MICROGLIAL-NEURONAL

INTERACTIONS PERTINENT TO

NEURONAL DEVELOPMENT

Sarah Claire Morgan

A dissertation submitted to

The Institute of Neurology, University College London,

in candidature for the degree of PhD in Neuroscience,

October, 2002
Microglia, the resident macrophage of the brain, were originally assumed to merely provide structural support for the surrounding neurones, however it is now accepted that microglia play more than a passive role during embryonic development and neural injury, for example in Alzheimer's Disease, multiple sclerosis, and AIDS associated dementia, but it is still unclear whether microglia aid neuronal survival or exacerbate neuronal death. Microglia are implicated in a variety of cellular functions including release of cytokines and cytotoxins, phagocytosing cellular debris and synaptic stripping, all of which can influence neuronal development, differentiation and survival. Such differential microglial responses may well depend on the signals received from the surrounding microenvironment.

In these studies the effect of microglia on the survival of cultured cerebellar granule neurones (CGCs) have been investigated. Conditioned medium (MG-CM) collected from primary microglial cultures, at either 1 day \textit{in vitro} or 7 days \textit{in vitro}, was used to treat 7 day \textit{in vitro} neurones and neuronal cell viability was assessed over the following 7 days. MG-CM enhanced the CGC survival compared with untreated CGCs, with the 7 day \textit{in vitro} MG-CM enhancing survival by up to 80\% above control levels. Addition of conditioned medium obtained from cultures where the microglia had been selectively depleted using L-leucine methyl ester removed this effect to produce control levels of CGC survival. Neuronal expression of the nuclear antigen Ki-67 was seen to be amplified following treatment with the MG-CM, compared with control, suggesting a proliferation of neuronal precursor cells.
These results show that microglia aged in culture are not only able to promote neuronal survival but also enhance neuronal precursor cell proliferation through the release of a microglial-derived neurotrophic factor. These results have ramifications for increasing the neuronal number in the developing or damaged brain through the modulation of neurotrophic factors.
# TABLE OF CONTENTS

| Title ........................................................................................... | 1 |
| Abstract .................................................................................... | 2 |
| Table of Contents ..................................................................... | 4 |
| List of Tables .......................................................................... | 8 |
| List of Figures ......................................................................... | 10 |
| Acknowledgements ..................................................................... | 14 |
| Abbreviations .......................................................................... | 15 |

## 1. INTRODUCTION

### 1.1. Overview of the central nervous system

#### 1.1.1. Structure of the brain

#### 1.1.2. Brain immunoprivileged

#### 1.1.3. Macrophage populations in the CNS

### 1.2. The microglial cell

#### 1.2.1. The origins of microglia

#### 1.2.2. Microglial identification and morphology

##### 1.2.2.1. Amoeboid microglia

##### 1.2.2.2. Ramified microglia

##### 1.2.2.3. Transformation into reactive phenotype

##### 1.2.2.4. Reactive microglia

#### 1.2.3. Characterisation of microglia

#### 1.2.4. Distribution of microglia in the CNS

#### 1.2.5. Functions of microglia

##### 1.2.5.1. Neurotoxic functions

##### 1.2.5.2. Neurotrophic functions

##### 1.2.5.3. Neuronal-microglial interactions

### 1.3. The cerebellum

#### 1.3.1. Circuitry of the cerebellum

#### 1.3.2. Development of the cerebellum

#### 1.3.3. Characterisation of developmental stages

#### 1.3.4. Control of the developing cerebellum

##### 1.3.4.1. Role of neurotrophic factors

##### 1.3.4.2. Role of glutamate and calcium

### 1.4. Transduction pathways involved in survival and proliferation

#### 1.4.1. G-protein signalling

#### 1.4.2. Glutamate receptors and transporters

##### 1.4.2.1. Glutamate release and function

##### 1.4.2.2. Ionotropic glutamate receptors

##### 1.4.2.3. Metabotropic glutamate receptors

##### 1.4.2.4. Glutamate transporters
1.4.3. Notch signalling pathway 79
1.4.4. Trk receptor signalling 85
  1.4.4.1. MAPK signalling cascade 85
  1.4.4.2. PI3K/Akt transduction pathway 87
1.4.5. Sonic hedgehog signal transduction pathway 90
1.5. Aims of thesis: Microglial effects on neuronal development .......... 92

2. MATERIALS AND METHODS ....................................................... 93

1.4.3. Notch signalling pathway 79
1.4.4. Trk receptor signalling 85
  1.4.4.1. MAPK signalling cascade 85
  1.4.4.2. PI3K/Akt transduction pathway 87
1.4.5. Sonic hedgehog signal transduction pathway 90
1.5. Aims of thesis: Microglial effects on neuronal development .......... 92

2. MATERIALS AND METHODS ....................................................... 93

2.1. Materials .................................................................................... 94

2.2. Methods ..................................................................................... 96
  2.2.1. Cell culture 96
    2.2.1.1. Cerebellar granule neurone culture 96
    2.2.1.2. Microglial cell culture 97
    2.2.1.3. Peritoneal macrophage culture 99
  2.2.2. Analysis of primary cultures using flow cytometry 100
  2.2.3. Preparation of microglial-conditioned medium 102
  2.2.4. Immunoprecipitation of individual factors from MG-CM 103
  2.2.5. Preparation of serum free microglial-conditioned medium 103
  2.2.6. Depletion of microglia in microglial cell cultures 104
  2.2.7. Preparation of peritoneal macrophage-conditioned medium 105
  2.2.8. Experimental Procedure 105
  2.2.9. Immunocytochemical analysis of culture composition 106
  2.2.10. Assessment of cell viability 108
    2.2.10.1. Live-dead assay 108
    2.2.10.2. Hoechst staining 109
  2.2.11. 'CellTiter 96 Aqueous One Solution Cell Proliferation Assay' 109
  2.2.12. Measurement of glutamate content 111
  2.2.13. Measurement of nitrite and total nitrite content 113
  2.2.14. Cell lysis and sample preparation 115
  2.2.15. Preparation of samples for immunoprecipitation 116
  2.2.16. Bradford protein assay 116
  2.2.17. Immunoprecipitation 117
  2.2.18. Denaturing polyacrylamide gel electrophoresis: SDS-PAGE 118
  2.2.19. Native polyacrylamide gel electrophoresis: PAGE 118
  2.2.20. Western blotting 119
    2.2.20.1. Protein transfer 119
    2.2.20.2. Immunoblotting 120
  2.2.21. Two dimensional electrophoresis 123
    2.2.21.1. Sample preparation 123
    2.2.21.2. First dimension: IEF Focusing 124
    2.2.21.3. Casting gradient PAGE gels 125
    2.2.21.4. Second dimension: 2D SDS-PAGE 126
    2.2.21.5. Visualisation 127
      2.2.21.5.1. Silver staining 127
      2.2.21.5.2. SYPRO® Ruby Fluorescent staining 128
  2.2.22. Statistical analysis 129
3. MICROGLIA RELEASE SOLUBLE FACTORS WHICH ENHANCE NEURONAL SURVIVAL AND PROLIFERATION... 130

3.1. Introduction .......................................................................................................... 131
3.2. Summary of results ............................................................................................ 133

3.3. Microglial survival in culture ............................................................................ 134
3.3.1. Assessment of microglial viability in culture 134
3.3.2. Immunocytochemical staining of microglial cultures 136
3.3.3. FACS analysis of microglial cultures 138
3.3.4. Measurement of metabolite levels in microglial supernatants 147
3.3.5. Selective depletion of microglia using L-LME 148
3.3.6. Culture of microglia in serum free medium 151

3.4. Effects of conditioned media on neuronal survival ...................................... 153
3.4.1. Addition of microglial-conditioned medium 153
3.4.2. Addition of microglial-conditioned serum free medium 157
3.4.3. Addition of LME treated microglial-conditioned medium 158
3.4.4. Addition of MG-CM from varying microglial plating densities 160
3.4.5. Addition of peritoneal macrophage-conditioned medium 162

3.5. Assessment of developmental stages of the CGC culture .......................... 164

3.6. Effects of conditioned media on proliferation ............................................... 167
3.6.1. Neuronal Ki-67 expression 167
3.6.2. ‘CellTiter’ proliferation assay 169

3.7. Discussion ........................................................................................................... 171
3.7.1. Survival of microglia in culture 171
3.7.2. Addition of conditioned media 173

4. SIGNAL TRANSDUCTION PATHWAYS REGULATED BY MICROGLIAL-CONDITIONED MEDIUM...................................... 180

4.1. Introduction.......................................................................................................... 181
4.2. Summary of results ............................................................................................ 183

4.3. Modulation of signalling pathways................................................................... 184
4.3.1. Modulation of Notch signalling 184
4.3.2. Modulation of cAMP/PKA and G-protein coupled receptor pathways 191
4.3.3. Modulation of glutamate receptors and calcium channels 194
4.3.3.1. Ionotropic glutamate receptors and L-type calcium channels 194
4.3.3.2. Metabotropic glutamate receptors 199
4.3.4. Modulation of intracellular calcium stores 204
4.3.5. Modulation of Trk receptors and their signalling cascades 206

4.4. Discussion ........................................................................................................... 211
4.4.1. Modulation of Notch signalling 211
4.4.2. Modulation of cAMP/PKA and G-protein coupled receptor pathways 214
4.4.3. Modulation of glutamate receptors and calcium channels 216
4.4.4. Modulation of Trk receptors and their signalling cascades 218
5. CHARACTERISATION OF DIFFUSIBLE FACTORS PRESENT IN MICROGLIAL-CONDITIONED MEDIUM.............. 221

5.1. Introduction .......................................................................................................... 222
5.2. Summary of results ............................................................................................. 223
5.3. Characterisation of MG-CM ............................................................................... 224
  5.3.1. Modification of MG-CM 224
  5.3.2. Fractionation of MG-CM 227
  5.3.3. MG-CM effects on neuronal protein synthesis 229
  5.3.4. Release of growth factors by microglia 230
  5.3.5. Analysis of conditioned medium by electrophoresis 234
     5.3.5.1. 1D native PAGE separation of proteins 234
     5.3.5.2. 2D PAGE separation of proteins 235
5.4. Discussion ........................................................................................................... 248

6. GENERAL DISCUSSION ......................................................................................... 253
  6.1. Evaluation of tissue culture ............................................................................... 254
  6.2. Microglial-neuronal interactions ...................................................................... 257
  6.3. Future work .......................................................................................................... 258
  6.4. General conclusions .......................................................................................... 260

7. REFERENCES .......................................................................................................... 261
## LIST OF TABLES

### 1. INTRODUCTION

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1.3</td>
<td>Characteristics of intrinsic brain macrophage populations</td>
<td>26</td>
</tr>
<tr>
<td>Table 1.2.3.a</td>
<td>Comparison of phenotypic cell surface and functional markers on microglia and peripheral blood monocytes</td>
<td>33</td>
</tr>
<tr>
<td>Table 1.2.3.b</td>
<td>Immunohistochemical staining of rat microglia in various states of activation</td>
<td>34</td>
</tr>
<tr>
<td>Table 1.2.4</td>
<td>Microglial cell numbers in different regions of the brain</td>
<td>35</td>
</tr>
<tr>
<td>Table 1.2.5.2</td>
<td>Summary of growth factors and cytokines released by microglia</td>
<td>40</td>
</tr>
<tr>
<td>Table 1.3.3</td>
<td>Markers of the developmental stages of the cerebellum</td>
<td>51</td>
</tr>
<tr>
<td>Table 1.4.1.a</td>
<td>Examples of ligands acting through G-protein coupled receptors</td>
<td>62</td>
</tr>
<tr>
<td>Table 1.4.1.b</td>
<td>Comparison of mammalian G-protein subfamilies</td>
<td>63</td>
</tr>
<tr>
<td>Table 1.4.2.3</td>
<td>Selective mGlu receptor agonists and antagonists</td>
<td>74</td>
</tr>
<tr>
<td>Table 1.4.4.2</td>
<td>Examples of Akt cellular substrates</td>
<td>89</td>
</tr>
</tbody>
</table>

### 2. METHODS

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.2.1.1</td>
<td>Solutions used in CGC culture</td>
<td>96</td>
</tr>
<tr>
<td>Table 2.2.1.2</td>
<td>Solutions used in microglial culture</td>
<td>98</td>
</tr>
<tr>
<td>Table 2.2.1.3</td>
<td>Solutions used in peritoneal macrophage culture</td>
<td>99</td>
</tr>
<tr>
<td>Table 2.2.2.a</td>
<td>Solutions used for FACS</td>
<td>102</td>
</tr>
<tr>
<td>Table 2.2.2.b</td>
<td>Antibody specificities, source and dilutions used for FACS analysis</td>
<td>102</td>
</tr>
<tr>
<td>Table 2.2.4</td>
<td>Antibodies used to immunoprecipitate factors from media</td>
<td>103</td>
</tr>
<tr>
<td>Table 2.2.5</td>
<td>Composition of G5 supplement</td>
<td>104</td>
</tr>
<tr>
<td>Table 2.2.9.a</td>
<td>Solutions used for immunocytochemical analysis</td>
<td>107</td>
</tr>
<tr>
<td>Table 2.2.9.b</td>
<td>Antibody specificities, source and dilutions used for immunocytochemistry</td>
<td>107</td>
</tr>
<tr>
<td>Table 2.2.13</td>
<td>Solutions for nitrite determination</td>
<td>114</td>
</tr>
<tr>
<td>Table 2.2.14</td>
<td>Solutions for cell lysis</td>
<td>115</td>
</tr>
<tr>
<td>Table 2.2.15</td>
<td>Solutions for immunoprecipitation cell lysis</td>
<td>116</td>
</tr>
</tbody>
</table>
Table 2.2.17. Primary antibody specificities and source used for immunoprecipitation 118
Table 2.2.18. SDS-PAGE solutions 118
Table 2.2.19. Native-PAGE solutions 119
Table 2.2.20.1. Solutions for protein transfer 120
Table 2.2.20.2.a. Solutions for immunoblotting 121
Table 2.2.20.2.b. Secondary antibody specificities and source 121
Table 2.2.20.2.c. Primary antibody specificities, source and dilutions used for Western blotting 122
Table 2.2.21.1. Lysis buffer for 2D electrophoresis 124
Table 2.2.21.2.a. First dimension electrophoresis protocol 124
Table 2.2.21.2.b. Equilibration buffer composition 124
Table 2.2.21.3.a. Composition of gradient polyacrylamide gels 125
Table 2.2.21.3.b. Composition of glass plate bonding solutions 126
Table 2.2.21.4. Solutions used of 2D SDS-PAGE 126
Table 2.2.21.5.1. Solutions used for silver staining 127
Table 2.2.21.5.2. Solutions for SYPRO® Ruby staining 128

3. MICROGLIA RELEASE SOLUBLE FACTORS WHICH ENHANCE NEURONAL SURVIVAL AND PROLIFERATION

Table 3.3.2.a. Immunoreactivity of microglial cultures 136
Table 3.3.2.b. Further immunoreactivity of microglial cultures 137

5. CHARACTERISATION OF DIFFUSIBLE FACTORS PRESENT IN MICROGLIAL-CONDITIONED MEDIUM

Table 5.3.5.2.a. Composition of standards used for 2D electrophoresis 238
Table 5.3.5.2.b. Identification of standards separated by 2D electrophoresis 241
Table 5.3.5.2.c. Identification and analysis of protein spots in 7 DIV SF-CM on 2D gels visualised using SYPRO Ruby staining 244
Table 5.3.5.2.d. Identification of proteins using SWISS-PROT database 246
# LIST OF FIGURES

## 1. INTRODUCTION

| Figure 1.1.1. | Overview of cell types present in the central nervous system | 19 |
| Figure 1.1.2. | Regulation of CNS T-cell responses by microglia | 24 |
| Figure 1.1.3. | Diagram depicting location of macrophage populations in the rat cerebral cortex | 25 |
| Figure 1.2.1.a. | Origin of amoeboid microglia | 28 |
| Figure 1.2.1.b. | Schematic to illustrate the regressive transformation of activated microglia into ramified microglia | 29 |
| Figure 1.2.2. | Primary cultures of rat microglia | 33 |
| Figure 1.2.5.3. | Schematic to demonstrate neuronal-microglial interactions | 44 |
| Figure 1.3. | Sagital view of the rat brain | 45 |
| Figure 1.3.1. | The circuitry of cerebellum | 46 |
| Figure 1.3.2. | Developmental stages of the cerebellum | 48 |
| Figure 1.3.4. | The eukaryotic cell cycle and its regulation | 52 |
| Figure 1.3.4.2. | Glutamate activates NMDA and AMPA receptors and results in an elevated intracellular Ca\(^{2+}\) and/or Na\(^+\) concentration | 56 |
| Figure 1.4. | Summary of signal transduction pathways involved in neuronal survival and proliferation | 60 |
| Figure 1.4.1.a. | Mechanism of G-protein signalling | 65 |
| Figure 1.4.1.b. | Schematic illustrating the multiple signalling pathways activated by receptors through coupling to different G-proteins | 66 |
| Figure 1.4.2.2. | Schematic representation of subtypes, subunits and splice variants of the ionotropic glutamate receptors | 69 |
| Figure 1.4.2.3. | Diagram illustrating the location of mGlu receptors relative to the synaptic cleft | 75 |
| Figure 1.4.2.4. | Stoichiometry of glutamate transport facilitated by the glutamate transporters | 78 |
| Figure 1.4.3.a. | Notch signalling pathway | 82 |
| Figure 1.4.3.b. | Notch and Wnt signalling pathway interactions | 84 |
| Figure 1.4.4.1.a. | Summary of MAPK signalling cascades | 85 |

10
Figure 1.4.4.1.b. The MEK1/2-ERK1/2 signalling cascade

Figure 1.4.4.2. The PI3K/Akt signalling cascade

Figure 1.4.5. The Shh signalling cascade

2. METHODS

Figure 2.2.1.2 Schematic of percoll gradient appearance following centrifugation

Figure 2.2.2. Schematic representation of the BDIS flow cytometer

Figure 2.2.8. Schematic of the experimental method

Figure 2.2.11. Structures of MTS tetrazolium and its formazan product

Figure 2.2.12. Representative standard curve of glutamate concentration against optical density

Figure 2.2.13.a. Summary of Griess reaction

Figure 2.2.13.b. Representative standard curve of nitrite and nitrate following conversion to nitrite

Figure 2.2.16. Representative standard curve of protein concentration against optical density

3. MICROGLIA RELEASE SOLUBLE FACTORS WHICH ENHANCE NEURONAL SURVIVAL AND PROLIFERATION

Figure 3.3.1. Isolated microglia survive up to 7 days in culture

Figure 3.3.3.a. Comparison of microglial and peritoneal macrophage size parameters

Figure 3.3.3.b Culture reactivity to CD45

Figure 3.3.3.c Culture reactivity to CD11b

Figure 3.3.3.d. Culture reactivity to ED1

Figure 3.3.4.a. Metabolite levels in the retained microglial supernatant

Figure 3.3.4.b. Unstimulated microglia express low levels of iNOS

Figure 3.3.5.a. Effect of adding 5 and 10 mM LME to microglial cultures

Figure 3.3.5.b. Survival of microglial cultures with and without LME treatment

Figure 3.3.6. Microglial survival in serum free media

Figure 3.4.1.a. Addition of MG-CM enhances neuronal survival
Figure 3.4.1.b. Neuronal cultures treated with MG-CM do not show enhanced nitrite and glutamate production

Figure 3.4.2. Addition of SF-CM enhances neuronal survival

Figure 3.4.3. Addition of LME-CM is not able to promote CGC survival

Figure 3.4.4. Plating density of the microglial cultures affects the efficacy of the conditioned media

Figure 3.4.5. PM-CM enhances neuronal survival but with a lower efficacy than MG-CM

Figure 3.5.a. CGCs cultures contain cells which represent neurones in all stages of development.

