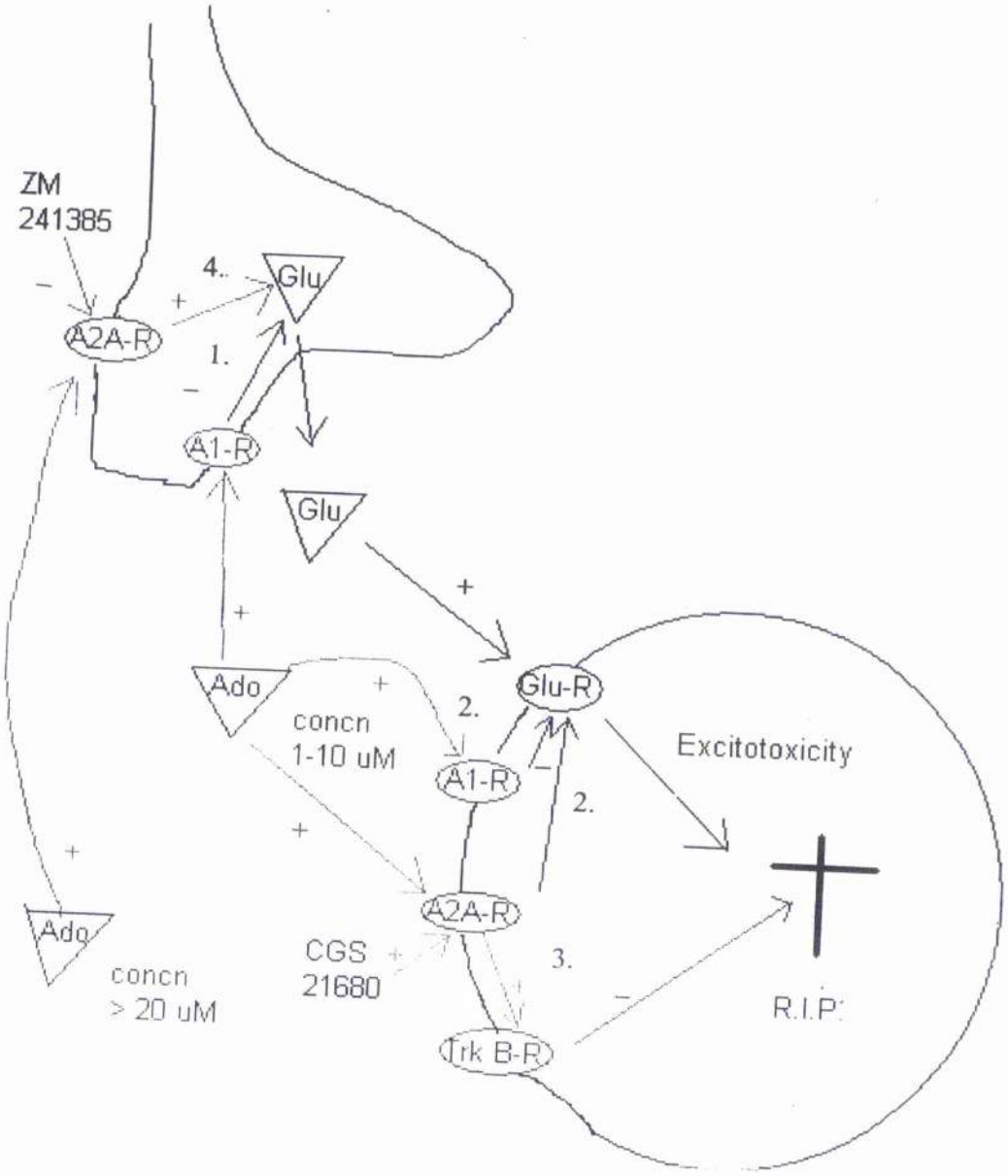

Figure 22. Summary of the mechanisms whereby adenosine and adenosine A_{2A} receptor ligands could modulate cell damage during an ischemic insult. When ischemia and cell damage is mild, the concentration of extracellular adenosine will only be between 1 and 10 μM (Latini & Pedata, 2001) and so the purine will exert neuroprotection. The main mechanisms whereby this would be achieved would be 1) attenuation of glutamate release (via presynaptic inhibitory A_1 receptors), 2) indirect modulation of glutamate receptors (both A_1 and A_{2A} mediated) and 3) transactivation of TrkB neurotrophin receptors. Adenosine A_{2A} receptor agonists would synergise in this latter action.