Figure 3.5.b. Expression of β-actin and β-tubulin remains constant during neuronal period in culture

Figure 3.6.1. Neuronal Ki-67 expression is enhanced following addition of MG-CM

Figure 3.6.2. Neuronal proliferation is enhanced following MG-CM treatment

Figure 3.7.2. Schematic representing effects of MG-CM on cultured CGCs

4. SIGNAL TRANSDUCTION PATHWAYS REGULATED BY MICROGLIAL-CONDITIONED MEDIUM

Figure 4.3.1.a. Neuronal cultures express delta and active Notch ICD

Figure 4.3.1.b. MG-CM modulates Notch signalling

Figure 4.3.1.c. MG-CM modulation of Notch signalling is specific

Figure 4.3.1.d. Chelation of extracellular calcium enhances neuronal survival but has minimal influence on neuronal proliferation

Figure 4.3.2. MG-CM acts via PTX sensitive G-protein coupled receptors and affects cAMP/PKA signalling

Figure 4.3.3.1.a. Modulation of AMPA receptor and L-type VOCCs affects the potency of MG-CM, but NMDA receptor modulation does not

Figure 4.3.3.1.b. Further modulation of AMPA receptor and L-type VOCCs following treatment with MG-CM

Figure 4.3.3.2.a. Modulation of mGlu receptors alters the effect of MG-CM on both neuronal survival and proliferation

Figure 4.3.3.2.b. Addition of neutralising mGlu receptor antibodies has minimal effect on the enhancement of neuronal survival caused by the addition of MG-CM, however neuronal proliferation is severely hindered
Figure 4.3.4. Thapsigargin-sensitive release of intracellular Ca$$^{2+}$$ is detrimental to both neuronal survival and proliferation 205

Figure 4.3.5.a. Neuronal cultures express TrkA, -B and -C 206

Figure 4.3.5.b. Blocking Trk receptors abates the effect of MG-CM on neuronal survival 207

Figure 4.3.5.c. Neuronal cultures express total ERK 1/2, p-ERK 1/2, Akt and p-Akt 209

Figure 4.3.5.d. Inhibition of PI3K signalling diminishes the effect of MG-CM on both neuronal survival and proliferation to a greater extent than MAPK signalling modulation 210

5. CHARACTERISATION OF DIFFUSIBLE FACTORS PRESENT IN MICROGLIAL-CONDITIONED MEDIUM

Figure 5.3.1. The active factor in MG-CM is heat-, protease- and acid- sensitive 226

Figure 5.3.2. The active component of MG-CM has a molecular weight of < 30 kD 228

Figure 5.3.3. The active factor in MG-CM functions by enhancing de novo protein synthesis in the neurones 229

Figure 5.3.4.a. Immunodepletion of growth factors from MG-CM diminishes MG-CM mediated neuronal survival 231

Figure 5.3.4.b. Inhibition of growth factor receptors blocks MG-CM mediated enhanced neuronal survival 233

Figure 5.3.5.1. Separation of MG-CM by 1D native PAGE indicates varied protein levels in control and conditioned media 235

Figure 5.3.5.2.a. Analysis of MG-CM using 2D electrophoresis and silver stain visualisation 237

Figure 5.3.5.2.b. Analysis of MG-CM using 2D electrophoresis and SYPRO Ruby visualisation 239

Figure 5.3.5.2.c. Further analysis of MG-CM using 2D electrophoresis and SYPRO Ruby visualisation 240

Figure 5.3.5.2.d. Identification of standards 242

Figure 5.3.5.2.e. Analysis of proteins in 7 DIV S-CM 243

6. GENERAL DISCUSSION

Figure 6.1. Development of cerebellar granule neurones in culture 256

Figure 6.3. Experimental protocol to investigate microglial-neuronal interactions 259
ACKNOWLEDGEMENTS

I would like to thank my Principal supervisor, Dr Jennifer Pocock, for her guidance and encouragement throughout the course of these studies and also Dr Deanna Taylor for her guidance, support and fun times in the lab. I wish to thank Dr Soren Naaby-Hansen, Dr Marketa Zvelebil and Akunna Akpan, Ludwig Institute, University College London, for all their help with the 2D electrophoresis, and also Dr David Baker and Gareth Pryce, Department of Neuroinflammation, Institute of Neurology, for all their guidance with the FACS analysis.

Thanks also goes to Amanda and Claudie for all their help, support and of course for all the fun times both in and out of the lab and to my friends and family for all their encouragement over the years. In particular I would like to extend a huge thank you to Mum, Dad, Phil and Matt for all their limitless support and belief in me.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>1 dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>2 dimensional</td>
</tr>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>AIDA</td>
<td>1-aminoindan-1,5-dicarboxylic acid</td>
</tr>
<tr>
<td>ADA</td>
<td>trans-azetidine-2,4-dicarboxylic acid</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid</td>
</tr>
<tr>
<td>AP4</td>
<td>2-amino-4-phosphobutryric acid</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytosine arabinoside (1-β-D-arabinofuranosylcytosine)</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CGA</td>
<td>chromogranin A</td>
</tr>
<tr>
<td>CGC</td>
<td>cerebellar granule cell</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CTX</td>
<td>cholera toxin</td>
</tr>
<tr>
<td>DCGIV</td>
<td>(2S, 1’R, 2’R, 3’R)-2-(2,3-dicarboxycyclopropyl) glycine</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>DPPI</td>
<td>dipeptidyl peptidase I</td>
</tr>
<tr>
<td>DSL</td>
<td>delta/serrate/lag2</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s balanced salts solution</td>
</tr>
<tr>
<td>ECD</td>
<td>extracellular domain</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGL</td>
<td>external germinal layer</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FAF-BSA</td>
<td>fatty acid free bovine serum albumin</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein-5-maleimide diacetate</td>
</tr>
<tr>
<td>GAP43</td>
<td>growth associated protein-43</td>
</tr>
<tr>
<td>GCP</td>
<td>granule cell precursors</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GPF</td>
<td>glial promoting factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>H89</td>
<td>N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride</td>
</tr>
<tr>
<td>HES</td>
<td>hairy and enhancer of split</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'bi-1-H-benzimidazol</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>ICD</td>
<td>intracellular domain</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin growth factor-I</td>
</tr>
<tr>
<td>IGL</td>
<td>internal granule cell layer</td>
</tr>
<tr>
<td>iGlu</td>
<td>ionotropic glutamate</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>INT</td>
<td>iodonitrotetrazolium chloride solution</td>
</tr>
<tr>
<td>KA</td>
<td>kainate</td>
</tr>
<tr>
<td>LME</td>
<td>L-leucine methyl ester</td>
</tr>
<tr>
<td>LME-CM</td>
<td>L-leucine methyl ester treated microglial-conditioned medium</td>
</tr>
<tr>
<td>L-NNA</td>
<td>N⁖-Nitro-L-arginine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule associated protein type 2</td>
</tr>
<tr>
<td>MAP4</td>
<td>(S)-2-amino-2-methyl-4-phosphonobutanoic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activating protein kinase</td>
</tr>
<tr>
<td>MCCG</td>
<td>2S,1'S,2'S-2-methyl-2-(2'carboxycyclopropyl)glycine</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum Essential Medium</td>
</tr>
<tr>
<td>MF</td>
<td>mossy fibres</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence index</td>
</tr>
<tr>
<td>MG</td>
<td>microglia</td>
</tr>
<tr>
<td>MG-CM</td>
<td>microglial-conditioned medium</td>
</tr>
<tr>
<td>mGlu</td>
<td>metabotropic glutamate</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MK-801</td>
<td>(+)-5-methyl-10,11-dihydro-5H-dinezo[a,d]cyclohepten-5,1-imine hydrogen maleate</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NIF</td>
<td>nifedipine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>nitrite</td>
</tr>
<tr>
<td>NT</td>
<td>neurotrophin</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide free radicals</td>
</tr>
</tbody>
</table>
ONOO\textsuperscript{−} \hspace{1em} peroxynitrite
PBS \hspace{1em} phosphate buffered saline
PCL \hspace{1em} Purkinje cell layer
PDGF \hspace{1em} platelet derived growth factor
PGE\textsubscript{2} \hspace{1em} prostaglandin E\textsubscript{2}
PI \hspace{1em} propidium iodide
PI3K \hspace{1em} phosphoinositol-3-kinase
PKA \hspace{1em} protein kinase A
PLC \hspace{1em} phospholipase C
PM\textsubscript{ϕ} \hspace{1em} peritoneal macrophage
PM-CM \hspace{1em} peritoneal macrophage-conditioned medium
PS \hspace{1em} presenilin
PTX \hspace{1em} pertussis toxin
SBTI \hspace{1em} soybean trypsin inhibitor
SCF \hspace{1em} stem cell factor
SDS \hspace{1em} sodium dodecylsulphate
SDS-PAGE \hspace{1em} SDS-polyacrylamide gel electrophoresis
SF-CM \hspace{1em} serum free-condition medium
SFM \hspace{1em} serum free medium
Shh \hspace{1em} Sonic hedgehog
TBS \hspace{1em} tris buffered saline
TGF \hspace{1em} transforming growth factor
TM \hspace{1em} transmembrane
TNF \hspace{1em} tumor necrosis factor
Trk \hspace{1em} tyrosine kinase
TTBS \hspace{1em} tween 20-Tris buffered saline
VOCC \hspace{1em} voltage operated calcium channels
1. INTRODUCTION
1.1 OVERVIEW OF THE CENTRAL NERVOUS SYSTEM

1.1.1 Structure of the brain

The central nervous system (CNS) is a complex network made up of approximately $10^{12}$ neurones, each connecting with between 10,000 – 100,000 other neurones (Alberts et al., 1994). Neurones are specialised for communication, conducting electrical signals along the axons away from the cell body. These signals are produced by a flux of ions across the nerve cell membrane. However, approximately 90% of the cells in the CNS are non-neuronal cells, termed glial cells. Originally it was assumed that these glial cells merely fulfilled a structural, passive role (Alberts et al., 1994) but it is now known that the neurone-glia unit determines not only neuronal function but also synaptic plasticity. Glia may be subdivided on the basis of size into macroglia and microglia (see figure 1.1.1.)

Figure 1.1.1. Overview of cell types present in the central nervous system

Ependymal cells are of neuroectodermal origin and line the ventricular compartments of the brain. Ependymal cells are densely ciliated and line the walls of the ventricles and aqueducts in the brain and the central canal of the spinal cord forming a barrier between
cerebrospinal fluid and neuronal tissue. Although their precise function is unknown their importance may lie in the continual, directional movement of cerebrospinal fluid (CSF). This action also helps to maintain a diffusion gradient, between the CSF and brain tissue, facilitating the movement of toxins and metabolites to the CSF for clearance. They may also have a host defence role, keeping the surface of the brain clear of debris and preventing margination of bacteria during meningitis. Recently it has been postulated that the ependymal cells may also represent the resident stem cell of the adult mammalian brain (Johansson et al., 1999).

Astrocytes, named from the Latin astrum, meaning star, can be technically divided into two types; protoplasmic astrocytes which are associated with gray matter or collections of neuronal cell bodies, and fibrous astrocytes which are associated with white matter or collections of axons (Aldskogius and Kozolova, 1998). Astrocytes are the largest of glial cells with an average diameter of 40-50 µM and account for approximately 30 - 40 % of the total glial population (Aldskogius and Kozolova, 1998). These star shaped cells possess long radial processes ending in numerous podia (or feet-like extensions) that connect to both neurones and capillaries. They fulfil a variety of functions including maintenance of normal brain homeostasis by the control of the chemical and ion extracellular environment and the uptake and metabolism of synaptically released glutamate (Anderson and Swanson, 2000). They also provide structural support and guidance for nerve cell growth (Kimbelberg and Norenberg, 1989), a function particularly assigned to a subtype of astrocyte termed radial glia (Bentivoglio and Mazzarello, 1999).
In disease they participate in glial scar formation and cause the upregulation of a variety of proteins (Kimbelberg and Norenberg, 1989).

Oligodendrocytes, from the Greek meaning ‘few tree cell’, make up a similar proportion of the glial cell number as astrocytes, approximately 30 - 40 % of the total glial population (Aldskogius and Kozolova, 1998). These cells have fewer processes and their main function is to form myelin around the axons of the nerve cells (Morell and Norton, 1980). This facilitates the fast propagation of electrical signals along the length of the axons. An unmyelinated neurone transmits a signal at a rate of 1 metre/sec, however a myelinated neurone propagates a signal at approximately 100 metre/sec. In multiple sclerosis (MS) the myelin becomes damaged which results in a decreased neural transmission rate. In the peripheral nervous system a comparable function is attributable to Schwann cells (Alberts et al., 1994).

The third type of glial cell, which will be described further in this study, is the microglial cell, from the Greek meaning ‘small glue’, which account for approximately 5 - 20 % of the total glial population (Gonzalez-Scarano and Baltuch, 1999; Zielasek et al., 1992; Banati and Graeber, 1994). Microglia are often referred to as the ‘resident macrophage of the brain’ for reasons that will be discussed later.

1.1.2 **Brain immunoprivileged**

The central nervous system is often described as an immunologically privileged site due to the inaccessibility of the organ to immune surveillance. The notion was suggested
following observations that transplanted tissue grafts were able to survive for relatively long periods of time in the brain without evidence of an immune response (Widner et al., 1989; Young et al., 1989). It has also been demonstrated that if the bacterium, mycobacterium, is injected into the brain the pathogen fails to initiate an immune response, however if injected into peripheral organs then an aggressive immune reaction is inaugurated (Matyszak and Perry, 1998). The immune privilege of the brain is maintained by the presence of the blood-brain barrier (BBB), whose tight endothelial junction prevent connections to the classical immune system, and due to the fact there is no conventional lymphatic drainage system. As a result the access for the classical blood borne immune cells, eg. B and T-lymphocytes and monocytes, is highly restricted (Andersson et al., 1992; Aloisi et al., 2000). Furthermore only low-level expression of major histocompatibility (MHC) antigens is observed (Perry and Gordon, 1988; Perry et al., 1997).

However such a system would inevitably present problems during disease where infectious agents are involved, so instead the immune system of the CNS is highly specialized (Shrikant and Benveniste, 1996). It has been shown that despite these immunological deficits the CNS is not in complete isolation from the peripheral immune system, as circulating monocytes and activated T-cells have been observed to cross the BBB in both health and disease states (Wekerle et al., 1986; Lawson et al., 1992). It has also been found that a resident populations of cells in the CNS, namely microglia, are able to upregulate their expression of MHC class II antigens following a pathological insult,
thereby enabling the presentation of antigens to peripherally primed T-cells (O'Keefe et al., 2001).

Normally upon infection, in peripheral tissues, macrophages act as antigen presenting cells (APCs), which activate lymphocytes and produce an immune cycle via MHC antigens. It has been suggested that initiation of such a T-cell response is unlikely to occur in the CNS and rather is initiated peripherally in the lymphoid organs. Once initiated the T-cells are then readily able to cross the BBB and be restimulated upon encounter of the target antigen presented by the local APCs (Shrikant and Benveniste, 1996; Aloisi et al., 2000). In the first instance it is the endothelial (or perivascular cells), which are thought to act as the APCs (Graeber et al., 1992) and function to restimulate the peripherally activated T-cells (Hickey and Kimura, 1988). These cells have been shown to exhibit both constitutive and inflammation upregulated expression of MHC class II and co stimulatory molecules (Graeber et al., 1992; de Simone et al., 1995). Once in the CNS parenchyma, further T-cell mediated inflammatory processes are interceded by microglial cells and to a lesser extent astrocytes, both of which can express MHC and complement proteins upon injury (Streit et al, 1989) and become effective APC (Aloisi et al., 2000) (figure 1.1.2.).

Upon activation microglia secrete a variety of pro-inflammatory cytokines, including interleukin-10 (IL-10), interleukin-12 (IL12), prostaglandin E₂ (PGE₂), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α), which provide signals to lymphocytes allowing their infiltration into the white matter (Gebicke-Haerter et al., 1996), they also proliferate and become phagocytic (Kreutzberg, 1996). As microglia are
found randomly throughout the brain, with some prevalence in the gray matter, the cerebral cortex and the hippocampus (see table 1.3.4.), and there is no area completely devoid of microglia, a quick response can be ensured when other cell types are compromised.

Figure 1.1.2. Regulation of CNS T-cell responses by microglia. CD4⁺ T-cells, activated in peripheral lymphoid organs, cross the blood-brain barrier and may be restimulated by MHC class II⁺ perivascular cells, acting as the first line immunocompetent cells of the CNS. Once in the CNS parenchyma, T-cells interact with microglia, which display upregulated MHC class II expression following activation by inflammatory stimuli. The microglia then restimulate the T-cells and secrete a variety of mediators which further exacerbate the inflammatory response of the T-cells (adapted from Aloisi et al., 2000; Becher et al., 2000).

It is therefore more commonly accepted that although conventional immune reactivity is prevented by the BBB, it is the perivascular cells and the microglia that form the network of immune effector cells in the CNS and provide the essential link between the CNS a barrier-protected organ, and the peripheral immune system (Kreutzberg, 1996). The
The microenvironment of brain has developed to down regulate potentially deleterious inflammatory reactions but is still able to generate a familiar immunological response albeit slightly muted (Kreuztberg, 1996; Becher et al., 2000).

### 1.1.3. Macrophage populations in the CNS

Macrophages are a unique class of cells in mammalian systems and are considered to be the scavengers of the body. Through their phagocytic and other functional properties they play significant roles in many biological processes, including inflammation, wound healing and immunity. Within the CNS there are a number of distinct subtypes of macrophages associated with specific regions (see review Jordon and Thomas, 1988).

**Figure 1.1.3.** Diagram depicting location of macrophage populations in the rat cerebral cortex (adapted from Jordon and Thomas, 1988).
Microglia constitute the largest population of brain macrophages and as will be discussed exist in a number of conformations associated with different morphological and functional characteristics. However other specialised macrophage populations can be located, such as those on the ventricular margins of the cerebral hemispheres (suparependymal macrophages), along the ventricular margin of the choroids plexus (epiplexus cells), along the peripheral margin of blood vessel (pericytes), within the subarachnoid space (meningeal macrophages) and finally associated with circulating blood (blood-derived macrophages).

**Table 1.1.3. Characteristics of intrinsic brain macrophage populations.** Properties of each class of brain macrophage are indicated including location and time of expression. Furthermore the degree of possession of typical macrophage structure is indicated by increasing numbers of plus (+) signs. The presence (+) or absence (-) of phagocytic activity is also indicated (adapted from Jordon and Thomas, 1988).

<table>
<thead>
<tr>
<th>macrophage type</th>
<th>location</th>
<th>time of expression</th>
<th>macrophage structure</th>
<th>phagocytic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>microglia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amoeboid</td>
<td>primarily white matter</td>
<td>pre / early postnatal</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>ramified</td>
<td>gray and white matter</td>
<td>postnatal to adult</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>reactive</td>
<td>areas of trauma</td>
<td>following trauma</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>supraependymal</td>
<td>ventricular margin</td>
<td>prenatal to adult</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>of ependymal epithelium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>epiplexus</td>
<td>ventricular surface</td>
<td>prenatal to adult</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>of choroids plexus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pericytes</td>
<td>external surface</td>
<td>prenatal to adult</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>of blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>meningeal</td>
<td>subarachoid space</td>
<td>prenatal to adult</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>
Each of these macrophage populations fulfils functions specialised to their location. The suparependymal macrophages are predominately observed in early postnatal animals and appear to function in tissue homeostasis. In the adult these cells remove metabolic debris of the ependyma and protect against contaminants in cerebrospinal fluid. The epiplexus cells perform a comparable function in the choroids plexus. Pericytes, which reside upon or are embedded in the basal lamina of capillary endothelium, offer protection from blood borne pathogens in the event of brain trauma, whilst the meningeal macrophages, also termed subarachnoid and leptomeningeal macrophages protect the pial surface. Finally monocytes can migrate out of circulating blood and differentiate into fully competent macrophages in response to injury, in this way the brain tissue has two populations of macrophages, the resident and the blood-derived populations.

1.2. THE MICROGLIAL CELL

1.2.1. The origins of microglia

The origin of amoeboid microglial cells has been a longstanding controversial issue with several theories being suggested (figure 1.2.1.a.), the main four being:

a. It was originally suggested by Rio Hortega in 1932 that microglia may be derived from an invasion of mesodermal cells, which normally form connective tissue in the periphery, into the CNS during embryonic development (see Giulian, 1987; Ling and Wong, 1993; Banati and Graeber, 1994).

b. Again in 1932 it was also suggested by Rydberg, and indeed supported by subsequent investigators, that microglia have a neuroectodermal origin arising from pluripotential subependymal glioblasts, cells which also give rise to
astrocytes and oligodendrocytes (see Lewis, 1968; Vaughn and Peters, 1968; Kitamura et al., 1984; Schelper and Adrian, 1986).

c. Another possibility was that microglia had a pericytal microglial origin, where pericytes associated with blood vessels are able to migrate into the CNS parenchyma (Mori and Leblond, 1969; Baron and Gallego, 1972).

d. The final possible source, originally postulated by Samtha and Juba in 1933 was for a monocytic microglial origin, where circulating monocytes migrate into the brain during the vascularisation of the brain early in embryonic development (see Imamato and Leblond, 1978; Ling, 1979).

Figure 1.2.1.a. Origin of amoeboid microglia  Amoeboid microglia have been postulated to arise from a number of different sources including monocytes, pericytes, glioblasts and mesenchymal cells. All have been suggested to enter the brain during early embryonic development and develop to give rise the amoeboid microglia (adapted from Giulian, 1987; Moore and Thanos, 1996).
It is now generally accepted that the last is the most likely source of amoeboid microglia. Ling and co-workers have demonstrated a monocytic origin for microglia by using carbon labelled monocytes (Ling et al., 1980). If these are injected into the peripheral bloodstream of postnatal rats, before complete development of the BBB, then labelled microglia can be identified in the recipient brain, indicating that the microglia are derived from the monocytes (Ling et al., 1980). Further experiments using bone marrow chimeras, where haematopoietic progenitor cells of the recipient were destroyed and replaced with donor derived cells, have demonstrated that microglia, along with meningeal and perivascular macrophages, are derived from the bone marrow (Hickey and Kimura, 1988). Developmental studies have also revealed that macrophages, initially observed in the choroid plexus and meninges, migrate along the ventricular surface and enter the brain parenchyma where they transform into amoeboid microglia (Sturrock, 1978; Boya et al., 1979), and ultimately via a regressive transformation into ramified microglia (figure 1.2.1.b.) (Ling 1979, 1982).

![Figure 1.2.1.b. Schematic to illustrate the regressive transformation of activated microglia into ramified microglia.](image)

The morphological transformation of amoeboid microglia into ramified microglia is a regressive phenomenon where there is a gradual diminution of the phagocytic activity of the amoeboid microglia observed by the gradual attenuation of their cytoplasm, reduction of lysosomes and surface markers (adapted from Ling and Wong, 1993).
1.2.2. Microglial identification and morphology

Microglia were discovered independently by Nissle (1899) and Robertson (1900) (see Banati and Graeber, 1994). The role of microglia in pathological conditions was first recognised and studied by del Rio-Hortega (1932) (see Giulian, 1987; Ling and Wong, 1993; Banati and Graeber, 1994). Microglia are initially identified as amoeboid cells and these transform to become ramified microglia. Following injury microglia become activated and further transform into reactive microglia. Under resting conditions microglia have approximately 1/7 the volume of astrocytes, however upon activation their volume increases 9 fold and becomes higher than that of astrocytes under the same conditions (Banati and Graeber, 1994).

1.2.2.1. Amoeboid microglia

Amoeboid microglia, which are specifically associated with the developing CNS, arise during the last week of gestation and disappear by the end of the second postnatal week (Ling et al., 1980). The cells have a rounded morphology possessing pseudopodia and thin filopodia-like processes and contain numerous lysosomes. During this early postnatal period, amoeboid microglia are believed to participate in gliogenesis and tissue histogenesis through the elimination of inappropriate axons and cells (Kreutzberg, 1996; Innocenti et al., 1983; Guilian, 1987).

1.2.2.2. Ramified microglia

Ramified microglia, which are associated with the matured CNS, first become evident during the second postnatal week (Kaur and Ling, 1991; Domaradzka-Pytel et al., 1999). Ramified microglia have a small flattened or angular nucleus with coarse chromatin
masses at the periphery when viewed by EM (Peters et al., 1991; Ling and Wong, 1993). The pale cytoplasm tends to accumulate at the poles and contains small golgi apparatus and a variable number of small granules, which are most likely to be lysosomes, and isolated rough ER. There is generally a long tapering process leading from the cell body, which is often branched. Ramified microglia were until recently considered to be an inactive cell type, however it has been demonstrated that these cells exhibit pinocytic activity and motility within a local area (Glenn et al., 1991; Booth and Thomas, 1991). As ramified microglia are distributed evenly throughout the brain parenchyma and express degradative enzymes it is thought that they function as a cleansing system, and aid the removal of cellular metabolites and dispose of toxic factors released by damaged neurones (Thomas, 1992). They may also limit the spread of diffusible neurotransmitters thereby contributing to nerve impulse transmission (Murabe and Sano, 1982; Biber et al., 1999; Noda et al., 2000; Nakajima et al., 2001).

1.2.2.3. Transformation into reactive phenotype

In response to neuronal injury, ramified microglia transform into active macrophages, termed 'reactive microglia'. Using the facial nerve model, Kreutzberg and colleagues have demonstrated that in response to neuronal damage ramified microglia proliferate and upregulate the expression of surface antigens e.g. CR3 complement receptors, MHC class I and II antigens and vimentin (Graeber et al., 1988a, 1988b, 1989; Streit et al., 1989). Such neuronal damage is sufficient to activate microglia resulting in an ‘active microglial’ phenotype. However, in response to lethal neuronal death, e.g. that caused by injection of the toxin ricin, it has been observed that the microglia transform into fully competent
phagocytic macrophages, termed ‘reactive microglia’, accompanied by further MHC class I antigen expression and synthesis of ED1 and ED3 macrophage specific antigens (Streit and Kreuzberg, 1987; Streit et al., 1989; Graeber et al., 1990).

1.2.2.4. Reactive microglia

Reactive microglia, when viewed by EM (Peters et al., 1991; Ling and Wong, 1993) have a rounded nucleus with margination of chromatin masses. They have a large amount of cytoplasm which contains well developed Golgi complexes, stringy rough ER and numerous lysosomal and phagosomal granules. Cytoplasmic vacuoles can be observed in the subplasmalemmal region and filopodial and pseudopodial processes project from the cell surface and they lack the branching processes associated with ramified microglia. Reactive microglia are a transient population of macrophages, specifically associated with the brain injury and neuroinflammation. Following an insult, reactive microglia migrate and rapidly accumulate at the site of injury and can persist in the tissue for several weeks, depending on the severity of the injury (Murabe et al., 1981). Reactive microglia secrete super oxide anions and nitric oxide along with a number of cytokines e.g. TNF-α, interleukin(IL)-1, and IL-6, consistent with their role as macrophages. The reactive microglia also express MHC class II antigens along with other surface molecules, e.g. CD40, B7 and ICAM-1, thus enabling them to fulfill the role of the endogenous antigen presenting cell (O’Keefe et al., 2001). The tissue damaging effects of activated microglia are under strict control and microglial activation normally occurs only as part of a constitutive surveillance function (Banati and Graeber, 1994).
Figure 1.2.2. Primary cultures of rat microglia. Primary microglia stained using fluorescein diacetate to show resting or ramified microglia (A) and activated or amoeboid microglia (B). Photographs: J. M. Pocock.

1.2.3. Characterisation of microglia

As discussed, microglia are generally accepted to be derived from monocytes, and therefore it is not surprising that they share many functional and antigenic markers with monocytes (figure 1.2.3.a.).

Table 1.2.3.a. Comparison of phenotypic cell surface and functional markers on microglia and peripheral blood monocytes. Microglia are ontogenetically related to peripheral monocytes and therefore express a large number of functional and antigenic markers (adapted from Williams et al., 1992).

<table>
<thead>
<tr>
<th>Antigen or functional marker</th>
<th>Antibody</th>
<th>Microglia</th>
<th>Peripheral blood monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic antigen ENM/11</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD11c</td>
<td>Leu-M5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-MHC class I</td>
<td>OX-18</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-MHC class II</td>
<td>OX-6</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-FcRI</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>α-FcRII</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

The level of expression of these cell surface and functional markers is dependent on the conformation of the microglia being investigated. As mentioned, under physiological conditions adult microglia assume a ramified appearance and are essentially quiescent.
cells (Lauro et al., 1995). Following moderate neuronal injury, microglia become activated and start to proliferate, marked by an alteration in their immunophenotype. In response to lethal injury, microglia become “super-activated” and assume a phagocytic nature, associated with upregulation of cell surface markers (Streit et al., 1988).

There are no antibodies which can distinguish between microglia and macrophages from other tissues, although differences in expression of certain cell surface antigens does occur, for example macrophages express high levels of CD45 whereas microglia are found to only express low levels (Ford et al., 1995). However it is possible to distinguish microglia from other macrophages by their unique pattern of ion channels. Rat microglia have been shown to lack the delayed outward potassium current which is displayed by peritoneal macrophages, and instead they show a large inwardly rectifying current which is activated by hyperpolarising voltage steps (Kettenmann, 1990).

Table 1.2.3.b. Immunohistochemical staining of rat microglia in various states of activation. Resting and activated microglia, despite arising from the same cell, express different surface markers according to their state of activation. It should be noted that here the term activated refers to microglia which are proliferating but are not phagocytic in order to delineate between these 2 conformation differences. Absent - ; weak -/+ ; well discernable + ; above normal ++ (adapted from Streit et al., 1988).

<table>
<thead>
<tr>
<th>antigen</th>
<th>antibody</th>
<th>resting</th>
<th>activated</th>
<th>super-activated/ phagocytic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR3 complement receptor</td>
<td>OX-42</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MHC class I</td>
<td>OX-18</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MHC class II</td>
<td>OX-6</td>
<td>-</td>
<td>-/+</td>
<td>+</td>
</tr>
<tr>
<td>Macrophage surface antigen</td>
<td>ED-1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
1.2.4. Distribution of microglia in the CNS

Microglia are present throughout the brain, indeed there is no region completely devoid of them, however they are not uniformly distributed (table 1.2.4.). There are a greater number of microglia in the gray matter than white matter and an estimate of the total number of microglia in the adult mouse brain is $3.5 \times 10^6$ cells (Lawson et al., 1990).

Table 1.2.4. Microglial cell numbers in different regions of the brain. The microglial distribution was evaluated in terms of density of microglial cell bodies per mm$^2$. The microglia were identified as cells which were F4/80 positive, a macrophage specific plasma membrane glycoprotein (adapted from Lawson et al., 1990).

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Number of microglia (cells mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>$65 \pm 2$</td>
</tr>
<tr>
<td>Hippocampal dentate gyrus</td>
<td>$120 \pm 7$</td>
</tr>
<tr>
<td>Caudal basal ganglia</td>
<td>$95 \pm 10$</td>
</tr>
<tr>
<td>Thalamus</td>
<td>$274 \pm 19$</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>$182 \pm 15$</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>$134 \pm 7$</td>
</tr>
<tr>
<td>Cerebellum granule layer</td>
<td>$32 \pm 2$</td>
</tr>
<tr>
<td><strong>TOTAL ADULT MOUSE BRAIN</strong></td>
<td><strong>$3.5 \times 10^6$</strong></td>
</tr>
</tbody>
</table>

1.2.5. Functions of microglia

As stated it is becoming increasingly evident that non-neuronal cells in the CNS play more than a passive role in the development and survival of neurones. Microglia, as the resident macrophage of the CNS are increasingly being seen to play an important part in this (Banati and Graeber, 1994). Historically microglia were commonly assumed to fulfil purely a role as a phagocytic scavenger. However more recently there have been a number of studies that demonstrate additional roles such as sources of the necessary
growth and/or differentiation factors for developing neurones (Jonakait et al., 1996), along with the identification of functions for ramified microglia (Ward et al., 1991). It is therefore important to look at both the neurotoxic and neurotrophic effects of microglia upon the CNS.

1.2.5.1. Neurotoxic functions

Microglia become activated *in vivo* in response to immunological challenges, a change which is characterised by morphological alterations, a shift in the expression of surface antigens (table 1.2.3.b.) and the production of immune modulators that impact on neurones to induce neurodegeneration (McMillian et al., 1995; Moore and Thanos, 1996). Microglia are the primary immune cell responsible for the detection of invading pathogens and neural injuries, and are associated with the pathogenesis of a wide variety of neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, AIDS associated dementia and amyotrophic lateral sclerosis (ALS). Whether the activation of microglia is aimed at reducing the damage caused in such diseases or whether they cause/exacerbate the damage is a debated issue.

*In vitro* such microglial activation may be triggered in a number of ways; stimulation with lipopolysaccharide (Kim et al., 2000), interferon-gamma (IFN-γ) (Woodroffe et al., 1989), chromogranin A (CGA) (Kingham et al., 1999), a neurotoxic prion protein fragment (Brown et al., 1996) or amyloid beta peptide (Noda et al., 1999) have all been shown to induce microglial activation. Such activation also results in the release of cytotoxic factors.
One study has shown that if microglia are overactivated, by LPS, then their apoptotic death is induced (Liu et al., 2001). A similar effect has been observed in microglia activated by CGA (Kingham et al., 1999). It is postulated that such activation-induced apoptosis of microglia may be a fundamental self-regulatory mechanism devised to limit bystander killing of vulnerable neurones. However in the extreme it can be foreseen that such depletion of the microglia from the brain may severely hamper its capacity for combating future inflammatory challenges and tissue repair.

Activated microglia have been demonstrated to release the excitatory amino acid glutamate (Piana et al., 1991; Kingham et al., 1999), a process which requires the synthesis of new protein (Kingham et al., 1999). Glutamate has been shown to be able to induce neuronal death (Marini and Paul, 1992) therefore through its release neuronal survival is compromised. Originally it was suggested that the release of glutamate could be blocked by the use of ionotropic glutamate (iGlu) receptor antagonists, e.g. MK-801 and APV, indicating the importance of these receptors (Piani et al., 1991), but more recently it has been shown that the glutamate is released via the low affinity sodium-independent $\mathbf{x}_c^-$ transporter pathway (Piani and Fontana, 1994), which leads to the activation of metabotropic glutamate (mGlu) receptors (Kingham et al., 1999). The glutamate released can either act directly upon the neurones to cause excitotoxic damage (Piani and Fontana, 1994) or can feed back onto the microglial cells with further detrimental results (Kingham et al., 1999).
Microglia are also able to convert L-tryptophan into quinolinic acid, which is a neurotoxic metabolite (Heyes et al., 1996). Quinolinic acid is normally present in the brain at nanomolar concentrations. However in a number of disease states, including Huntington’s disease (Heyes et al., 1991), HIV infection (Brouwers et al., 1993), Lyme borreliosis (Halperin and Heyes, 1992) and spinal cord injury (Blight et al., 1995), the concentration is seen to rise to micromolar concentrations and it is likely that microglia are the source.

Microglia have also been shown to be a source of nitric oxide (NO') (Chao et al., 1992; Boje et al., 1992), synthesised by the enzyme inducible nitric oxide synthase (iNOS; Minghetti et al., 1997). Although under normal physiological conditions NO is involved in inter- and intra-cellular signalling (Chen et al., 2000) at excessive levels NO can induce neuronal injury (Kim et al., 2000). Furthermore, it can readily react with superoxide free radicals (O_2'), also known to be released by microglia (Woodrooffe et al., 1989), to form the highly reactive peroxynitrite (ONOO') species which is capable of inflicting additional neuronal damage (Kim and Ko, 1998). Such reactive species are well known to initiate multiple cellular lesions including lipid peroxidations, DNA strand breaks and protein alterations (Théry et al., 1991; Alberts et al., 1994).

In addition microglia have also been found to secrete a number of other proinflammatory factors including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), arachidonic acid, eicosanoids and a low molecular weight neurotoxin (Liu et al., 2001; Giulian et al., 1986, 1993). Microglia also release a number of proteases including metalloproteases (Colton and Gilbert, 1993), elastase (Giulian and Baker, 1986) and cathepsin B (Kingham
and Pocock, 2000). Microglia also secrete a variety of astroglial growth factors, such as glial promoting factor (GPF) -2 and -4, and interleukin-1 (IL-1), thereby enhancing the astrocytic contribution to the inflammatory process (Giulian, 1987).

Some investigations have observed that there is a region-specific differential susceptibility of neurones to microglial neurotoxicity, especially that caused by LPS stimulation (Kim et al., 2000). It has been noted that neurones from microglial rich regions, such as the substantia nigra, are more sensitive to bacterial-toxin induced neurotoxicity compared with neurones from regions with less microglia such as the hippocampus and cortex, a difference observable even at high LPS concentrations. Such a result may reflect the levels of inflammation related factors produced by microglia.

1.2.5.2. Neurotrophic functions

Microglia release a number of growth factors and cytokines, both when ramified and following activation (see Barron, 1995) which aid neuronal survival, differentiation and indeed regeneration. Such factors are growth factors, which are low molecular weight (30 kDa) proteins that bind to receptors on the cell surface resulting in the activation of intracellular signalling cascades, ultimately affecting cellular processes such as proliferation, survival and differentiation. Cytokines are a unique family of growth factors that are able to stimulate immune responses. Microglia have been reported to release a wide number of growth factors and cytokines (see table 1.2.5.2.), which affect surrounding cell types and also, via feedback loops, effect the further secretion of growth factors and cytokines from the microglia themselves.
Table 1.2.5.2.  Summary of growth factors and cytokines released by microglia

<table>
<thead>
<tr>
<th>GROWTH FACTORS:</th>
<th>main functions</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>crucial for development and maintenance of neurones; microglia require stimulation to induce both gene and protein expression</td>
<td>Mallat et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lindholm, 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heese et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Krenz and Weaver, 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jonakait et al., 2000</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>anti-inflammatory action; promotes proliferation and growth; mediator of ontogenetic neurone death</td>
<td>Constam et al., 1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kreutzberg, 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottner et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dunker et al., 2001</td>
</tr>
<tr>
<td>PDGF</td>
<td>promotes proliferation of precursor cells; enhanced phosphoinositol turnover</td>
<td>Nishiyama et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicholas et al., 2001</td>
</tr>
<tr>
<td>EGF</td>
<td>promotes proliferation; inhibits carcinogenic cell proliferation</td>
<td>Nagata et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Briers et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nolte et al., 1997</td>
</tr>
<tr>
<td>bFGF</td>
<td>promotes proliferation of astrocytes and oligodendrocytes, promotes neuronal survival</td>
<td>Morrison et al., 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shimojo et al., 1991</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Eccleston and Silberg, 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Walicke, 1998</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CYTOKINES:</th>
<th>main functions</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>modulation of immune response; increases responsiveness to growth factors; induces proliferation and cytokine expression; stimulation of cell destruction</td>
<td>Dickson et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Uno et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fine et al., 1999</td>
</tr>
<tr>
<td>IL-1</td>
<td>enhance activation of immune response; promotes proliferation; induces cytokine expression</td>
<td>Giulian et al., 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Banati and Graeber, 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagai et al., 2001</td>
</tr>
<tr>
<td>IL-6</td>
<td>enhances immune response activation; promotes differentiation; augments cellular response to cytokines</td>
<td>Frei et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dickson et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Veerhuis et al., 2002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagai et al., 2001</td>
</tr>
<tr>
<td>IL-12</td>
<td>promotes Th1 responses in progenitor cells following infection</td>
<td>Prinz et al., 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nagai et al., 2001</td>
</tr>
</tbody>
</table>
Microglia have also been found to secrete extracellular glycoproteins (Baenziger et al., 1972; Lawler, 1986) which have been shown to stimulate neuronal differentiation and regeneration in vitro (Neugebauer et al., 1991; O'Shea et al., 1991; Osterhout et al., 1992; Chamak et al., 1994).

The neurotrophin family, including the members NGF, BDNF, NT-3, NT-4/5 and NT-6 all play a very important role in neuronal growth, regeneration and survival. Miwa et al. (1997) reported that microglia are able to synthesize NGF, BDNF and NT-4/5. They also found that following LPS stimulation the production of BDNF and NT-4/5 could be enhanced in the microglia. As it is already known that elevated BDNF and NT-4/5 levels play an important role in neuronal regeneration this work demonstrates that diffusible factors from microglia could be very beneficial to the CNS environment.

A number of investigators have reported a survival-enhancing effect of media conditioned in the presence of microglia on the survival of neuronal cultures. Jonakait et al. (1996) have shown that MG-CM is able to promote cholinergic (Ch-AT) differentiation in the basal forebrain. They observed that MG-CM could increase the proportion of Ch-AT positive cells with the same efficacy as IFN, suggesting the possibility that the MG-CM contained the molecule(s) which mediate the action of IFN, which had been previously demonstrated to increase Ch-AT activity (Jonakait, 1994).

MG-CM has also been shown to protect neurones from damage induced by increased glutamate levels, which was taken as one of the actions of an ischaemic insult (Wantanabe
et al, 2000). The results indicated that the MG-CM was able to offer neuroprotection immediately after the glutamate insult but the effect dissipated during the late phase of the insult. The effect of the MG-CM was concluded to be due to cytokines present in the conditioned media, including IL-6 and TNF-α.