However, during a heavier ischaemic insult, cell damage will be more severe and the concentration of adenosine could rise to 20 μM or more, a situation in which activation of A_{2A} receptors would increase the release of glutamate (4). The cell damage caused by release of the excitatory amino acid would overcome the neuroprotective modulatory effects of A_1 and A_{2A} receptor activation. In this scenario, adenosine A_{2A} receptor antagonists would be beneficial.

7. Discussion of the effects of glutamate and adenosine receptor ligands on bone cell cultures.

7.1. Differentiation of MC3T3-E1 cells.

Cells maintained in medium with the osteogenic supplements ascorbic acid and β-glycerol phosphate showed a 2-fold enhanced expression of alkaline phosphatase compared to cells maintained in medium without these supplements, an obervation in agreement with the literature (Quarles et al., 1992). As alkaline phosphatase expression is commonly used as a marker of osteoblast differentiation, it is clear that the MC3T3-E1 cultures used were capable of expressing the osteoblast phenotype and therefore represent a good *in vitro* model for examining the effects of potential osteogenic agents.

7.2. Effect of NMDA on MC3T3-E1 cell development.

7.2.1. Effect of NMDA on MC3T3-E1 cell proliferation.

In the present study, NMDA enhanced MC3T3-E1 cell proliferation. There are conflicting reports on the role of glutamate in bone cell viability. Gray and colleagues (2001) showed that NMDA did not have an effect on bone formation in primary rat osteoblasts, whereas others have shown that glutamate acts as a trophic factor in bone cell development as assessed by markers of differentiation and reduction of apoptosis (Taylor & Skerry, 2001; Genever & Skerry, 2001). In agreement with the latter observations, the current data suggests strongly that the transmitter acts as a mitogen on bone cells via the NMDA receptor.

7.2.2. Effect of N-methyl-D-aspartate on MC3T3-E1 cell differentiation.

NMDA increased alkaline phosphatase expression in MC3T3-E1 cells, an effect which the NMDA selective antagonist AP5 trended to reduce. Although Genever & Skerry (2001) reported that glutamate increased MC3T3-E1 cell differentiation, the present data are the first to demonstrate that the transmitter mediates some of this trophic effect via the NMDA receptor. It is believed that this is the first study to report that this glutamate receptor subtype mediates the trophic effects of the transmitter on bone cells. It is known that glutamate acting on NMDA receptors is essential as a neurotrophic factor during early development of the CNS (Burgoyne et al., 1993) and thus, it seems that this property of the transmitter extends to bone cells.

7.3. Effect of QA on MC3T3-E1 cell development.

7.3.1. Effect of QA on MC3T3-E1 cell proliferation.

Quinolinic acid was also mitogenic for MC3T3-E1 cells. Again, as far as it is known this is the first study to demonstrate that the compound has a mitogenic effect on bone cells. However, it is not the first observation to demonstrate a role for the kynurenine in cell proliferation. Rzeski and colleagues (2001) showed that tumour cells express glutamate receptors and the kynurenine pathway is expressed in local immune cells during tumour growth (Moffett et al., 1997). The same occurs in bone cells (Stone & Darlington, 2002). Thus, it is possible that kynurenine pathway activation during bone remodelling disorders could influence proliferation in this tissue.

7.3.2. Effect of QA on MC3T3-E1 cell differentiation.

As with NMDA, quinolinic acid increased alkaline phosphatase in MC3T3-E1 cells, an effect which AP5 showed an apparent tendency to reduce. However, the top concentration of QA showed a tendency to decrease cell differentiation. As far as it is known, this is the first report demonstrating a role of the kynurenine in bone cell differentiation.

The mechanisms of action for these trophic effects of QA and NMDA remain to be determined. However, it has been shown that QA can induce NGF activity in astroglia (Dong-Ruyl et al., 1997) and this neurotrophin induces differentiation of MC3T3-E1 cells (Yada et al., 1994) so perhaps this same growth factor induction by the kynurenine is occurring in osteoblasts. Also, it is known that in the CNS, there is a close synergistic relationship between glutamate and neurotrophic factors (Lessmann, 1998) so perhaps this property extends to non-neuronal cells. Interestingly, it has been shown that 4-hydroxynonenal, one of the major products of lipid peroxidation can have opposing effects on cell proliferation and differentiation (Parola et al., 1999). Furthermore, at 3000 μM, quinolinic acid has been suggested to have a negative effect on lipid peroxidation in rat brain (Stipek et al., 1997). The present data show that at this concentration quinolinic acid was able to induce cell proliferation but tended to inhibit differentiation. The latter action at least was not mediated via the NMDA receptor as AP5 did not have any significant effect on it. Thus, it is pleasing to hypothesise that byproducts of lipid peroxidation have a negative effect on MC3T3-E1 cell proliferation but a positive effect on their differentiation. Finally, the data also raises the tantalising question of whether the kynurenine pathway itself (Stone, 1993; 2001) is actually active in bone cells.