Nakajima et al (1989) reported that MG-CM had a neurotrophic effect on cultured rat neocortical neurones. They further demonstrated that the MG-CM could not only enhance mesencephalic neuronal survival and maturation but could furthermore promote neurite extension in culture (Nagata et al, 1993). They identified that the neurotrophic molecule in the MG-CM was heat labile, suggesting that it might be a protein factor and discounted the possibility that the effect was due to the presence of NGF, IL-1β, IL-6 or TNF-α. In another study it was shown that MG-CM released soluble factor(s) which supported the survival of cerebral cortical neurones (Zhang and Fedoroff, 1996). They observed that the unidentified soluble factors present in the MG-CM had a dose related effect on neuronal survival and above a certain threshold neuronal survival could not be further potentiated.

Diffusible factors released by microglia have also been shown to have a stimulatory role during myelinogenesis (Hamilton and Rome, 1994) by inducing the synthesis of sulfatide, a myelin-specific galactolipid, in oligodendrocytes, as well as the expression of the myelin-specific proteins myelin basic protein and proteolipid protein.

It should however be acknowledged that some investigators have found that MG-CM is in fact neurotoxic. Zietlow et al (1999) observed that when microglia were co-cultured with
embryonic dopaminergic neurones there was enhanced neuronal survival compared with control cultures of dopaminergic neurones. However if MG-CM was added instead to the dopaminergic neurones then the neuronal survival was seen to be decreased. This discrepancy may be due to the possibility that when co-cultured in the presence of neurones microglia release survival enhancing factors, but when cultured in isolation the microglia release factors which are detrimental to neuronal survival. However the study did not observe a difference between MG-CM collected from non-activated microglial cultures and from zymosan A activated microglial cultures, which may indicate that the microglial preparations used were already considerably activated, and therefore the microglial were releasing proinflammatory, neurotoxic factors. Such an observation further underlines the probability that the actions of microglia are tightly controlled in the brain, and are able to perform different functions under different circumstances, be that neurotrophic or neurotoxic.

1.2.5.3. Neuronal-microglial interactions

Taken together the above discussions demonstrate that microglia have a complex part to play in the homeostasis of the brain and have the potential to fulfil a neurotrophic role and a neurotoxic role. It has been suggested that the microglial influence on neuronal populations may involve a sustained neurite-promoting stimulation which can however be modulated by their neurotoxic activities triggered as a response to local signals (Chamak et al., 1994). There is also the premise that microglial activation is a reactive process, and that microglia respond to signals which arise from injured neurones (Bruce-Keller, 1999; Streit et al., 1999). The microglial activation which accompanies neuronal injuries is
likely to primarily serve a beneficial purpose. It is postulated that in reversible injuries, such as motorneurone axotomy, the microglia may receive neuronal signals which induce production of trophic factors. In the case of irreversible damage, such as ischaemic damage, the neurone-derived signals may cause the microglia to release toxic factors to facilitate the rapid ‘finishing off’ of the non-viable cell, and its phagocytic removal (Streit et al, 1999). Thus there may well be a fine line between generation of survival-promoting and death promoting signals.

Figure 1.2.5.3. Schematic to demonstrate neuronal-microglial interactions. An injured, but viable neurone, may emit different signals to those released by irretrievably injured neurones. These signals may then cause a differential response of the microglia to release either toxic or neurotrophic signals (adapted from Streit et al., 1999).
1.3. THE CEREBELLUM

The cerebellum, from the Latin meaning ‘little brain’, provides one of the most extensively characterised regions of the brain. The cerebellum is composed of predominately two basic neuronal cell types, namely granule and Purkinje cells, along with other constituent cells including glial cells, all of which have distinct cell morphologies, migration patterns and antigenic markers. The spatially and temporally well-defined differentiation of the two neurone populations from this midbrain/hindbrain region of the neural tube epithelium means that the cerebellar neuroepithelium provides an ideal system to study neurogenesis, lineage determination and survival in the brain (Burgoyne et al., 1993). The most abundant type of cell in the cerebellum is the granule cell with the murine cerebellum contains ~ $10^8$ granule cells, which is more than the number of neurones in the rest of the brain combined (Wechsler-Reya and Scott, 1999; Miyazawa et al., 2000). The importance of these glutamate-secreting cells (Van Vliet et al., 1989) in normal cerebellar function is evident from mutant mice, in which the loss of granule cells leads to severe ataxia (Kofuji et al., 1996; Hamre and Goldwitz, 1997; Mullen et al, 1997).

![Figure 1.3. Sagital view of the rat brain.](http://www.ufbi.ufl.edu/~joneslab/ratcsf.htm)
1.3.1. Circuitry of the cerebellum

The cerebellar cortex is located at the rear base of the brain and its ultrastructure is composed of four distinct layers. The molecular layer is predominately composed of cell processes with relatively few cell bodies. The cells which do reside in this layer are stellate and basket cells. The Purkinje cell layer is a layer of neurones with large somata, which send dendritic trees into the molecular layer, and send their axons down through the sub-cortical white matter to the deep cerebellar nuclei. Purkinje cells are the only output cells of the cerebellar cortex and are inhibitory in nature (Saab and Willis, 2001).

![Image of cerebellar circuitry](image)

Figure 1.3.1. The circuitry of cerebellum. The cerebellum is composed of a limited number of neuronal types, producing excitatory (+) and inhibitory (-) synapses as depicted (adapted from Kiernan, 1998).

The granule cell layer is composed of densely packed small neurones called granule cells, which are the only excitatory neurones in the cerebellum, and send axons into the
molecular layer, where they turn and run parallel with the layer and synaptically contact thousands of Purkinje cell dendrites. The overall ratio of granule cells to Purkinje cells is 449:1 in the rat, and as high as 3300:1 in the human brain (Korbo et al., 1993). There is also a small proportion of non-granule cells in the granule cell layer, namely the Golgi cells. These cells also send dendrites into the molecular layer and send inhibitory axons to granule cells. The medulla layer is the innermost layer and contains myelinated fibres from other regions of the brain. The application of an electrical current to the parallel fibres in the molecular layer is conducted to all connecting cells, and as all the connecting fibres are inhibitory, the overall effect of granule cell excitation is the inhibition of discharges by their target neurones. Such stimulation is caused by the excitatory neurotransmitter glutamate (Kaneko et al., 1987). Granule cells also receive GABAergic input from the golgi cells (Zheng et al., 1993).

The cerebellar cortex also contains a number of types of glia including; (1) astrocytes are found in the granular layer; (2) oligodendrocytes in the medullary layer and (3) Bergmann glia, a specialised form of astrocyte, which are localised in a row with or just below the Purkinje cell layer. Microglia are also found throughout the cerebellum (Maslinska et al., 1998; Vela et al., 1995), though at a lower density than observed in other regions of the CNS (Lawson et al., 1990).
1.3.2. Development of the Cerebellum

The process of granule cell differentiation has been studied in detail by many investigators, with Ramon y Cajal (1911) (see Kuhar et al., 1993) being one of the first to describe the sequential stages of development of the cerebellum (figure 1.3.2.)

**Figure 1.3.2. Developmental stages of the cerebellum.** By postnatal day 8 the developing cerebellum contains cells in each of the 4 major stages of development. *(A)* EGL precursor cells undergoing mitosis are located at the pial surface. *(B)* Postmitotic cells undergoing axon extension and glia-guided migration through the ML are located below the proliferating precursor cell population. *(C)* Postmigratory cells are transiting the Purkinje cell layer. *(D)* The IGL is established following synaptogenesis with the Purkinje cells. EGL: external germinal layer; ML: molecular layer; PCL: Purkinje cell layer; IGL: internal granule cell layer; MF: mossy fibres (adapted from Kuhar et al., 1993).
The granule cell precursors (GCPs) are generated on the outside of the cerebellum in a region known as the external germinal layer (EGL). This region only gives rise to one class of neurone, the cerebellar granule cell (CGC). In contrast, the other cerebellar cell types, including the Purkinje cells, are derived from a separate pool of precursors and become postmitotic by embryonic day 13 (Alder et al., 1996) evidenced in work where both GCPs and rhombic lip cells are shown to express the granule cell marker RU49. These GCPs are most likely to be derived from early embryonic precursors from the rhombic lip of the dorsal aspect of the midbrain / hindbrain region 13 (Alder et al., 1996). The EGL is best developed by postnatal day 7 and has all but disappeared by postnatal day 18 (Mares et al., 1970). In the murine cerebellum the cells of the EGL are seen to proliferate rapidly during these 2-3 weeks after birth, generating a large pool of GCPs. The EGL zone expands from a single cell layer to a layer of about 8 cells in thickness. The rate of proliferation of these GCPs, i.e. the length of time to divide, has been estimated to be approximately 16 - 20 hr (Alder et al., 1996).

The developing GCPs then exit the cell cycle, extend axons and begin to migrate through the molecular layer (ML). This migration is thought to be guided by resident Bergmann glia cells. The postmigratory cells then continue through the Purkinje cell layer (PCL) and establish the internal granule cell layer (IGL). The terminal differentiation of the granule cells is then finally dependent on the interaction with their afferent axons, the mossy fibres (MF) (Kuhar et al., 1993; Wechsler-Reya and Scott, 1999; Miyazawa et al., 2000) which exert a neurotrophic effect on the developing neurones. Mossy fibres are glutamatergic and when stimulated produce glutamate (Ottersen et al., 1990) which leads to glutamate
receptor activation (Garthwaite and Garthwaite, 1988; Garthwaite and Brodbelt, 1989) on the cerebellar granule neurones and plasma membrane depolarisation (Resink et al., 1994). The release of glutamate from the mossy fibres can be inhibited by blocking their 5-HT2 presynaptic receptors (Maura et al., 1991). The amount of dendritic process outgrowth is proportional to the extent of the mossy fibre input (Burgoyne et al., 1993).

1.3.3. Characterisation of developmental stages

It is possible to define which developmental stage CGCs represent by assessing the changing expression of characteristic receptors. For example during the stages of development the CGCs have been shown to display characteristic changes in the expression of the N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor subunits. The NMDA receptor is composed of a number of subunits, which belong to either the NR1, NR2 or NR3 family (review McBain and Mayer, 1994). Each family consists of a number of different functional isoforms. Though the exact number of subunits in a native NMDA receptor has not been determined, there is evidence they are heteromultimeric assemblies formed from NR1 subunits in combination with at least one type of NR2 subunit (Monyer et al., 1994). The NR2 subunit containing NMDA receptors have been shown to give rise to low conductance openings (Cull-Candy et al., 1998) and it is the composition of these NMDA receptors in particular which have been shown to change subunit expression over time. In the immature cerebellar granule cell the NR2B is expressed whilst in the mature cerebellar granule cell this is switched to NR2A and NR2C (Cathala et al., 2000). This change in expression may be due to interactions at the mossy fibre - granule cell synapse (Ueda et al., 1995).
Another method has employed the use of in situ hybridisation analysis to isolate developmentally regulated clones (Kuhar et al., 1993). Such an approach has revealed the presence of non-overlapping sets of granule cell cDNAs expressed by cells and when used in conjunction with the expression of the axonal glycoprotein TAG-1 and the α6 subunit of the GABA\(_A\) receptor, it is possible to identify which neurones are undergoing proliferation, axon extension, migration or synaptogenesis with afferent neurones. This method offers a way to allow the four stages of CGC development to be clearly delineated (table 1.3.3.).

Table 1.3.3. Markers of the developmental stages of the cerebellum. Summary of in situ localisation of mRNAs encoded by novel cDNAs, as well as those encoding the axonal glycoprotein TAG-1 and the α6 subunit of the GABA\(_A\) receptor, to distinguish between the developmental stages of the cerebellum (adapted from Kuhar et al., 1993).

<table>
<thead>
<tr>
<th>Developmental Stage</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. proliferation</td>
<td>cDNA: GC10, GC27, GC60, GC61</td>
</tr>
<tr>
<td>2. axon extension</td>
<td>TAG-1</td>
</tr>
<tr>
<td>3. migration</td>
<td>cDNA: GC9, GC44</td>
</tr>
<tr>
<td>4. synaptogenesis</td>
<td>GABA(_A)R</td>
</tr>
</tbody>
</table>

1.3.4. Control of the developing cerebellum

The mechanisms for the control of this sequence of events are still unclear. It has been demonstrated that the proliferation of the GCPs may be driven in part by Purkinje cell-derived Sonic hedgehog (Shh) (Wetts and Herrup, 1983; Sonmez and Herrup, 1984; Herrup and Sunter, 1987; Vogel et al., 1989; Smeyne et al., 1995), which is a potent mitogen for GCPs (Wechsler-Reya and Scott, 1999).
The eukaryotic cell cycle and its regulation. The cell cycle is regulated by the interplay of cyclins, which are expressed and degraded throughout the progression of the cell cycle, with cyclin dependent kinases (CDKs) to form activated kinases that phosphorylate targets leading to cell cycle regulation. Different CDKs control the onset of S and M phase and increasing activity of these CDKs can advance both events. CDKs are themselves regulated by various mechanisms including the availability of the cyclin regulatory unit, the phosphorylation of the catalytic subunit, by the presence of CDK inhibitors (e.g. p15, p16, p18, p19, p21 and p27), and Rb, all of which act to prevent the cell cycle from progressing until all events within a particular phase are completed (adapted from Cell Cycle Methods, Biosource International and www.biocarta.com).
The messages to stop the proliferation of the GCPs and initiate differentiation have also been investigated and are thought to involve the actions of cyclin-dependent kinase (CDK) inhibitors, eg. p27/Kip1 (Miyazawa et al., 2000). This protein inhibits some of the various cyclin-CDK complexes that control G1 progression and entry into S phase (figure 1.3.4.). It has been shown that cells in the EGL express p27 more strongly than cells in the more superficial zone suggesting that p27 accumulates in GCPs as they proliferate and eventually causes the arrest of division (Miyazawa et al., 2000). Depolarising neurotransmitters also appear to be able to inhibit DNA synthesis whilst being permissible to differentiation, an effect that can be mimicked *in vitro* by culturing in depolarising concentrations of KCl (Cui and Bulleit, 1998).

1.3.4.1. **Role of neurotrophic factors**

There are a number of neurotrophins that are known to act on neurones to enhance their survival both *in vivo* and *in vitro*. This has led to the concept of the neurotrophic theory for the establishment of neuronal networks. In this theory the very survival and differentiation of CNS neurones is said to be dependent upon their interactions with multiple neurotrophins derived from a neuronal target (Purves, 1986), this idea has now been extended to all include interactions also from afferent neurones, neighbouring neurones and the surrounding glial cells.

IGF-I has been demonstrated to increase CGC survival in pure cultures and withdrawal of IGF-I results in neuronal death of differentiated CGCs (Lin and Bulleit, 1997). IGF-I has been therefore identified as a potent mitogen and trophic factor for developing CGCs.
The same study also found that the effect of IGF-I was not mediated via Trk (tyrosine kinase receptor) signalling neurotrophins e.g. BDNF, NT-3. It was noted that the IGF-I could stimulate GCP proliferation and also permit terminal differentiation. It is postulated that the source of the IGF-I may be the Purkinje cells which form the synaptic targets of CGCs. *In vivo* studies have further confirmed that IGF-I is able to increase granule cell number by inducing GCP proliferation (Ye et al., 1996).

NGF has been demonstrated to have functions as a target-derived survival factor for both peripheral and central neurones. BDNF, NT3 and NT-4/5 have been shown to exhibit wider neurotrophic effects across the developing and mature CNS (Segal et al., 1992). Such neurotrophins have functions for the survival and differentiation of the different cell types in the cerebellum. NGF is a known survival factor for Purkinje cells, whilst developing granule cells, which express TrkB (tyrosine kinase B) receptor, respond to BDNF and NT-4/5. It has been observed, *in vitro*, that BDNF has a specific neurotrophic effect early in development, whilst NT3 exerts its actions on more mature CGCs (Segal et al., 1992). *In vivo* however NT3 has been found to have no physiological effect due to the lack of TrkA receptors on CGCs, but both BDNF and NT-4/5 have been shown to promote neurite extension and survival by acting through the TrkB receptor (Gao et al., 1995). This effect could be blocked with the use of K-252a, a specific Trk inhibitor. This progression in neurotrophin responsiveness may be a mechanism for the fine control of neuronal differentiation.
It has been noted that neurotrophins do not stimulate the proliferation of GCPs. They are also unable to influence the initiation of neuronal differentiation (Gao et al., 1995) and only have a trophic role on the maturation and maintenance of differentiated CGCs. Although there is a low level of expression of the Trk receptors (the neurotrophin receptors) in GCPs, the expression is only seen to be increased after differentiation has begun. Thus it is thought that a membrane bound signal, encoded by the weaver gene, is required for the initial stages of differentiation and then the neurotrophins, BDNF and NT-4/5, act downstream to influence maturation and maintenance (Gao et al., 1995). In the murine weaver mutant, the GCPs proliferate as normal (Rezai and Yoon, 1972), but fail to initiate neuronal differentiation and neurite extension (Willinger and Margolis, 1985; Gao et al., 1992; Gao and Hatten, 1993), and therefore mouse mutant represents a good model for studying signals that initiate neuronal differentiation.

**1.3.4.2. Role of glutamate and calcium**

Glutamate, an excitatory amino acid, is also thought to exert important neurotrophic actions on CGCs via the activation of the N-methyl-D-aspartate receptor (NMDA) (Schramm et al., 1990). It is thought that glutamate may form the basis of the neurotrophic factor released by the mossy fibres (Burgoyne et al., 1993). The main evidence for this is that the mossy fibre terminals are known to be glutamatergic and that the synaptic transmission to the CGCs involves glutamate acting on NMDA and non-NMDA receptors, namely AMPA (2-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and kainate receptors (figure 1.3.4.2.). Also CGCs have a high sensitivity to NMDA receptor activation early in development, a time when the interaction with the mossy
fibres is essential for development. As CGCs mature they develop a magnesium (Mg\(^{2+}\)) block of the NMDA receptor which is induced by the continued chronic activation of the NMDA receptor. This high sensitivity to NMDA receptor activation demonstrates that Ca\(^{2+}\) entry through the NMDA receptor also plays a key role in regulating neuronal growth, differentiation and survival and furthermore activation of glutamate receptors results in increased BDNF expression (Lindholm et al., 1993) which, as already mentioned, is a CGC neurotrophin. BDNF is also, via a feedback mechanism, able to partially protect the neurones against neurotoxicity mediated by chronic exposure to a high glutamate concentration.

**Figure 1.3.4.2.** Glutamate activates NMDA and AMPA receptors and results in an elevated intracellular Ca\(^{2+}\) and/or Na\(^{+}\) concentration. Glutamate is a major neurotransmitter in the CNS. Following its release from a transmitter cell it diffuses across the synapse and activates the ionotropic glutamate receptors, NMDA and AMPA receptors, on the receiving cell. This activation subsequently allows the flux of Ca\(^{2+}\) and Na\(^{+}\). The flux of these ions then has downstream effects on a variety of cell functions including ultimately gene expression (adapted from Garthwaite, 1991).
An important consideration in the survival and differentiation of neurones is the interplay between neuronal activity and calcium channel activation. Voltage operated calcium channels (VOCCs) are expressed early in development and it is changes in their localisation and subtype expression that can be observed during maturation. The activity of these VOCCs is very important for development. The mediator of these effects is the cytoplasmic concentration of free ionic calcium, \([\text{Ca}^{2+}]_c\). For CGCs to survive in culture conditions have to be optimal for the activation of these channels. In general high depolarising \(K^+\) concentrations (Kingsbury et al., 1985; Yu and Chaung, 1997) or a low NMDA concentration has been found to be optimal and these are thought to mimic the endogenous activity at the mossy fiber – granule cell glutamatergic synapse. The benefits of these systems can be reversed by removal of the stimulus or by suppression of the VOCC channel directly with inhibitors, either way the result is apoptotic neuronal death (Toescu, 1999).