7.4. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell development.

7.4.1. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell proliferation.

CGS 21680 had a mitogenic effect on MC3T3-E1 cells. This is not the first evidence for the adenosine A_{2A} receptor inducing cell proliferation. Sexl et al. (1995) showed that the adenosine A_{2A} receptor could induce capillary outgrowth and endothelial cell proliferation. Furthermore it has been demonstrated that the adenosine A_{2A} receptor can induce proliferation in thyroid cells (Ledent et al., 1998). Also, the receptor has been suggested to play a role in trout male germ cell proliferation (Loir, 2001).

ZM 241385 was also proliferative for MC3T3-E1 cells. That the response is not attenuated by the presence of ZM 241385 but is in fact enhanced by it is most likely due to an effect on receptor upregulation (Varani, 1999; 2000) as the cells were exposed to the agents for 72 hr. As ZM 241385 alone was able to cause a response, this suggests that there is a basal release of adenosine which mediates proliferation. However, it should be noted that Klinger and colleagues (2002) demonstrated that the adenosine A_{2A} receptor can show constitutive activation of MAP kinases so it is possible that the upregulated receptor was inducing proliferation in the absence of the purine.

That there is adenosine signalling in bone cells has been shown previously. Shimegi (1996;1998) reported that adenosine could induce proliferation in these cells. It was suggested that adenosine mediated some of this effect via A_1 receptors as it was sensitive to cyclopentylxanthine and pertussis toxin but some of the effect was insensitive to these agents. The present data are the first to reveal using adenosine A_{2A} receptor ligands that this adenosine receptor subtype is capable of inducing proliferation of MC3T3-E1 cell, although the data would have to be confirmed by cell counts. However, it should be mentioned there is a possibility that in these cells, there is a site recognised by CGS 21680 that is not the "classical" A_{2A} receptor as reported for the CNS neurones (Lindstrom et al., 1996). Nevertheless, the present data strongly supports the existence of functional adenosine A_{2A} receptors in this cell line.

7.4.2. Effect of adenosine A_{2A} receptor ligands on MC3T3-E1 cell differentiation.

CGS 21680 showed a trend of increasing alkaline phosphatase expression in MC3T3-E1 cells which ZM 241385 trended to reduce. Although as mentioned above the adenosine A_{2A} receptor is known to have a proliferative effect on a number of cell types. As far as it is known, this is the first data to report that this receptor has an additional trophic role and is capable of enhancing cell differentiation.

The signal transduction pathway that underlies this trophic effect of the A_{2A} receptor ligands remains to be determined. It is established that in the CNS, the adenosine A_{2A} receptor enhances glutamate release (Marchi et al., 2002). Skerry and Genever (2001) reported that glutamate induces alkaline phosphatase expression in osteoblasts so the obvious mechanism whereby the receptor can induce differentiation of MC3T3-E1 cells is by increasing glutamate release.

However, it is known that the adenosine A_{2A} receptor has a close synergistic relationship with growth factors, especially NGF (Arslan et al., 1997; Arslan & Fredholm, 2000; Lee et al., 2002) and this neurotrophin can induce differentiation of MC3T3-E1 cells (Yada et al., 1994). In addition, as just mentioned, it is apparent that the adenosine A_{2A} receptor can activate MAP kinases so it is possible that the proliferation observed was mediated via this pathway. Thus, it is possible that the adenosine A_{2A} receptor was enhancing differentiation of MC3T3-E1 cells by this mechanism or a combination of these mechanisms.