An interesting aside is that if CGCs \textit{in vitro} are preincubated in the presence of low concentrations of NMDA (or glutamate) then this offers a level of protection against subsequent exposure to toxic concentrations of glutamate. This suggests that the neuroprotection mechanisms evoked under such circumstances requires the synthesis of new mRNA and protein and is possibly mediated by the expression of a NMDAR mediated and transcriptionally directed neuroprotective protein (Marini and Paul, 1992). This neuroprotective state induced by NMDA can be blocked by co-incubation with either actinomycin D (RNA synthesis inhibitor) or cyclohexamide (protein synthesis inhibitor) (Damschroder-Williams et al., 1995), which adds further credence to the suggestion that
there is increased expression of a neuroprotective protein. This effect appears to be
developmentally regulated and can only be observed in a restricted time frame, primarily
prior to 10 days *in vitro*.

Depolarising concentrations of KCl have been observed to decrease the proliferation of
GCPs but allow their differentiation to proceed (Cui and Bulleit, 1998). It is postulated
that the KCl antagonises the mitogenic effect of IGF-I on GCPs and is not merely an
effect of selective death of the dividing GCPs. This is corroborated by the observation
that the expression of Brn-5, an early terminal differentiation marker which is expressed
soon after withdrawal from the cell cycle, was seen to be increased.
1.4. TRANSDUCTION PATHWAYS INVOLVED IN NEURONAL SURVIVAL AND PROLIFERATION

All cells in multicellular organisms are continually exposed to a wide variety of extracellular stimuli that needs to be interpreted and translated into an appropriate cellular response. The signals received can be locally derived soluble factors (e.g. ion changes during synaptic transmission), or distantly produced factors (e.g. hormones and growth factors), ligands on the surface of neighbouring cells (e.g. Notch signalling), or indeed the extracellular matrix itself. To achieve this cells express a repertoire of specific receptors that are sensitive to individual stimuli. These receptors may be grouped into families based on the method by which they generate the intracellular signals that give rise to the functional response. It needs to be remembered that these linear pathways are not free standing entities but are parts of a larger network and as such can be modulated by other signalling pathways and thus generate the flexibility required to respond to a wide array of factors (Bhalla and Iyengar, 1999; Jordan et al., 2000).

A number of receptor families have implications and roles to play in the development of neurones, through the activation and inhibition of pathways which regulate process such as cell death and survival, cell proliferation and cell differentiation. In this thesis a number of such pathways and their activity has been assessed including glutamate receptors, voltage-operated calcium channels, G-protein coupled receptors, delta-Notch signalling and tyrosine kinase receptors (see figure 1.4).
Figure 1.4. Summary of signal transduction pathways involved in neuronal survival and proliferation. A number of pathways are known to have implications in neuronal survival and proliferation, a variety of which have been investigated during the course of thesis by modulating their action through the addition of selective agonists and antagonists (written in red). The pathways investigated were (i) Notch signalling (red), (ii) ionotropic glutamate receptors (iGlu receptor; yellow), (iii) voltage operated calcium channels (VOCC; orange), (iv) metabotropic glutamate receptors (mGlu receptor; blue), (v) G-protein coupled receptor signalling (GPCR; purple) and (vi) receptor tyrosine kinase (RTK) mediated signalling (green).

Abbreviations: AC, adenylate cyclase; AIDA, 1-aminoindan-1,5-dicarboxylic acid; AKT, also known as protein kinase B; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ATP, adenosine-5′-triphosphate; BDNF, brain derived neurotrophic factor; Ca^{2+}, calcium; cAMP, cyclic adenosine-5′-monophosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; DAG, diacylglycerol; DCGIV, (2S,TR,2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine; EDTA, ethylenediaminetetra-acetic acid; ER, endoplasmic reticulum; H89, N-(2-(P-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide hydrochloride; ICD, intracellular domain; InsP3, Inositol-1,4,5-triphosphate; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; Lyn, Lyn tyrosine kinase; MAP4, (S)-2-amino-2-methyl-4-phosphonobutanoic acid; MAPK, mitogen-activated protein kinase; MCCG, (2S,3S,4S)-2-methyl-2-(carboxycyclopropyl)glycine; MEK, MAP kinase kinase; MK801, (5R,10S)-(+) -5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine hydrogen maleate; Na^+, sodium; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; NT3, neurotrophin 3; PI3K, phosphatidylinositol 3-kinase; PKA, cAMP dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; PLC-γ, phospholipase C-γ; PTX, pertussis toxin; tADA, trans-azetidine-2,4-dicarboxylic acid.
growth factors e.g. BDNF, NGF, NT3
extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

mGlu receptor
GROUP I
Notch ligands e.g. delta, jagged

mGlu receptor
GROUP II

Notch

calcium chelation e.g. EDTA

VOCC
L-type

iGlu receptor
AMPA

iGlu receptor
NMDA

CNQX

L-AP4

MK801

thapsigargin

DCGIV

mGlu receptor
GROUP III

glutamate

sOS
Grb2
Ras
Raf

PI3K
SHC

GTP

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

PTX

mGlu receptor
GROUP II

MCCG

iGlu receptor
AMPA

iGlu receptor
NMDA

VOCC
L-type

CNOX

L-AP4

MK801

thapsigargin

intracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neuropeptides, hormones

extracellular stimuli e.g. neurotransmitters, neopep...
1.4.1 G-protein signalling

The majority of cell-cell communication involves the release of soluble signalling molecules, including peptide and glycoprotein hormones, neuropeptides, biogenic amines, eicosanoids or neurotransmitters (e.g. glutamate), which bind to cell surface receptors on a neighbouring cell and trigger a cascade leading to the formation of an intracellular response. Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) are commonly used to transduce many of these extracellular signals, received at a cell surface receptor, into intracellular responses. Indeed approximately 80% of all signals may function via such G-protein coupled receptors (GPCR) (Birnbaumer and Brown, 1990) functioning via a number of receptors (table 1.4.1.a.). G-proteins reside predominately at the intracellular face of the plasma membrane where they interact upstream, with their receptor, and downstream with components of various signalling systems.

Table 1.4.1.a. Examples of ligands acting through G-protein coupled receptors

<table>
<thead>
<tr>
<th>class of ligand</th>
<th>receptors</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>neurotransmitters</td>
<td>dopamine D1, D2</td>
<td>Ohara et al., 1998; Sidhu and Niznik, 2000</td>
</tr>
<tr>
<td>serotonin</td>
<td>5HT</td>
<td>Andrade et al., 1986; Raymond et al., 2001</td>
</tr>
<tr>
<td>GABA</td>
<td>GABAB</td>
<td>Morishita et al., 1990; Couve et al., 2000</td>
</tr>
<tr>
<td>glutamate mGlul, II, III</td>
<td>De Blasi et al., 2001; Hermans and Challis, 2001</td>
<td></td>
</tr>
<tr>
<td>hormones</td>
<td>purines</td>
<td>P&lt;sub&gt;2Y&lt;/sub&gt;</td>
</tr>
<tr>
<td>tachykinins</td>
<td>NK1, NK2, NK3</td>
<td>Helke et al., 1990; Maggi, 2000</td>
</tr>
<tr>
<td>vasopressin</td>
<td>V-1a, V-1b, V-2</td>
<td>Xuan et al., 1987; Kolaj and Renaud, 1998</td>
</tr>
<tr>
<td>bradykinin</td>
<td>BK1, BK2</td>
<td>Etscheid and Villereal, 1989; Yang et al., 1999</td>
</tr>
<tr>
<td>sensory</td>
<td>light rhodopsin</td>
<td>Bennett and Dupont, 1985; Albert and Yeagle, 2000</td>
</tr>
</tbody>
</table>
G-proteins are trimeric entities, consisting of α (20 mammalian isoforms), β (5 mammalian isoforms) and γ (10 mammalian isoforms) subunits, which function via GTP hydrolysis. There are four major subfamilies of G-proteins, termed Gs, Gi, Gq and G12, which vary in the composition of the α subunit and its susceptibility to different bacterial toxins (Fields and Casey, 1997; table 1.4.1.b).

**Table 1.4.1.b. Comparison of mammalian G-protein subfamilies.** G-proteins can be divided into four subfamilies, according to a subunit composition, and exhibit different tissue distributions. Each of the subfamilies can be further characterised according to further variations within the α subunit. The groups can be classified according to their toxin susceptibility (Adapted from Fields and Casey, 1997).

<table>
<thead>
<tr>
<th>subfamily</th>
<th>Gα subunit</th>
<th>toxin substrate</th>
<th>signalling pathway</th>
<th>distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gi</td>
<td>Gz</td>
<td>none</td>
<td>↓AC</td>
<td>brain, adrenal medulla, platelets</td>
</tr>
<tr>
<td></td>
<td>Gι1</td>
<td>PTX</td>
<td>↓AC</td>
<td>ubiquitous</td>
</tr>
<tr>
<td></td>
<td>Gι2</td>
<td>PTX</td>
<td>↓AC</td>
<td>ubiquitous</td>
</tr>
<tr>
<td></td>
<td>Gι3</td>
<td>PTX</td>
<td>↓AC</td>
<td>ubiquitous</td>
</tr>
<tr>
<td></td>
<td>Gζ</td>
<td>PTX</td>
<td>Ca²⁺ channels</td>
<td>brain</td>
</tr>
<tr>
<td></td>
<td>Gκ</td>
<td>PTX</td>
<td>K⁺ channel</td>
<td>retina</td>
</tr>
<tr>
<td></td>
<td>Gι</td>
<td>PTX, CTX</td>
<td>↑cGMP-PDE</td>
<td></td>
</tr>
<tr>
<td>Gs</td>
<td>Gα</td>
<td>CTX</td>
<td>↑AC</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Gι2</td>
<td>Gι2</td>
<td>none</td>
<td>?</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Gι3</td>
<td>Gι3</td>
<td>none</td>
<td>?</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Gq</td>
<td>Gq</td>
<td>none</td>
<td>↑PLC-β</td>
<td>ubiquitous</td>
</tr>
<tr>
<td>Gι4</td>
<td>Gι4</td>
<td>none</td>
<td>↑PLC-β</td>
<td>spleen, lung kidney</td>
</tr>
<tr>
<td>Gι5</td>
<td>Gι5</td>
<td>none</td>
<td>↑PLC-β</td>
<td>myeloid and lymphoid cells</td>
</tr>
<tr>
<td>Gι6</td>
<td>Gι6</td>
<td>none</td>
<td>↑PLC-β</td>
<td>myeloid and lymphoid cells</td>
</tr>
</tbody>
</table>
Certain $G_\alpha$ subunits contain site(s) for NAD$^+$-dependent ADP-ribosylation which can be catalysed by either cholera toxin (CTX) or pertussis toxin (PTX). CTX catalyses the ADP-ribosylation of a conserved arginine residue on the $G_\alpha$ unit, resulting in the inhibition of both GTPase activity and interaction with $G_{\beta\gamma}$. PTX catalyses the ADP-ribosylation of a cysteine residue at the c-terminus of $G_{i\alpha}$ subunits and results in the inhibition of receptor-G protein coupling.

The activation of G-proteins follows a stepwise mechanism. Firstly an agonist-bound receptor interacts with a particular inactivated G-protein, the contact drives the release of GDP from the $G_\alpha$ subunit. GTP subsequently binds which results in a conformational change in the $G_\alpha$ subunit, which in turn decreases its affinity for the $G_{\beta\gamma}$ and the receptor and so dissociates. This in turn results in decreased affinity of the receptor for the agonist and so the bound agonist is released. The dissociated/activated $G_\alpha$ or $G_{\beta\gamma}$ subunits can then interact with specific effector molecules to mediate the production of soluble intracellular secondary messengers e.g. adenylate cyclase (AC) and phospholipase C (PLC). The signal is terminated by the intrinsic GTPase activity of the $G_\alpha$ subunit which hydrolyses the bound GTP to GDP and re-associates with the $G_{\beta\gamma}$. The re-formed G-protein is then available to interact with an agonist bound receptor.
G-proteins are known to activate a number of effector signalling pathways. The first pathway which was identified to be activated by G-protein was adenylate cyclase (AC). Initially it was found that AC activation was dependent on GTP (Rodbell et al., 1971) and was subsequently found to be due to a GTPase protein (Cassel and Selinger 1976, 1977a and 1977b). Since that time a number of other pathways have been found to be targets of G-proteins, including PLC (Abbracchio et al., 1995; Baek et al., 1996), PI3K (Kue and Daaka, 2000; Filardo, 2002; Jo et al., 2002), MAPK (Blaukat et al., 2000) and ion channels (Diverse-Pierluissi et al., 1996; Postma et al., 1996; Komwatana et al., 1998). The different subfamilies of G-proteins differentially modulate these pathways either by activating them or inhibiting them (see table 1.4.1.b. and figure 1.4.1.b.).
Figure 1.4.1.b. Schematic illustrating the multiple signalling pathways activated by receptors through coupling to different G-proteins. Following receptor activation G-proteins can modulate a number of signalling pathways, either by enhancing the activity (solid line) or by downregulating the activity (dotted line). Furthermore the different classes of $\alpha$ subunits and $\beta\gamma$ subunits exert differential effects on these transduction pathways (adapted from Birnbaumer and Brown, 1990; Selbie and Hill, 1998; Hermans and Challiss, 2001; Liebmann, 2001).
1.4.2. **Glutamate receptors and transporters**

Glutamate is the principal excitatory neurotransmitter in the brain, the action of which was first recognised in the 1950's by Hayashi and coworkers (see Takagaki, 1996) and Curtis and Watkins (see Watkins, 2000). A number of glutamate receptors and transporters have been identified and the action and/or suppression of these exert important roles in neuronal plasticity, development and degeneration.

1.4.2.1. **Glutamate release and function**

Glutamate is released from presynaptic vesicles by Ca\(^{2+}\) dependent mechanisms involving N and P/Q voltage operated calcium channels (VOCC). In some circumstances glutamate can also be released by a reverse operation of glutamate transporters, for example in cerebral ischaemia, the Na\(^{+}\) and K\(^{+}\) gradient across the membrane is reduced thus allowing glutamate release (Phillis et al., 2000; Rossi et al., 2000). The glutamate concentration in a presynaptic vesicle is approximately 100 mM whereas the glutamate concentration in the synaptic cleft is approximately 2-1000 μM (Meldrum, 2000).

Once released glutamate can act on two major groups of receptors, either (i) ligand gated receptor channels, termed ionotropic glutamate receptors (iGlu receptors) or (ii) G-protein coupled receptors, metabotropic glutamate receptors (mGlu receptors). Furthermore, glutamate can be moved through the action of the glutamate transporters present on both the glia and the neurones. The different glutamate receptors can also be activated by other endogenous ligands, for example L-aspartate, sulfonic and sulfinic glutamate or aspartate.
anallogues (e.g. L-cysteine sulfinate, L-homocytsteine sulfinate, L-homocysteate, L-cysteate) and quinolinic acid, however glutamate is the most potent (Mayer et al., 1984).

Glutamate plays a variety of roles at different developmental stages from embryo to adult. During early development glutamate plays a vital role in the differentiation, migration and survival of newly formed neurones, primarily through facilitating the entry of Ca$^{2+}$ (Hack and Balazs, 1994). If glutamate receptors, e.g. NMDA iGlu receptors, are blocked during the perinatal period then the level of neuronal death by apoptosis is dramatically increased (Ikonomidou et al., 1999). Glutamate is also involved in both acute and chronic neurodegeneration. There are a number of possible mechanisms by which glutamate and related compounds may cause degeneration. Endogenous glutamate released from the neurones, and other cell types such as microglia, may contribute to acute neurodegeneration occurring in cerebral ischemia, status epilepticus or traumatic brain injury (see review Bittigau and Ikonomidou, 1997). In each of these disorders the primary mechanism involved is loss of ionic equilibrium due to the excessive release of glutamate and the associated entry of Na$^{+}$ and Ca$^{2+}$ through ligand-gated and voltage-operated channels. The raised intracellular Ca$^{2+}$ is then in turn able to activate a variety of enzymes which can ultimately result in neuronal death (Meldrum and Garthwaite, 1990). Glutamate also plays a role in a number of late onset neurological disorders, e.g. Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, motor neurone disease (see reviews Nakao and Brundin, 1998; Tapia et al., 1999). Each of these disorders is dependent to some extent on endogenous glutamate excessively activating a variety of glutamate receptors. Excessive exogenous glutamate, or related compounds, consumed in
the diet, have also been shown to be able to result in damage in the brain due to excessive glutamate receptor activation (for review see Meldrum, 1993).

1.4.2.2. Ionotropic Glutamate receptors

Ionotropic glutamate (iGlu) receptors are ligand gated cationic channels, which cause neuronal depolarisation by generating fast excitatory postsynaptic potentials. To date 15 different genes have been identified that encode iGlu receptor subunits (Watkins, 2000; Koles et al., 2001) (see figure 1.4.2.2.). These genes determine the molecular composition of the three pharmacologically defined families of glutamate-gated receptor channels (Myers et al., 1999), named according to the compound which stimulates them, (1) N-methyl-D-aspartate (NMDA) receptors, (2) α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and (3) kainate (KA) receptors.

Figure 1.4.2.2. Schematic representation of subtypes, subunits and splice variants of the ionotropic glutamate receptors (adapted from Watkins, 2000).

Ionotropic glutamate receptors are widely distributed throughout the CNS (Hollman and Heinemann, 1994; Scherzer et al., 1998), and have been found to be co-localised in some
neurones (Bekkers and Stevens, 1989; Jones and Baughman, 1991). All iGlu receptors are permeable to Na$^+$ and K$^+$ cations (Korogod and Saytchenko, 1997; Gill et al., 1998; Miller, 1998), however whilst all NMDA receptors are also Ca$^{2+}$ permeable, only certain AMPA and KA receptors are Ca$^{2+}$ permeable (Hollmann et al., 1991; Ozawa et al., 1998).

Ionotropic glutamate receptors mediate fast excitatory postsynaptic potentials (EPSPs), which are critical in allowing rapid communication between neurones (Rho and Strorey, 2001), unlike the mGlu receptors which mediate slower, more persistent communication between neurones (Dodd et al., 1979; Rho and Storey, 2001). When the electrophysiology of these fast EPSPs is analysed two components can be routinely observed allowing the NMDA and AMPA/KA receptors to be distinguished from each other. AMPA/KA receptors are linked to channels that display rapid opening and closing kinetics which are highly permeable to Na$^+$ and K$^+$, whilst NMDA receptors display slower voltage-dependent kinetics. The reason for this is that near the neuronal resting potential (~ -60 mV) the channels linked to AMPA receptors are more permeable to Na$^+$ and K$^+$, resulting in neuronal depolarisation, whilst the channels linked to NMDA receptors are blocked by extracellular Mg$^{2+}$ (Mayer et al., 1984; Nowak et al., 1984). Once the AMPA receptors have initiated neuronal depolarisation the Mg$^{2+}$ block of the NMDA receptor is relieved and so the slow activating NMDA receptors can participate in continuing the synaptic current. This results in a large influx of Ca$^{2+}$ through the NMDA receptor, which can in turn trigger intracellular signalling cascades and thereby produce long-lasting changes in neuronal functions (Nakanishi et al., 1998; Dingledine et al., 1999).
As such iGlu receptors possess the ability to produce not only short term excitation but also significantly alter the effectiveness of synaptic transmission. Such changes can persist for several days, resulting in specialised forms of synaptic plasticity which are thought to underlie learning and memory (Collingridge and Singer, 1990; Kaczmarek et al., 1997). Both AMPA and NMDA receptors appear to be intrinsically involved in this process (Kullmann et al., 1996) and are therefore postulated to play a pivotal role in learning and memory (Nakanishi, 1992).

Overactivity of the iGlu receptors results in uncontrolled Ca\(^{2+}\) influx and leading to a marked elevation of intracellular Ca\(^{2+}\) concentrations, which in turn results in excitotoxicity and neuronal death (Doble, 1995). Such dysfunction may be correlated with the pathology of a number of neurological disorders including Parkinson’s disease (Yung, 1998; Greenamyre, 2001), Alzheimer’s disease (Sze et al., 2001), Huntington’s disease (Kieval et al., 2001) and epilepsy (Loscher, 1998).

Peptide growth factors are vital regulators of CNS development, synaptic plasticity and neuronal survival (Lewin and Barde, 1996), exerting both short and long term effects on synaptic plasticity (Thoenen, 1995; Katz and Shatz, 1996), through the modulation of the different glutamate receptors both in vivo and in vitro (Mattson et al., 1989; Fernandez-Sanchez and Novelli, 1993; Boxer et al., 1999; Blanc et al., 1999; Gonzalez de la Vega et al., 2001; Numakawa et al., 2002). Prolonged treatment with bFGF can prevent NMDA receptor-mediated cell death in ischaemia (Fisher et al., 1995; Kirschner et al., 1995) by enhancing the ability of extracellular calcium to inactivate NMDA receptor currents (Fernandez-Sanchez and Novelli, 1993; Boxer et al., 1999). It has also been shown that
bFGF can regulate internal calcium concentrations following NMDA receptor activation (reviewed Lindholm, 1994), and modulate those AMPA receptors which are linked to the inositol phosphate cascade in hippocampal cultures (Blanc et al., 1999).

Insulin growth factor-1 (IGF-1) has been shown to induce a depression of AMPA receptor-mediated currents (Wang and Linden, 2000) and kainate receptor-mediated currents (Gonzalez de la Vega et al., 2001), but not NMDA receptor-mediated currents. This potentiation of AMPA and kainate responses by IGF-1 can be blocked by the addition of phosphatidylinositol 3-kinase (PI3K) inhibitor indicating a role for this kinase in the effect that IGF-1 exerts on neuronal plasticity (Gonzalez de la Vega et al., 2001).

Conversely it has also been found that activation of iGlu receptors can in turn modulate the production of active growth factors. For example it has been found that activation of both NMDA and AMPA/KA receptors downregulates the expression of TGF-β2 (Dobbertin et al., 2000). Interestingly the activation of mGlu receptors on astrocytes has also been found to stimulate the production of TGF-β (Bruno et al., 1998). AMPA receptors are also known to interact with the protein tyrosine kinase Lyn (Hayashi et al., 1999), and through this association are able to regulate the expression of BDNF, which in turn may contribute to synaptic plasticity. Overall such observations combined could provide a mechanism that would allow for fine tuning of growth factor levels, allowing neurones and astrocytes to compensate for each other.
All these results combined reinforce the notion that iGlu receptors have an intimate role in the regulation of a number of growth factors, and provide evidence for a mechanism by which excitatory amino acids in conjunction with growth factors can influence the development and survival of glial and neuronal cells in the CNS.

1.4.2.3. Metabotropic glutamate receptors

The mGlu receptors are coupled to intracellular signal transduction pathways via G proteins (see review Nakanishi, 1994). The mGlu receptor family comprises eight subtypes, divided into three groups, (I, II and III) on the basis of sequence similarity and transduction pathways. In general the activation of group I results in increased neuronal excitation and excitability whereas activation of group II/III leads to depressed synaptic excitation. There are a large number of commercially available agonists and antagonists of the different mGlu receptors (see table 1.4.2.3.) and the use of these has aided the clarification of the localisation, distribution and effects of mGlu receptor activation.

Group I includes mGlu1 (splice variants: a, b, c, d, e, g) and -5 (splice variants a and b), which are coupled to polyphosphoinositide (PI) hydrolysis. The two subtypes can be characterised by their intracellular calcium mobilization kinetics. Activation of mGlu5 results in an oscillatory increase in cytosolic Ca\(^{2+}\), whilst activation of mGlu1 induces a single-peaked increase in intracellular Ca\(^{2+}\) (Kawabata et al., 1996). Both receptors share the same subcellular localisation in the periphery of the postsynaptic densities (Abe et al., 1992; Lujan et al., 1996) but their anatomical expression is also different; mGlu1 is particularly abundant in cerebellar Purkinje cells, and mGlu5 is highly expressed in the
and cerebral cortex. They both function primarily to amplify postsynaptic responses to glutamate concentrations that are sufficiently high to spread to the periphery of the synapse and are thus stimulatory in nature and reinforce excitatory synaptic transmission. Both receptors are also expressed on microglia (Biber et al., 1999).

Table 1.4.2.3. Selective mGlu receptor agonists and antagonists. There are a large number of commercially available selective agonist and antagonists for the different members of mGlu receptor family. The specificity of these compounds for group or subtype is indicated below (adapted from Schoepp et al., 1999).

<table>
<thead>
<tr>
<th>Group</th>
<th>Agonist</th>
<th>Antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>(R, S)-3,5-DHPG, tADA</td>
<td>AIDA, DL-AP3</td>
</tr>
<tr>
<td>mGlu1</td>
<td>-</td>
<td>CPPCOET, LY367385</td>
</tr>
<tr>
<td>mGlu5</td>
<td>CHPG</td>
<td>MPEP, SIB 1757, SIB 1893</td>
</tr>
<tr>
<td>Group II</td>
<td>(2R,4R)-APDC, DCGIV, L-CCG-I</td>
<td>MCCG, MTPG, LY 341495</td>
</tr>
<tr>
<td>mGlu2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mGlu3</td>
<td>NAAG</td>
<td>-</td>
</tr>
<tr>
<td>Group III</td>
<td>L-AP4, L-SOP</td>
<td>MSOP, MAP4, CPPG</td>
</tr>
<tr>
<td>mGlu4</td>
<td>ACPT-I</td>
<td>-</td>
</tr>
<tr>
<td>mGlu6</td>
<td>HomoAMPA</td>
<td>-</td>
</tr>
<tr>
<td>mGlu7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mGlu8</td>
<td>(R,S)-PPG, (S)-3,4-DCPG</td>
<td>-</td>
</tr>
</tbody>
</table>

Group II includes mGlu 2 and -3, which are negatively coupled to adenylate cyclase (AC) through a PTX-sensitive GTP-binding protein (G-protein). Both subtypes are diffused throughout the brain and are preferentially localised in the preterminal region of the neuronal axon, far from the active zone of neurotransmitter release (Tanabe et al., 1993; Shigemoto et al., 1996; Lujan et al., 1997). Activation of these receptors serves to inhibit the release of glutamate, but only in response to glutamate concentrations that are high.
enough to spread back to the remote regions of the axon. Both mGlu2 and -3 have been shown to be expressed on microglia (Taylor et al., 2002), but only the mGlu3 receptor is expressed by astrocytes (Tanabe et al., 1993). The function of this expression is not fully understood but activation may lead to the release of neurotrophic factors of astroglial origin (Bruno et al., 1997).

Group III includes mGlu 4 (splice variants a and b), -6, -7 (splice variants a and b) and -8, which are distributed throughout the brain and are localised on nerve terminals, in close proximity to the active zone of neurotransmitter release (Ohishi et al., 1995; Shigemoto et al., 1996). It is hypothesised that these subtypes behave as glutamate autoreceptors and act as a primary fail-safe mechanism to avoid excessive glutamate release.

**Figure 1.4.2.3. Diagram illustrating the location of mGlu receptors relative to the synaptic cleft.** Group I mGlu receptors (mGlu1 and -5; red) are located predominately perisynaptically on the postsynaptic membrane and also on microglia. mGlu5 is also expressed on astrocytes. Group II mGlu receptors (mGlu2 and -3; blue) are localised on the preterminal axon and on microglia. mGlu3 expression is also observed on astrocytes. Group III mGlu receptors (mGlu4, -6, -7, -8; green) are located on the nerve terminal.
The mGlu receptors are of considerable therapeutic interest as alterations in their expression may contribute to a wide number of disorders associated with the CNS, therefore the identification of factors that can regulate the expression of these receptors would be of the utmost interest. Group I mGlu receptors may serve as potentially important therapeutic targets for the control of inflammatory pain, anxiety, Parkinson's disease and drug abuse (Spooren et al., 2001; Walker et al., 2001; Gasparini et al., 2002), as well as being potential targets for anticonvulsant and neuroprotective therapies (Kingston et al., 1999; Gasparini et al., 2002; Zhang et al., 2002). Indeed, activation of group I mGlu receptors has been shown to be required for normal development, suggesting that their antagonism might be neuroprotective in brain ischaemia and in other forms of acute neurodegeneration. Activation of group II mGlu receptors has been found to protect neurones against excitotoxic degeneration, suggesting that agonists may serve as neuroprotective drugs (Battaglia et al., 1998). Whilst antagonists of group I and agonists of group II and III mGlu receptors have been found to have antianxiety-like effects (Klodzinska et al., 1999; Marek et al., 2000; Tatarczynska et al., 2001).

1.4.2.4. Glutamate transporters

The neurotransmitter glutamate is synthesised and stored in specialised glutamatergic neurones and upon stimuli is released into the synaptic cleft and initiates the activation of a wide number of receptors which transduce signals that affect neuronal survival and development (Balazs et al., 1988). Extracellular levels of glutamate are regulated by non-vesicular transporting proteins, which reduce the synaptic cleft glutamate concentration (Attwell et al., 1993) and thus terminate the glutamatergic transmission, thereby
preventing excessive glutamate receptor stimulation which is neurotoxic (Choi et al., 1987; Rosenberg et al., 1992).

To date a number of glutamate transporters have been identified in the CNS. The glutamate-aspartate transporter (GLAST), also termed excitatory amino acid transporter-1 (EAAT-1), and the glutamate transporter-1 (GLT-1), also termed EAAT-2, are astroglial associated glutamate transporters (Pines et al., 1992; Storck et al., 1992; Arriza et al., 1994), whilst the excitatory amino acid carrier-1 (EAAC-1), also termed EAAT-3, and EAAT-4 and -5 are the neuronally associated glutamate transporters (Kanai and Hediger, 1992; Fairman et al., 1995; Velaz-Faircloth et al., 1996; Nakayama et al., 1996; Bjoras et al., 1996; Arriza et al., 1997; Eskandari et al., 2000).

The expression of each of these glutamate transporters have been shown to change during development (Furuta et al., 1997), with the expression of GLT-1 and GLAST and the neuronal transporter EAAT4 increasing with maturation, whilst the expression of EAAC1 is greatest in the newborn. The glutamate transporters are sodium (Na⁺) dependent and function using a Na⁺ and K⁺ electrochemical gradient as the driving force for transport (see figure 1.4.2.4. for stoichiometry). In addition the neuronal transporter EAAT4, may be functionally linked to Cl⁻ channels to diminish synaptic transmission (Fairman et al., 1995; Kataoka et al., 1997).
The glutamate transport system may also play a more active role in glutamatergic signalling mechanisms (see review Gegelashvili and Schousboe, 1997), especially during periods of elevated extracellular glutamate concentrations, where the effective removal of glutamate is a crucial rescue mechanism and the failure or loss of the glutamate transport system may aggravate glutamate induced neurotoxic damage (Frandsen and Schousboe, 1990). Under conditions of energy failure, such as that observed in ischemia, the electrochemical gradients are reduced or disrupted, the glutamate transporters may function in a reversed mode and carry glutamate out from the cytoplasm to the exterior and thus contribute to the excitotoxic levels of glutamate (Nicholls and Attwell, 1990). Such alterations in glutamate transporters have been reported to occur in a number of neurodegenerative conditions, including ALS, where it has been observed that astroglial glutamate transporters are downregulated (Rothstein et al., 1992), and in the perinatal model of hypoxia ischemia, where transiently elevated levels of extracellular glutamate have been postulated to be due to a reversal of glutamate transporter function (Szatkowski and Attwell, 1994). Conversely it has been suggested that excessive uptake of glutamate is detrimental as it results in hypofunction of the glutamate receptors, a mechanism that
has been hypothesised to be involved in the development of psychoses such as schizophrenia (Carlsson and Carlsson, 1990).

The glutamate transporters are of considerable therapeutic interest as their regulation may enhance the action of drugs (Kanner, 1993). There may be various points of interception at which the glutamate transporters may be upregulated. Glutamate itself can regulate the expression of the transporters via activation of mGlu receptors and also kainate receptors (Gegelashvili et al., 1996) and also by activity dependent trafficking of transporters without involving glutamate receptor activation (Duan et al., 1999). It has also been demonstrated that a number of neuronally derived diffusible factors can lead to the upregulation of expression and function of astroglial glutamate transporters, e.g. BDNF (Gegelashvili et al., 1997), pituitary adenylate cyclase-activating polypeptide (PACAP; Figiel and Engele, 2000) and vasoactive intestinal peptide (VIP; Brown, 2000). Furthermore, recent studies have demonstrated an involvement of both the PI3 kinase and p42/44 MAP kinase pathways (Gegelashvili et al., 2000) and NF-κB transcription factors (Zeleniaia et al., 2000).

1.4.3. Notch signalling pathway

The Notch proteins, originally identified in Drosophila, are highly conserved transmembrane receptors that are involved in cell fate regulation, asymmetry and lateral inhibition, influencing proliferation, differentiation and apoptotic events at all stages of development, in both invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999). The Notch receptor, initially synthesised as a single polypeptide chain with a molecular weight
of 300 kD, is proteolytically processed to result in the formation of a heterodimeric receptor with an N-terminal extracellular domain (ECD) which is non-covalently linked to its transmembrane (TM) and intracellular domains (ICD). The Notch receptor is activated by association with transmembrane ligands, expressed on an adjacent cell, via a Delta/Serrate/Lag2 (DSL) domain present on the extracellular domains of both receptor and ligand (figure 1.4.3.a.).

In mammals four subtypes of Notch receptors have been identified, Notch-1, -2, -3 and -4, whilst five DSL ligands have been described jagged1/serrate1, jagged2/serrate2, delta1, -2 and -3 (Hicks et al., 2000). The presence of multiple receptors and ligands provides potential for combinational complexity with respect to both distribution and ligand-receptor specificities both of which remain undefined at present. The extracellular factors which modulate this activation also remain to be fully determined, however, recently proteins encoded by the fringe family of genes, (including Lunatic fringe, Lfng, Manic fringe, Mfng, and Radical fringe, Rfng), have been shown to be specific modulators of ligand-receptor binding (Hicks et al., 2000). The effects of Notch are also suggested to be modulated by signals, namely cytokines and growth factors, received by the cells. Cells stimulated with cytokines which support cell-growth, such as stem cell factor (SCF) or interleukin-6 (IL-6) may choose self-renewal, whilst cells treated with interleukin-2 (IL-2) or interleukin-7 (IL-7) may be stimulated to differentiate (Chou et al., 1999; Varnum-Finney et al., 2000). It has also been demonstrated that fibroblast growth factor (FGF) can regulate both receptor and ligand expression (Faux et al., 2001).
Once the Notch receptor has been activated by the relevant ligand, the ICD is released by proteolytic cleavage, via a pathway that is at present poorly understood, but which is facilitated by a membrane bound protein, presenilin (PS) (Fraser et al., 2000). Presenilin mutations have been found to be one of the causes of familial Alzheimer's disease (FAD), which accounts for approximately 5% of all Alzheimer's cases (Rogaev et al., 1995; Sherrington et al., 1995). In this subtype of FAD the mutated form of PS results in a gain of function leading to an overproduction of a toxic amyloid peptide. Despite this enhanced proteolytic activity of PS, in FAD there is an accompanying diminution in Notch signalling (Capell et al., 2000; Zhang et al., 2000) whether there is a role of decreased Notch signalling in the aetiology of Alzheimer's disease remains to be determined.

Once cleaved, the active ICD is translocated to the nucleus, a process believed to be facilitated by Deltex which is a positive regulator of Notch (Diederich et al., 1994; Matsuno et al., 1995). Once in the nucleus the ICD-Deltex complex binds to and transactivates the transcriptional repressor C promoter Binding Factor-1 (CBF-1)/Recombination signal Binding Protein-J kappa (RBPJk) resulting in the activation of CBF-1/RBPJk-regulated genes, namely Hairy and Enhancer of Split (HES)-1 (Jarriault et al., 1995), which is a family of basic helix-loop-helix (bHLH) transcription factors, which in turn regulate the expression of other genes concerned with proliferation, differentiation and apoptosis. The mechanism of transactivation whereby CBF-1/RBPJk switches from a function of transcriptional repression to one of transcriptional activation is not understood but may involve the mastermind-like gene MAML-1 (Wu et al., 2000).
Figure 1.4.3. **Notch signalling pathway.** Notch activation, caused by fringe assisted ligand association results in the proteolytic cleavage of the ICD by presenilin. ICD is translocated to the nucleus, facilitated by Deltex, where it binds with a site-specific DNA binding transcriptional protein CBF-1/RBPJk, which in turn upregulates the expression of a variety of genes e.g. Hes genes. In this manner Notch can inhibit differentiation and so be permissive to continued proliferation. Notch activation can be downregulated by the association with the protein Numb. Abbreviations: DSL, Delta/Serrate/Lag2 domain, TM, transmembrane domain; ICD, intracellular domain; CBF-1, C promoter Binding Factor-1.
The activation of Notch signalling through its ligands is indispensable, as studies using knockout mice have demonstrated embryonic lethality following deletion of Notch family genes (reviewed Kojika and Griffin, 2001) and furthermore forms a pivotal regulator of neurone proliferation and differentiation. Notch activation is in general non-permissive of precursor differentiation and functions through lateral inhibition (Heitzler et al., 1996), a process by which two identical cells can be induced in vivo to adopt different developmental fates (Artavanis-Tsakonas et al., 1999). The Notch signalling pathway can be inhibited by the binding of a membrane-associated protein, called Numb, to the cleaved ICD (Guo et al., 1996), thereby preventing its nuclear translocation (Frise et al., 1996). Inhibition of Notch signalling in this way may act to control neuronal differentiation and proliferation possibly by the process of lateral inhibition (Verdi et al., 1996).

Notch can also be activated in a manner not reliant on DSL ligand binding, but through an interaction with Wnt signalling (Anderton et al., 2000; De Strooper and Annaert, 2001). Wnt signalling plays an important role in cell fate decisions and pattern formation via a family of membrane receptors known as frizzled (Cook et al., 1996). Dysregulation of this pathway may be involved in the pathogenesis of Alzheimer's disease (Lovestone et al., 1994; Lovestone and Reynolds, 1997). The Notch and Wnt signalling pathways are thought to converge at separate two points (figure 1.4.3.b.); firstly the extracellular Wnt signalling molecule appears to associate with the ECD of the Notch receptor and trigger the release of an intracellular transcriptional activator without the involvement of ICD cleavage (Wesley and Saez, 2000), and secondly the cleaved ICD appears to interact with downstream components of the Wnt pathway (Axelrod et al., 1996).
Figure 1.4.3.b. Notch and Wnt signalling pathway interactions. Notch and Wnt are believed to be linked at several points. Firstly the extracellular domain of Notch receptor can be activated by Wnt. This causes the release of an intracellular transcriptional activator which is not linked to cleavage of the ICD. Alternatively once the activated ICD, following receptor activation by a DSL ligand, may be able to associate with dishevelled and result in nuclear transcription of Wnt-induced genes. Abbreviations: A, transcriptional activator.
1.4.4. Trk receptor signalling

1.4.4.1. MAPK signalling cascade

The transmission of extracellular signals into intracellular responses can also be modulated by the activity of mitogen-activated protein (MAP) kinases. The activation of a MAP kinase involves a three step cascade of kinases culminating in the activation of the MAP kinase itself, which in turn phosphorylates a variety of intracellular targets including transcription factors, transcriptional adapter proteins, and other protein kinases. Since the discovery of the first MAP kinase system, the Raf-1-MEK1/2-ERK1/2 cascade in the early 1980s, a number of other MAP kinases have been identified including c-Jun N-terminal kinases (JNKs), p38 and ERK5 (figure 1.4.4.1.a).

Figure 1.4.4.1.a. Summary of MAPK signalling cascades. The ERK, JNK and p38 signalling pathways regulated by MEKK family members are illustrated. Dotted arrows indicate presumed coupling (Hagemann and Blank, 2001).

The Raf-1-MEK1/2-ERK1/2 axis is of great interest in the control of cellular proliferation and survival, via the activation of the Trk receptors. The binding of the appropriate ligand to the Trk receptor stimulates homo and/or hetero-dimerization, resulting in increased
Following ligand stimulated activation of the Trk receptor, phosphorylated tyrosine residues on the receptor serve as docking sites for Grb2 which becomes complexed with Sos which leads to the activation of Ras, which thereby recruits Raf-1 to the plasma membrane where it becomes activated. Activated Raf-1 signals via MEK to ERK1/2, which regulates transcription factor expression to control neuronal proliferation and differentiation (adapted from Hagemann and Blank, 2001).
bound conformation through the action of SOS, Ras is able to interact with its downstream effectors including the MAPKKK, Raf-1. Raf-1 next activates MEK1/2, which in turn activates ERK1/2, which controls downstream processes including proliferation, synaptic plasticity and cell differentiation.