7.5. Effect of nitrosative and oxidative stress on MC3T3-E1 cell viability.

7.5.1. Effect of nitrosative stress on MC3T3-E1 cell viability.

As neither of the glutamatergic ligands caused excitotoxicity in MC3T3-E1 cells alone, it was decided to see if they were able to cause cell damage under conditions of nitrosative and oxidative stress as has been demonstrated recently in the CNS (Behan & Stone, 2002). In the present study, the nitric oxide donor, S-nitroso-N-acetylpenicillamine (SNAP) failed to induce death of MC3T3-E1 cells contrary to the report of Damoulis & Hauschka (1997). However, these authors did not use ascorbate supplemented media in their study whereas in the current study, ascorbate and β-glycerolphosphate was used to enhance cell differentiation. Ascorbate is a natural antioxidant (see e.g. Halliwell, 1999) and it is possible that the presence of it in the media was preventing SNAP from causing cell damage to MC3T3-E1 cells.

7.5.2. Effect of oxidative stress on MC3T3-E1 cell viability.

However, cell damage using hydrogen peroxide (H_2O_2), a well established donor of oxidative stress was obtained in the current study, observations in agreement with the literature (Mody et al., 2001). That H_2O_2 was able to induce cell injury whereas SNAP was not suggests that the formation of reactive oxygen species by the H_2O_2 was able to overwhelm the antioxidant effects of ascorbate and explain the insensitivity of MC3T3-E1 cells to low concentrations of H_2O_2.

In support of this idea, Chiarugi et al. (2001) showed that ascorbate cannot protect neuronal cells from all forms of oxidative injury so it is possible this lack of a protective effect of ascorbate could extend to bone cells as well. Neither NMDA or quinolinic acid were able to modulate hydrogen peroxide induced cell damage. However, it is interesting to note that 10 μM H_2O_2 non-significantly increased cell proliferation. Furthermore, 100 μM H_2O_2 did not induce statistically significant cell death but was able to reduce the proliferative effect of the A_{2A} ligands to near control levels. These observations are in agreement with the literature that low concentrations of hydrogen peroxide have a stimulatory effect on cell growth whereas high concentrations (>100 μM) tend to induce cell death (Burdon, 1995).

It has been reported that the adenosine A_{2A} receptor has an antioxidant effect in certain cell types. Lee & Emala (2002) demonstrated that the receptor attenuated hydrogen peroxide-induced injury in proximal tubular cells using HK-2 cells. Furthermore, Fredholm et al. (1996) showed that the receptor mediated the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. However, as CGS 21680 was unable to affect cell damage induced by H_2O_2 in this study, these results suggest that this antioxidant effect does not extend to bone cells.

7.6. Effect of serum withdrawal on caspase-3 expression in MC3T3-E1 cells.

Serum withdrawal increased the amount of apoptosis in MC3T3-E1 cells as assessed by immunostaining for caspase-3. Previous studies with this cell line have shown that caspase-3 expression is induced by staurosporine (Chae et al., 2000) and hypoxia (Chae et al., 2001). Furthermore, Jilka and colleagues (1998) demonstrated that serum withdrawal from MC3T3-E1 cells increased apoptosis as assessed by terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling. However, the present data is the first to demonstrate that removal of growth factor support induces apoptosis as assessed by caspase-3 expression in this cell line. Neither adenosine A_1 or A_{2A} receptor ligands, nor quinolinic acid had an effect on apoptosis. This could either be because the receptor is not yet expressed at this early stage of treatment *in vitro* or simply that adenosine receptors do not have an anti-apoptotic effect in these cells.

However, glutamate acts as a trophic factor in osteoblasts (Genever & Skerry, 2001) and expression of the phenotype for glutamate is differentiation dependent (Bhangu et al., 2001). Adenosine A_{2A} receptors increase glutamate release in the CNS (Cunha, 2001). As discussed below, A_{2A} receptors appear to have a mitogenic effect on MC3T3-E1 cells and so it would be surprising if they did not have additional trophic actions. Therefore, to confirm that adenosine receptors and quinolinic acid do not have a modulatory effect on caspase-3 dependent apoptosis in MC3T3-E1 cells it would be interesting in the future to treat them at a later stage *in vitro* when they are starting to differentiate.

8. General discussion.

8.1. Discussion of a cell line vs. the use of a primary culture for examining the effects of glutamate on cell development.