1.4.4.2. PI3K/Akt transduction pathway

Cellular survival is an active decision that is monitored continuously and regulated by input signals that promote either survival or programmed cell death, i.e. apoptosis. The phosphatidylinositol 3-kinase (PI3K) pathway represents an important pathway whereby survival signals are transduced from activation of the growth factor receptors and Trk receptors into intracellular signals. One of the major focal points of this pathway is the serine/threonine protein kinase Akt kinase (Akt), (also termed Protein Kinase B (PKB) and Rac (related to the A and C kinases)) which was originally identified by Staal and coworkers (1977; 1987; 1988). A number of different isoforms of Akt have been identified, including PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Coffer and Woodgett, 1991; Jones et al., 1991; Cheng et al., 1992; Konishi et al., 1995) which have been found to be implicated in a number of diverse cellular processes such as glucose metabolism, transcription, apoptosis, proliferation, survival, growth, and migration.

Following ligand mediated activation of the Trk receptor (figure 1.4.4.2.), the enzyme PI3K, originally isolated by the group of Cantley (Carpenter et al., 1990), becomes activated resulting in the generation of membrane phospholipids, phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) and phosphatidyl-inositol-4,5-bisphosphate (PI-4,5-P2)
Figure 1.4.4.2. The PI3K/Akt signalling cascade. Following ligand stimulated activation of the Trk receptor, PI3K becomes activated and promotes the formation of PI-3,4,5-P$_3$ from PI-4,5-P$_2$. This subsequently promotes Akt to become membrane associated. Akt is activated by phosphorylated by PDK1 and interacts with a variety of downstream targets. Akt is deactivated by the action of protein phosphatase e.g. PP2A. PTEN acts as a negative regulator of the pathway, by limiting the availability of phospholipids. **Abbreviations:** BAD, Bcl-2 pro-apoptotic family member; eNOS, endothelial nitric oxide synthase; FKHLR1, forkhead transcription factor family member 1; GSK-3, glycogen synthase kinase-3; NFkB, nuclear factor κB; P, phosphate; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI-3,4,5-P$_3$, phosphatidylinositol-3,4,5-triphosphate; PI3K, phosphatidylinositol 3-kinase; PI-4,5-P$_2$, phosphatidylinositol-4,5-bisphosphate; PP2A, protein phosphatases 2A; PTEN, phosphatase and tensin homologue deleted on chromosome ten; Trk, tyrosine kinase (adapted from Brazil and Hemmings, 2001; Katso et al., 2001; Manning et al., 2002).
Akt is recruited to the plasma membrane following its association with PI-3,4,5-P$_3$ via interaction with its PH (pleckstrin homology) domain (Haslem et al., 1993; Frech et al., 1997). Akt is subsequently activated by phosphorylation on two regulatory sites, Thr-308 and Ser-473. Whilst 3-phosphoinositide-dependent protein kinase-1 (PDK1) has been identified to be the kinase responsible for Thr-308 phosphorylation (Alessi et al., 1997), the mechanism of the Ser-473 phosphorylation remains controversial (Hill et al., 2001). Once fully activated Akt is able to mediate its cellular effects on downstream targets including BAD, GSK3 and members of the Forkhead family (see table 1.4.4.2).

**Table 1.4.4.2. Examples of Akt cellular substrates.** Substrates of Akt are involved in a number of processes including apoptosis, metabolism and many other varied processes. Abbreviations: BAD, Bcl-2 pro-apoptotic family member; CBP, cAMP binding protein; CREB, cAMP responsive element binding protein; eNOS, endothelial nitric oxide synthase; FKhRL1, forkhead transcription factor family member 1; GDK-3, glycogen synthase kinase-3; I-kB, inhibitor of NFkB; NFkB, nuclear factor xB; NO, nitric oxide (adapted from Brazil and Hammings, 2001).

<table>
<thead>
<tr>
<th>Akt substrates</th>
<th>Effect of phosphorylation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAD</td>
<td>blocks BAD-induced apoptosis</td>
<td>del Peso et al., 1997; Datta et al., 1997</td>
</tr>
<tr>
<td>eNOS</td>
<td>activates eNOS and leads to NO production</td>
<td>Dimmeler et al., 1999</td>
</tr>
<tr>
<td>FKhRL1</td>
<td>inhibits transcriptional activity of FKhRL1</td>
<td>Brunet et al., 1999</td>
</tr>
<tr>
<td>CREB</td>
<td>increased association with CBP and p130</td>
<td>Du and Montminy, 1998</td>
</tr>
<tr>
<td>GSK-3</td>
<td>inactivates GSK-3 activity</td>
<td>Cross et al., 1995</td>
</tr>
<tr>
<td>I-kB kinase</td>
<td>activates transcriptional activity of NFkB</td>
<td>Kane et al., 1999</td>
</tr>
</tbody>
</table>

The activity of Akt can be controlled by reversible phosphorylation on both Ser-473 and Thr-308 residues, and indeed inactivation is mediated by protein phosphatases inhibitors,
e.g. c (PP2A) (Andjelkovic et al., 1996). Furthermore the activity of Akt can be regulated by limiting the lipid substrate PI-3,4,5-P$_3$ necessary for its activation, a process regulated by a tumor suppressor protein termed PTEN (phosphatase and tensin homologue deleted on chromosome ten) (Stambolic et al., 1998; Cantley and Neel, 1999). Mutations in PTEN, which render it inactive, allow Akt activity to go unchecked and therefore results in uncontrolled proliferation commonly associated with cancer (Haas-Kongan et al., 1998).

1.4.5. **Sonic Hedgehog signal transduction pathway**

Sonic hedgehog (Shh), a member of the Hedgehog (Hh) family of secreted proteins, carries out a diverse range of functions during brain development, including neural tube patterning (Ericson et al., 1997; Briscoe et al., 2000; Patten and Placzek, 2000; Briscoe et al., 2001), precursor cell proliferation (Rowitch et al., 1999; Wechsler-Reya and Scott, 1999), and neuronal and glial survival (Ahlgren and Bronner-Fraser, 1999; Britto et al., 2000; Davies and Miller, 2001; Rangarajan et al., 2001), and restraining growth in the adult brain (Goodrich and Scott, 1998; Charytoniuk et al., 2002). These responses are controlled by regulating the production and amount of Shh protein (Ho and Scott, 2002).

Shh acts through the Patched-1 (Ptc1)-Smoothened (Smo) receptor complex, resulting in the activation of an intricate signalling pathway (figure 1.4.5.), the full details of which are still to be determined, but involves PKA activity (Hynes et al., 1997; Lee et al., 1997; Ruiz i Altaba, 1998, 1999a, 1999b). As a result inhibition of Shh responses can be achieved through increase in cAMP and PKA activity (Hynes et al., 1995; Concordet et al., 1996;
Epstein et al., 1996; Ungar and Moon, 1996). To date, only one factor capable of enhancing the Shh has been identified, the chemokine SDF-1α (Klein et al., 2001), which functions in a PTX sensitive manner, suggesting receptor coupling to a Gα-protein.

**Figure 1.4.5. The Shh signalling cascade.** In the absence of Sonic hedgehog (Shh), Patched 1 (Ptc1) inhibits smoothened (Smo), thereby inhibiting the downstream transduction cascade. On Shh binding to the Ptc1-Smo receptor this inhibition is released and the downstream transduction cascade is initiated (adapted from Klein et al., 2001; Ruiz i Altaba et al., 2002; Ho and Scott, 2002).

Regulation and maintenance of the Shh pathway is of utmost importance for normal brain function. In the adult brain Shh is postulated to play a survival role and as such may function in repair mechanisms after brain injury (Pepinsky et al., 2002). Therefore, the ability to control this pathway by altering the pharmacokinetic properties of Shh may provide a window of opportunity in therapeutic treatment. Therefore the factors able to alter the dynamic properties of Shh need to be identified and it appears likely that growth factors may have a part to play (Pirskanen et al., 2000; Wada and Nohno, 2001; Alvarez et al., 2002; Reilly et al., 2002).
1.5. **Aims of thesis: Microglial effects on neuronal development**

Microglia release a wide variety of growth factors and cytokines (see 1.2.5.) which are capable of directly affecting the development of the surrounding neurones. Over the past decade the functions of microglia have been found to go beyond their classical portrayal as neurotoxic cells and it appears more plausible that microglia can also exert neurotrophic effects upon the CNS, and may enable neurones to recover from injury and regenerate (Kreutzberg, 1996). Indeed several investigators have reported beneficial effects of microglial-derived diffusible factors on neuronal survival (Nagata et al., 1993; Zhang and Fedoroff, 1996; Cunningham et al., 1998; Zietlow et al., 1999) though little data has been presented as to the mechanism(s) of action. Many of the survival pathways, as discussed, are not solely controlled by ligand binding but also by extracellular factors acting as co-stimulators. This therefore highlights the probability that growth factors and cytokines in the CNS may have functions which also contribute to maintaining and/or upregulating receptor expression. The aim of this thesis was to further explore the effects that microglia have on the growth and survival of developing neurones, *in vitro*, by investigating the effects of microglial-derived diffusible factors and their mechanisms of action.
2. MATERIALS AND METHODS
2.1. MATERIALS

Wistar rats were bred and reared in-house from stock animals obtained from Charles River UK Ltd (Kent, UK). Foetal calf serum, Earle’s balanced salts solution (EBSS), minimum essential medium with Earl’s salt, Neurobasal-A medium and G5 media supplement were obtained from Life Technologies Ltd (Paisley, UK). Glass coverslips, Whatmann filter paper and tissue culture plasticware was obtained from Scientific Laboratory Supplies Ltd (Nottingham, UK). Phosphate-buffered saline (PBS) powder was from ICN Biomedicals Inc (Maidenhead, UK). Solutions used for FACS analysis were from BD BioSciences (Oxford, UK). The ED1 (MCA341), CD11b (MRC OX-42), CD11b-RPE, CD45-FITC and ED1-FITC antibodies were from Serotec (Oxford, UK). The Boehringer Mannheim L-glutamate assay kit was purchased from Digen Ltd (Oxford, UK). The ‘Cell Titer 96 AQeous One Solution Cell Proliferation assay’ and MAPK inhibitors, U0126 and PD98059, were purchased from Promega (Southampton, UK). The BrdU, nNOS, iNOS and nestin antibodies were from BD PharMingen (Oxford, UK). Vimentin, Ki-67 and all biotinylated secondary antibodies were from Dako (Ely, UK). Protein A/G agarose beads and also Delta1, p-ERK, p53, TrkB, TrkC, TGFβ1, TGFβ3, EGF, bFGF antibodies and all horse radish peroxidase (HRP) secondary antibodies were purchased from Autogen BioClear (Wilt, UK). MAP2 antibody was purchased from TCS Biological Ltd (Bucks, UK). PDGF antibody was obtained from CN Biosciences UK, Ltd (Notts, UK). Cleaved notch1, Akt, pAkt, p44/42 MAPK and TrkA antibodies were purchased from New England BioLabs UK Ltd (Herts, UK). Avidin-biotin complex and Vectorshield mounting medium was from Vector Laboratories Ltd (Peterborough, UK). All mGlu receptor agonists and antagonists were purchased from Tocris Cookson (Bristol, UK). The Pall Gellman NANOSEP microconcentrators and syringe
filters were obtained from Pall Europe Ltd (Portsmouth, UK). Bradford protein assay reagent kit was obtained from Perbio Science UK (Cheshire, UK). Pre-cast Tris-HCl gels and 2D SDS PAGE molecular weight standards were purchased from BioRad (Herts, UK). DPX mountant, formaldehyde, perchloric acid and methanol were purchased from VWR International (Leics, UK). Dialysis tubing was obtained from Medicell (London, UK). Immobiline Dry Strip immobilized pH gradient (IPG) gels were obtained from Amersham BioSciences (Bucks, UK). Ampholytes were purchased from Pharmacia Biotech Ab (Sweden). Re-Blot, stripping reagent, was from Chemicon International Ltd (Harrow, UK). The enhance chemiluminescence (ECL) substrate solutions were prepared in-house using chemicals purchased from Sigma (Dorset, UK). The percoll and the GAP-43, GFAP, β-tubulin and β-actin antibodies, Bandeiraea simplicifolia isolectin B4, Immobilon PDVF membrane and the coloured molecular weight markers, fluorescein diacetate (FDA), propidium iodide (PI) and bisbenzimide trihydrochloride (Hoechst 33342), along with all other chemicals, were purchased from Sigma (Dorset, UK).
2.2. METHODS

2.2.1. Cell culture

2.2.1.1. Cerebellar granule neurone culture

Primary cultures of cerebellar granule neurones (CGCs) were isolated from 3-5 day old Wistar rat pups and prepared as previously described (Pocock et al., 1993). The rat pups were killed by carbon dioxide induced anoxia followed by decapitation, in accordance with the Scientific Procedures Act 1986.

Table 2.2.1.1. Solutions used in CGC culture

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>phosphate buffered saline (PBS), 0.3 % fatty acid free bovine serum albumin (FAF-BSA), 10 mM glucose, 1.5 mM MgSO₄·7H₂O</td>
</tr>
<tr>
<td>Solution B</td>
<td>16 U/ml DNase, 0.24 mM MgSO₄·7H₂O, 8 μg/ml soybean trypsin inhibitor (SBTI) dissolved in solution A</td>
</tr>
<tr>
<td>Solution C</td>
<td>100 U/ml DNase, 50 μg/ml SBTI, 1.5 mM MgSO₄·7H₂O dissolved in solution A</td>
</tr>
<tr>
<td>Solution D</td>
<td>Earle’s balanced salts solution (EBSS) with 26 mM NaHCO₃, 3 mM MgSO₄·7H₂O, 4 % FAF-BSA</td>
</tr>
<tr>
<td>Media</td>
<td>Minimum Essential Medium (MEM) with Earle’s salts supplemented with 25 mM KCl, 30 mM glucose, 25 mM NaHCO₃, 1 mM glutamine, 10 % heat inactivated foetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 6 μg/ml ampicillin</td>
</tr>
</tbody>
</table>

Briefly, the cerebella were removed into filter-sterilised solution A and the tissue was chopped using a flame-sterilised blade. The chopped tissue was then enzymatically digested, using 0.5 mg/ml trypsin dissolved in solution A, for 20 min at 37 °C, followed by the addition of solution B. The DNase in solution B prevents the suspension becoming gelatinous and the SBTI inhibits any further action of the trypsin thereby allowing the continued processing of the sample. The solution was mixed and centrifuged at 500 g for 5 min. The loose pellet was resuspended in 2 ml of solution C
using sterile, flame polished (to prevent cell adherence) glass pasteur pipettes of decreasing bore size, until smooth. The suspension was then pipetted on top of solution D and centrifuged at approximately 500 g for 5 min. The pellet was resuspended in medium and cell number counted using a haemocytometer. Approximately 8 x 10^6 cells were harvested from each rat pup. The volume was then adjusted to give an end cell plating density of 0.6 - 0.75 x 10^6 neurones/coverstlip on 13 mm poly-D-lysine coated coverslips. After 24 h in vitro the medium was changed to cytosine furanoarabinoside (Ara-C) supplemented medium (10μM) to prevent the proliferation of non-neuronal cells. The cultures were maintained at 37 °C in 5 % CO₂. Cells were routinely used after 7 days in vitro (DIV) unless otherwise indicated.

The coated coverslips were prepared from sterile glass coverslips which had been sterilised by soaking in 100 % ethanol for 4 days, followed by baking at 250 °C for a further 4 days. These sterile coverslips were then added to filter-sterilised poly-D-lysine solution (15 mg/L) and incubated for 1 hour at 37 °C, with frequent gentle inversion to ensure even coating. The coverslips were separated and allowed to air dry in a sterile hood before being put in the wells of a 24 well plate.

2.2.1.2. Microglial cell culture

Primary cultures of microglial cells were isolated from 3-5 day old Wistar rat pups and prepared as previously described (Kingham and Pocock, 2001). The rat pups were killed by carbon dioxide induced anoxia followed by decapitation, in accordance with the Scientific Procedures Act 1986.
Table 2.2.1.2 Solutions used in microglial culture

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x PBS</td>
<td>1.4 M NaCl, 54 mM KCl, 30 mM NaH₂PO₄·2H₂O, 260 mM Na₂HPO₄, 110 mM D (+) glucose, 0.2% fatty acid free-BSA, pH 7.4 (NaOH / HCl)</td>
</tr>
<tr>
<td>1 x PBS</td>
<td>1:9 (v/v) 10 x PBS ; distilled H₂O</td>
</tr>
<tr>
<td>Isotonic Percoll</td>
<td>9:1 (v/v) percoll : 10 x PBS</td>
</tr>
<tr>
<td>Media</td>
<td>Minimum Essential Medium (MEM) with Earle's salts supplemented with 25 mM KCl, 30 mM glucose, 25 mM NaHCO₃, 1 mM glutamine, 10 % heat inactivated foetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 6 µg/ml ampicillin</td>
</tr>
</tbody>
</table>

Briefly, brains (minus cerebellum) were removed into ice cold PBS and homogenised. The homogenate was centrifuged at 500 g and the pellet resuspended in 10 ml 70 % isotonic percoll diluted with PBS. This was overlaid with 10 ml 35% isotonic percoll, followed by 10 ml 1 x PBS. The gradient was centrifuged at 1250 g for 45 min at 20 °C. The cells were collected from the 35 / 70 % interface and washed once in PBS at 500 g for 10 min (figure 2.2.1.2.).

Figure 2.2.1.2 Schematic of percoll gradient appearance following centrifugation. Following centrifugation the mixed cells separate to form distinct layers, red blood cell collect at the base, myelin and other debris at the 35 % / PBS interface and microglia at the 35 / 70 % interface.

After washing the pellet was resuspended in medium and cell number counted using a haemocytometer. Approximately 1 x 10⁶ microglial cells were harvested from each rat pup. The volume was then adjusted to give an end cell plating density of 0.6 x 10⁶
microglial cells per ml and plated onto sterile, uncoated 13 mm coverslips. Approximately 1 hour after plating the medium was changed. The cultures were maintained at 37 °C in 5 % CO₂. Cells were routinely used after 1 or 7 days in vitro.

2.2.1.3. Peritoneal macrophage culture

Primary cultures of peritoneal macrophages (PMϕ) were collected from adult rats as previously described (Soares et al., 1998; Gjomarkaj et al., 1999; Wang et al., 2000). The rats were killed by carbon dioxide induced anoxia followed by decapitation, in accordance with the Scientific Procedures Act 1986.

Briefly the peritoneal cavity was lavaged with 25 ml of filter sterilised Earle’s Balanced Salt Solution (EBSS). Following centrifugation at 500 g for 10 mins at room temperature, the cells were resuspended in culture medium and counted. Approximately 15 x 10⁶ peritoneal cells were harvested from each adult rat. The volume was then adjusted to give an end cell plating density of 0.6 x 10⁶ cells per ml and plated onto sterile coverslips. Approximately 1 hour after plating the medium was changed. The cultures were maintained at 37 °C in 5 % CO₂.

<table>
<thead>
<tr>
<th>Table 2.2.1.3.</th>
<th>Solutions used in peritoneal macrophage culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>Minimum Essential Medium (MEM) with Earle’s salts supplemented with 25 mM KCl, 30 mM glucose, 25 mM NaHCO₃, 1 mM glutamine, 10 % heat inactivated foetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 6 μg/ml ampicillin</td>
</tr>
</tbody>
</table>
2.2.2. Analysis of primary cultures using flow cytometry

Cell surface markers on the microglial cells were compared with those present on peritoneal macrophages using a Becton Dickinson Immunocytometry Systems (BDIS) FACSCalibur modular flow cytometer.

A flow cytometer measures and analyses the optical properties of single cells passing through a focused laser beam. As cells pass through the laser beam they disrupt and scatter the laser light, which is detected as forward and side scattered light. Forward scatter (FSC) light is related to the size of the cell and side scatter (SSC) light is an indication of a cell's internal complexity. In addition to scatter, a cytometer also measures fluorescence parameters. Cells can be stained with fluorescent dyes or fluorochromes that are coupled to antibodies directed against specific cell surface antigens. The fluorochromes absorb the laser light and emit a portion of this light in different regions of the spectrum. The cytometer measures the relative amounts of each dye on an individual cell, generating data about the molecular properties of the cells. Analysis is performed on many hundreds of cells within a sample, providing statistically significant information of the physical and biochemical composition.