It was mentioned in the Introduction what the general advantages and disadvantages of primary cultures vs. cell lines for the assessment of neurotoxicity were. However, the merits and pitfalls of each based upon experience in their use is now discussed. On the whole, it was felt that the advantages of the cell line over the primary culture system were numerous. The former system on the whole was easier to set up and maintain and the cells in general seemed a lot more robust. Furthermore, they turned out to be more economical than the primary culture system as they did not require the use of an expensive substrate (laminin) and were easier to set up in 96 well plates than the neuronal cultures. Furthermore, the setting up of a breeding colony for the establishment of primary cultures is an expensive operation and this is obviously not necessary when a cell line is used. Furthermore, less time, effort and expense is needed for the characterization of the cell line as regards cell type is required as previous studies have revealed that the cell line consists of a homogenous cell population. (Quarles et al., 1992). Therefore, it was only necessary to ensure they differentiated in accordance with the literature by assaying for alkaline phosphatase.

8.2. Osteoblasts appear not to be vulnerable to excitotoxicity.

In the present study, MC3T3-E1 cells did not succumb to excitotoxicity as did primary neuronal cultures. NMDA and quinolinic acid had a mitogenic effect on MC3T3-E1 cells. However, as mentioned earlier, it is generally appreciated that a primary cell culture more closely represents the situation *in vivo* than a cell line. Therefore there is always the possibility that due to the homogeneity of the cells in the MC3T3-E1 system, the cellular interactions would be restricted and the cells are not exposed to the same complicated network of messages typical of neuronal circuits that is present in primary neuronal cultures.

However, this is unlikely to be the case as to why MC3T3-E1 cells did not succumb to excitotoxicity as Skerry & Taylor (2001) reported that primary rat osteoblasts, SaoS-2 cells and MG 63 cells did not exhibit cell death in response to mM quantities of AMPA, glutamate or NMDA. Furthermore, the expression and developmental regulation of glutamate receptors and NMDA receptor subunits in the latter cell line and in primary osteoblasts occurs as it does in the CNS (Itzstein et al., 2001). Also, NMDA receptors in bone cells have the same electrophysiological characteristics as their neuronal counterparts (Skerry & Taylor, 2001; Gu et al., 2002).

However, glutamate is known to be essential as a neurotrophic factor during CNS development (Burgoyne et al., 1993). In cerebellar granule cell culture, Hunt & Patel (1990) showed that quinolinic acid at concentrations as high as 2 mM promoted the differentiation of these neurons when they were treated at an early stage. Yet concentrations as low as 100 μM can be toxic to these cells if exposed to the agent at 7 days *in vitro* (Hossain et al., 2002). The same is true of NMDA, 30 μM of the compound can have a trophic action on these cells at an early stage of development e.g. within hrs of plating (Pearce et al., 1987) but this concentration can also be neurotoxic to the cells when exposed after 8 days *in vitro* (Hossain et al., 2002). Thus, glutamate can be a neurotrophic factor but it can also be an excitotoxin depending on the stage of development of the cells.

Expression of the glutamate phenotype is differentiation dependent in MC3T3-E1 cells (Genever & Skerry, 2001) and appearance of alkaline phosphatase is not evident until the onset of growth arrest until about day 9 in culture (Quarles et al., 1992). The expression of alkaline phosphatase in the cultures used in the project was in agreement with this. The present data showed that NMDA and quinolinic acid appeared to be acting as trophic factors in MC3T3-E1 cells when the cells were treated as late as 11 DIV. However, it is certainly possible that this is still an age at which glutamate is a trophic factor in bone cell development. Thus, it is possible that had the cells been treated at a later stage than this they would have been vulnerable to excitotoxicity.

There is also the possibility that the incubation period used for the excitotoxins simply was not long enough to induce cell death as it was observed that a 24 hr exposure to NMDA and quinolinic acid induced proliferation. However, after a 72 hr exposure to the compounds this mitogenic effect was not evident. Thus, it is possible that had the cells been incubated with the agents longer, excitotoxicity might have occurred. However, it is possible osteoblasts cannot succumb to excitotoxicity at all. Osteoblastic glutamate release is negatively regulated by depolarization and voltage-dependent calcium entry (Genever & Skerry, 2001) whereas central glutamate release is positively coupled to voltage dependent entry of Ca^{2+}. (Nizhikawa, 2001). Thus in the CNS, NMDA and QA (Tavares et al., 2002) acting on autoreceptors contributes to the pathological accumulation of glutamate in the extracellular space as does depolarization induced reversal of the glutamate transporter. However, it is unlikely that the same mechanisms come into play when osteoblasts are exposed to these agents due to this inate difference in voltage-regulated calcium dependent glutamate release.

8.3. Further work.

First, it should be stressed that it would be necessary to repeat the experiments for each data point in triplicate over 3 different culture preparations for the neuronal data and over 3 different subcultures for the bone cell data in order to confirm that the data is indeed truly statistically significant.

Further work could be performed examining the neuroprotective mechanism of action of the A_{2A} receptor ligands. There is evidence that CGS 21680 binds to two different sites in rat brain (Lindstrom et al., 1996). Therefore, initially, it would be interesting to characterise the cells as regards the existence of the "classical" A_{2A} receptor in culture using ligand binding, Western Blotting, RT-PCR and immunocytochemistry. Also, it would be necessary to ascertain whether the A_{2A} receptor mediated protective effect is mediated via the "classical" receptor by seeing if chemically dissimilar antagonists attenuated the effect of CGS 21680 and pA2 values for the antagonists calculated. In addition, unfortunately, the project only assessed the effects of chronic treatment with the A_{2A} ligands on excitotoxicity.

It is known that adenosine receptors can experience "effect inversion" (Jacobson et al., 1996) and in the clinic and potential therapeutic situations, the compounds could be used either acutely or chronically. Thus it would be necessary to assess what effects different treatment times have on the neuroprotective effects of the A_{2A} ligands. Furthermore, it would be very exciting to elucidate the signal transduction mechanisms responsible for the A_{2A}-receptor mediated neuroprotection. These are likely to be inhibition of NMDA channel function and transactivation of neurotrophin receptors.

It is possible that with neuronal cultures some of the protection exhibited by the adenosine A_{2A} receptor could be due to indirect effects on glial cell function, it would be needed to explore this possibility in order to gain a full understanding of the neuroprotective modulatory action these compounds show by using pure glial cultures.

With regard to the work on osteoblasts, it would be interesting to see if osteoblasts succumb to excitotoxicity when exposed to higher concentrations of glutamate receptor ligands than were used in the project. Also, it would be useful to see, using the aforementioned techniques if glutamate receptors do show the same developmental expression in MC3T3-E1 cells *in vitro* as they do in cultured central neurons and if they need to be treated at a certain stage to succumb to excitotoxicity. In addition, it would be interesting to fully elucidate the role of adenosine A_{2A} receptors in osteoblastic function and pathology. The present data strongly suggest the existence of functional A_{2A} receptors on MC3T3-E1 cells *in vitro*. However the existence of the receptor should be confirmed using the molecular biological and immunocytochemical techniques mentioned above and if so, it would be fruitful to use this cell line to see if the adenosine A_{2A} receptor has the capacity to protect against excitotoxicity if this occurs in bone cells.

8.4. Conclusions.

To summarize this project has indicated that the adenosine A_{2A} receptor is neuroprotective against a mild excitotoxic insult but is neurodetrimental when cell damage becomes more severe. The choice of which ligand to use in the whole animal and the clinic could depend upon the degree of excitotoxicity and cell damage. The use of a low dose of an adenosine A_{2A} receptor agonist which did not induce any haemodynamic changes might be useful in the treatment of chronic neurodegenerative disorders where cell death occurs primarily via an apoptotic mechanism e.g schizophrenia.

However, when excitotoxicity and cell damage are more pronounced as occurs during ischaemia, the use of an adenosine A_{2A} receptor antagonist would be the better choice. There is always the risk that an antagonist might block beneficial A_{2A} receptor-mediated effects on blood pressure and on platelet aggregation (Jones et al., 1998). Although encouraging data from Monopoli and colleagues (1998a) have revealed that after i.p. administration in rats, SCH 58261 did not induce hemodynamic changes up to a dose of 0.1 mg/kg but increased blood pressure and heart rate starting from a dose of 1 mg/kg. Therefore, when used at the "correct dose", an adenosine A_{2A} receptor antagonist would exert protection via blocking central A_{2A} receptor evoked neurodetrimental effects without compromising beneficial A_{2A} receptor mediated peripheral effects. However, more studies in the whole animal are needed to clarify this.

With regard to the work on bone cells, this project has revealed that the NMDA receptor and the A_{2A} receptor have a trophic effect on osteoblasts *in vitro*. Furthermore, it has supported the idea that MC3T3-E1 cells are a good *in vitro* system for examining the role of adenosine A_{2A} receptors in osteogenesis. Adenosine A_{2A} receptors and the kynurenine pathway could offer a therapeutic target in the treatment of bone remodelling disorders marked by a loss of osteoblastic function e.g. osteoporosis and periodontal disease.

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