The argon-ion laser produces light at a wavelength of 488 nm (see figure 2.2.2.), with a beam which is both large enough to fully illuminate the cells and provide uniform excitation across the sample stream. The laser is directed through a focusing lens onto the cell. The forward scatter (FSC) signal is collected by the forward scatter diode. The side scatter (SSC) and fluorescence parameters are gathered by the collection lens, which is positioned at 90°, and are subsequently focused into a series of optical filters and spectrally split by a collection of dichroic mirrors (DM) and filters as indicated.
Green/yellow light passes to the FL1 photomultiplier tube (PMT), i.e. those cells labelled with fluorescein isothiocyanate (FITC) conjugate. Red light passes to the FL3 PMT, i.e. cells labelled with peridinin chlorophyll protein (PerCP), whilst yellow and orange light is reflected to the FL2 PMT, i.e. cells labelled with R. Phycoerythrin (RPE) conjugate. A 10 % fraction split of the FL1 signal provides the SSC signal. The optical signals received are finally converted to proportional electronic signals by the PMT, and the data can be analysed using the CELLQUEST software.

**Figure 2.2.2. Schematic representation of the BDIS flow cytometer.** The laser light passes through a focusing lens and is directed onto the cell surface. Resultant emitted light is spectrally split and collected as indicated. Optical signals are finally converted into electronic signals that can be analysed.

Briefly, following their isolation and purification, MG or PMφ cells were cultured for either 1 or 7 days *in vitro*, at a density of $0.6 \times 10^6$ cells/ml, before being retrieved into medium using a cell scraper. Cells were pelleted and resuspended in fix solution (table
2.2.2.a) and incubated for at least 15 mins at room temperature. Samples which were to be analysed using the ED1 antibody were solubilised by the addition of 0.1 % triton into the fixative and incubated for 30 min at room temperature. Following fixing/solubilisation the cells were pelleted and resuspended in an appropriate primary antibody (table 2.2.2.b.) and incubated for 1 hr at room temperature. Following appropriate antibody treatments, cells were pelleted and resuspended in sheath fluid (Becton Dickinson), an optimised PBS solution and analysed by flow cytometry according to the manufacturers instructions.

<table>
<thead>
<tr>
<th>Table 2.2.2.a.</th>
<th>Solutions used for FACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>fix</td>
<td>8 % formaldehyde in PBS; 140 mM NaCl, 5.4 mM KCl, 3 mM NaH₂PO₄·2H₂O, 26 mM Na₂HPO₄, pH 7.4 (NaOH / HCl)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2.2.2.b.</th>
<th>Antibody specificities, source and dilutions used for FACS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>antibody</td>
<td>details</td>
</tr>
<tr>
<td>mouse anti-rat CD11b(OX-42):RPE</td>
<td>clone MRC OX-42</td>
</tr>
<tr>
<td>mouse anti-rat CD45:FITC</td>
<td>clone MRC OX-1</td>
</tr>
<tr>
<td>mouse anti-rat ED1:FITC</td>
<td>clone ED1</td>
</tr>
</tbody>
</table>

2.2.3. Preparation of microglial-conditioned medium

Microglia were used at two different stages; 1 day in vitro and 7 days in vitro. To obtain microglial conditioned medium (MG-CM), supernatant from the cell culture wells was collected after 1 day in vitro (1 DIV MG-CM) or 7 days in vitro (7 DIV MG-CM), centrifuged at 10,000 g to remove contaminating cellular material and stored sterile at -20 °C before being subsequently transferred to CGCs.
2.2.4. **Immunoprecipitation of individual factors from MG-CM**

Individual growth factors were immunodepleted according to the method of Zhao and Eghbali-Webb (2001). Briefly aliquots (600 μl) of either control media or 7 DIV MG-CM were incubated with 1 mg of each specific antibody (table 2.2.4.) and 30 μl of Protein A/G-agarose (Autogen Bioclear, Wilts, UK) at 4 °C for 16 hr with constant rotation. The immunocomplex was removed by centrifugation at 10,000 rpm for 1 min, the supernatant was collected and stored at -20 °C prior to use.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>details</th>
<th>source</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit anti-PDGF</td>
<td>polyclonal, clone Ab-1</td>
<td>CN BioSciences, UK</td>
<td>PC21</td>
</tr>
<tr>
<td>rabbit anti-TGFβ1</td>
<td>polyclonal, clone V</td>
<td>Autogen Bioclear, UK</td>
<td>Sc-146</td>
</tr>
<tr>
<td>rabbit anti-TGFβ3</td>
<td>polyclonal, clone III</td>
<td>Autogen Bioclear, UK</td>
<td>Sc-83</td>
</tr>
<tr>
<td>goat anti-rat EGF</td>
<td>polyclonal, clone R-20</td>
<td>Autogen Bioclear, UK</td>
<td>Sc-1343</td>
</tr>
</tbody>
</table>

2.2.5. **Preparation of serum free microglial-conditioned medium**

Conditioned media was collected from microglial cultures grown in a serum free, defined media, Neurobasal-A medium supplemented with G5 (Life Technologies, UK; for composition see table 2.2.4.) (SFM). Following the standard 1 hour adhesion step, culture medium was replaced with SFM. The conditioned media was collected as previously described after 1 day *in vitro* (1 DIV SFM-CM) or 7 days *in vitro* (7 DIV SFM-CM)
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>Transferrin</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Selenite</td>
<td>5.2 ng/ml</td>
</tr>
<tr>
<td>Biotin</td>
<td>10 ng/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>3.6 ng/ml</td>
</tr>
<tr>
<td>FGF</td>
<td>5.2 ng/ml</td>
</tr>
<tr>
<td>EGF</td>
<td>10 ng/ml</td>
</tr>
</tbody>
</table>

### 2.2.6. Depletion of microglia in microglial cell cultures

To ascertain that the effects of the MG-CM on the neuronal cultures were due to the microglia and not as a result of other cell types possibly present in the microglial cultures, microglia were selectively depleted using L-leucine methyl ester (LME) (Brown et al., 1996; Vincent et al., 1997). The selective toxicity of LME is dependent on its intracellular conversion to membranolytic metabolites by the acyl transferase activity of dipeptidyl peptidase I (DPPI) which is enriched in these cells (Thiele et al., 1983; Thiele and Lipsky, 1990, 1992).

Briefly, microglial cultures were incubated with 10 mM LME following the 1 hour adhesion step. After 24 hours the medium was removed and replaced with fresh culture medium. The conditioned medium (LME-CM) was collected as previously described after a further 1 or 7 days *in vitro*.
2.2.7. Preparation of peritoneal macrophage-conditioned medium

Peritoneal macrophages were used at two different stages; 1 day \textit{in vitro} and 7 days \textit{in vitro}. To obtain peritoneal macrophage-conditioned medium (PM-CM), supernatant from the cell culture wells was collected as previously described after 1 day \textit{in vitro} (1 DIV PM-CM) or 7 days \textit{in vitro} (7 DIV PM-CM).

2.2.8. Experimental Procedure

Conditioned medium from non-stimulated microglial cultures was added to primary CGC cultures as depicted (figure 2.2.8.). Briefly 250 µl of medium was removed from each well containing the 7 day \textit{in vitro} neurones and replaced with the appropriate conditioned medium. Controls were set up in which either (a) 250 µl of fresh CGC medium or (b) 250 µl of fresh microglial medium was added. It should be noted that the microglial medium is identical to the CGC medium but lacks the AraC, which halts the proliferation of non-neuronal cells. The neurones were then cultured for a further 7 days, during which time cell viability and proliferation were assessed.
Figure 2.2.8. Schematic of the experimental method. Microglia were isolated and cultured supernatant retrieved after 1 or 7 days. Cerebellar granule neurones were prepared and cultured for 7 days. After 7 days in vitro, 250 μl of media was removed from each well of the neurones and replaced with the appropriate conditioned medium. The neurones were then cultured for a further 7 days during which cell survival and proliferation was assessed.

2.2.9. Immunocytochemical analysis of culture composition

Immunolocalisation was used to assess the composition of both the microglial and cerebellar granule neurone cultures. Antibodies against specific cell markers were utilised, followed by incubation with a biotinylated secondary antibody and detected using an avidin-biotin peroxidase method, which amplifies the signal obtained from primary antibody binding. Staining was visualised using DAB, which results in a brown precipitate observable using light microscopy.
Table 2.2.9.a. Solutions used for immunocytochemical analysis

<table>
<thead>
<tr>
<th>Fix</th>
<th>100 % ice cold methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>137 mM NaCl, 5.4 mM KCl, 2.9 mM NaH_{2}PO_{4},2H_{2}O, 25.8 mM Na_{2}HPO_{4},</td>
</tr>
<tr>
<td>DAB</td>
<td>0.7 mg/ml DAB, 0.2 mg/ml urea hydrogen peroxide, 0.06 M tris buffer in 5 ml distilled H_{2}O</td>
</tr>
<tr>
<td>Dehydration</td>
<td>45 sec 90% ethanol, 45 sec 100 % ethanol, 45 sec 100 % ethanol, 45 sec 100 % xylene</td>
</tr>
<tr>
<td>Carbonate buffer</td>
<td>100 mM NaHCO_{3}, 150 mM NaCl; pH 8.5 adjusted with 100 M Na_{2}CO_{3}</td>
</tr>
</tbody>
</table>

Table 2.2.9.b. Antibody specificities, source and dilutions used for immunocytochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Details</th>
<th>Source</th>
<th>Catalogue number</th>
<th>Primary antibody dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-CD11b</td>
<td>monoclonal, clone MRC OX42</td>
<td>Serotec</td>
<td>MCA275G</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse anti-ED1</td>
<td>monoclonal, clone ED1</td>
<td>Serotec</td>
<td>MCA341B</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-GFAP</td>
<td>monoclonal, clone GA5</td>
<td>Sigma</td>
<td>G3893</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-vimentin</td>
<td>monoclonal, clone Vim3B4</td>
<td>DAKO</td>
<td>M7020</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-Ki-67</td>
<td>monoclonal, clone MIB-5</td>
<td>DAKO</td>
<td>M7248</td>
<td>1:50</td>
</tr>
</tbody>
</table>

Briefly, following treatment, the cells were fixed for at least 10 min at -20 °C. Following washing 3 times in PBS, the cells were blocked using normal horse serum diluted at 1:40 in PBS (PBS-NHS) and incubated for 30 min at room temperature. This was followed by the addition of 50 μl of the appropriately diluted primary antibody (table 2.2.8.b.) in PBS-NHS and incubated overnight at 4 °C. Cells were washed 3 times with PBS prior to the application of the anti-mouse biotinylated secondary antibody, diluted at 1:200 in PBS-NHS. Following 1 hr incubation, the cells were washed in PBS and incubated for 1 hr with avidin:biotinylated enzyme complex (ABC). The ABC was diluted according to the manufacturer’s instructions 30 min prior to addition. Following 3 further PBS washes the cells were stained with 3,3-
diaminobenzidine tetrahydrochloride peroxidase substrate (DAB; Sigma Fast™ DAB tablet), washed in distilled H₂O, dehydrated in ethanol and xylene and mounted.

In some experiments following the final wash in distilled H₂O the cells were incubated with Bandeiraea simplicifolia isolectin B4, diluted at 1:100 in carbonate buffer, for 1 hour at room temperature. Following 3 washes in PBS the cells were immersed in a 1:1000 solution of 4,6-diamidino-2-phenylindole (DAPI) for 10 seconds to stain all cell nuclei. The cells were washed finally three times with PBS and mounted using Vectorshield mounting medium.

2.2.10. Assessment of cell viability
2.2.10.1. Live-dead assay

Cell viability was assessed by a double stain method using fluorescein-5-maleimide diacetate (FDA) (34 µM) and propidium iodide (PI) (6 µM) (Bonfocco et al., 1996; Kingham et al., 1999).

The principle of the assay is that live cells have intact plasma membranes, whereas dead cells have a permeable membrane. Only live cells are able to take up and retain fluorescein, a fluorescent cell permeant dye which is converted into an intense green fluorescence (530 nm fluorescence) upon entry into the cell. However dead cells, with permeable membranes, allow entry of propidium, which then binds to nucleic acids and produces a bright red fluorescence (> 600 nm fluorescence). The numbers of live cells, FDA-positive, and number of dead cells, PI-positive, were scored using a fluorescence microscope, at a magnification of 40 times. Approximately 5 - 10 fields
of view per coverslip were scored and 2 - 3 coverslips per treatment were assessed. This was repeated on at least 3 different cultures.

2.2.10.2. **Hoechst staining**

The number of apoptotic cells was assessed by 2'-[4-Ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazol (Hoechst 33342) staining (Yan et al., 1994; Kingham et al., 1999).

Briefly, cells were fixed in phosphate buffered saline with 8 % formaldehyde for 10 min at 4 °C, and then stained with Hoechst 33342 (5 mg/ml) for 10 - 15 min. The morphology of the nucleus was viewed using a fluorescence microscope with excitation at 365 nm and emission at > 490 nm. Apoptotic cells contained small, brightly stained pyknotic nuclei when compared with control non-pyknotic nuclei. The number of apoptotic and non-apoptotic cell were scored using an Olympus IX70 inverted fluorescence microscope. Five fields of view per coverslip were scored and 3 coverslips per treatment were assessed. This was repeated on at least 3 separate cultures.

2.2.11. **'CellTiter 96 Aqueous One Solution Cell Proliferation Assay'**

The number of proliferating cells was determined using a commercially available colorimetric assay “CellTiter 96 Aqueous One Solution Cell Proliferation Assay” (Cell Titer Assay; Promega).

The assay uses a tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophen-yl)-2H-tetrazolium inner salt; MTS], which is
bioreduced by proliferating cells into a coloured formazan product that is soluble in tissue culture medium. The presence of an electron coupling reagent (phenazine ethosulfate; PES) maintains the stability of the MTS in solution. The quantity of formazan can be monitored spectrophotometrically by measuring the absorbance at 492 nm and is directly proportional to the number of living cells in culture. Proliferating cells reduce the MTS compound to the coloured formazan end product at a greater rate than quiescent cells. This increased rate of reduction is attributable to changes in the intracellular environment; specifically the altered ratios of reduced to oxidized metabolic intermediates, e.g. NADPH/NADP and NADH/NAD, in proliferating cells. The MTS compound is able to accept electrons from these reduced metabolic intermediates.

![MTS and Formazan Structures](image)

**Figure 2.2.11. Structures of MTS tetrazolium and its formazan product.** MTS is bioreduced by proliferating cells into a coloured formazan end product with absorbance spectrophotometrically measurable at 492 nm.

The kit was used according to manufacturers instructions. Briefly, the assays were set up as described. After 20 hours the media volume on the cells was reduced from 500 μl to 250 μl and 50 μl of the Cell Titer Assay reagent was added. The cells were incubated for 4 hours at 37 °C in a humidified, 5 % CO₂ atmosphere, after which time
a 50 μl sample was removed and the absorbance recorded at 492 nm using a 96 well plate Anthos HTll microplate absorption photometer reader. The reaction was stopped by the addition of 12.5 μl 10 % SDS to each well, and the absorbance reread.

As there is a slight amount of spontaneous 492 nm absorbance with culture medium incubated with Cell Titer Assay reagent controls were set up. The control wells contained the same volume of culture media, which had not been in contact with cells, and Cell Titer Assay reagent as in the experimental wells. The average 492 nm absorbance from the “no cell” control wells was subtracted from all other absorbance values to yield corrected absorbances.

2.2.12. Measurement of glutamate content

The determination of glutamate levels in the supernatants was carried out using a colorimetric method coupled to glutamate dehydrogenase and a formazan end product using a commercially available kit (Boehringer Mannheim).

The principle of the assay is that L-glutamate is deaminated oxidatively by nicotinamide-adenine dinucleotide (NAD) to 2-oxoglutarate in the presence of the enzyme glutamate dehydrogenase (GDH).

L-glutamate + NAD$^+$ + H$_2$O $\overset{GDH}{\longrightarrow}$ 2-oxoglutarate + NADH + NH$_4^+$

In the further reaction catalysed by diaphorase, the NADH formed converts iodonitrotetrazolium chloride (INT) to a coloured formazan end product, the absorbance of which measured at 492 nm.

NADPH + INT + H$^+$ $\overset{diaphorase}{\longrightarrow}$ NAD$^+$ + formazan
The equilibrium of the first reaction lies far on the side of L-glutamate. Therefore by trapping the formed NADH with INT, the equilibrium is displaced in favour of 2-oxoglutarate.

Briefly, diaphorase (0.032 U), NAD (0.22 mg/ml), INT solution and sample supernatant were combined in a 96-well plate and incubated for 1 min, followed by the addition of 4 U of glutamate dehydrogenase solution. The absorbance was measured at 450 nm every 3 minutes, using an Anthos HTII microplate absorption photometer, until the reaction reached a steady state. Medium that had not been exposed to cells was used as a blank. A standard curve was constructed by adding known amounts of a glutamate standard to culture medium (figure 2.2.13.).

**Figure 2.2.12.** Representative standard curve of glutamate concentration against optical density. Glutamate standards (0-3 mM) were treated as described and the optical density at 450 nm measured. The reaction was found to reach steady state after 30 min and a linear standard curve was produced.
2.2.13. Measurement of nitrite and total nitrite content

The determination of nitrite in the supernatants was carried out employing a colorimetric detection with Griess reagent. The principle of this assay requires the formation of a chromophore from a diazotization of sulfanilamide by acidic nitrite, followed by coupling with the bicyclic amine N-(-1-naphthyl)-ethylenediamine (figure 2.2.14.a.).

Figure 2.2.13.a. Summary of Griess reaction (Miranda et al., 2001).

![Diagram of Griess reaction](image)

However, nitric oxide can also be broken down into another stable end product as well as nitrite (NO$_2^-$), namely nitrate (NO$_3^-$).

\[
4\text{NO}^- + \text{O}_2 \rightarrow 4\text{NO}_2^- \xrightarrow{\text{H}_2\text{O}} \text{NO}_2^- + 3\text{NO}_3^- + 2\text{H}^+ \]

Nitrate can be stoichometrically reduced to nitrite by an enzymatic method, using nitrate reductase and β-NADPH (Green et al 1982). Lactate dehydrogenase and pyruvate are subsequently added into the system to consume any residual NADPH, as
NADPH interferes with the Griess reaction (Hevel and Marletta, 1994). The treated sample can then be assayed with by Griess reagent to give total nitrite.

Briefly, nitrite (NO$_2^-$) concentration was determined by combining Griess Reagent with an equal volume of supernatant to be examined and incubated for 15 - 30 min in the dark at room temperature (Ding et al., 1988; Kingham et al., 1999). Nitrate was first converted to nitrite, to give total nitrite, before the Griess assay was performed, by incubating the samples with nitrate reductase (0.025 U/ml) and $\beta$-NADPH (100 $\mu$M) for 15 min at 37°C. Lactate dehydrogenase (1111 U/ml) and sodium pyruvate (110 mg/ml) were added prior to the addition of Griess reagent (Green et al., 1982). Medium that had not been exposed to cells was used as a blank. The optical density was measured at 540 nm using an Anthos HTII microplate absorption photometer.

Standard curves using known concentrations of nitrite and nitrate (to give total nitrite) were also constructed (figure 2.2.13.b.). The conversion of nitrate to nitrite was not found to be 100 % hence each standard curve has an independent trendline, conversion was approximately 75 % (see trendlines on figure 2.2.14.b).

**Table 2.2.13. Solutions for nitrite determination**

| Greiss Reagent | 0.1 % (w/v) N-(-1-napthyl)-ethylenediamine, 1 % (w/v) sulphanilamide in 5 % H$_3$PO$_4$ (v/v) |

114
Figure 2.2.13.b. Representative standard curve of nitrite and nitrate following conversion to nitrite. Nitrite standards (squares) were incubated with an equal volume of Griess reagent for 15 min and the optical density recorded at 540 nm. Nitrate standards (circles) were converted to nitrite with nitrate reductase and β-NADPH, and subsequently incubated with Griess reagent to yield the optical density of total nitrite.

2.2.14. Cell lysis and sample preparation

Cells were maintained in 24 well tissue culture plates, experimentally treated and then whole cell lysates were prepared for analysis by SDS-PAGE and Western blotting. Briefly, culture medium was removed and the cells were scraped into 15 µl of ice cold lysis buffer. This was pooled from at least three 13 mm coverslips to give approximately 40 µl of sample. The lysis buffer contained detergents to solubilise proteins and a mixture of protease inhibitors to prevent protein breakdown. The mixture was incubated on ice for 10 min and the nuclei and membranes pelleted by centrifugation at 13,000 g for 10 min. The supernatant, which contained the solubilised proteins, were subsequently assayed for protein.

<table>
<thead>
<tr>
<th>Table 2.2.14. Solutions for cell lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
</tr>
<tr>
<td>1 µM microcystin LR, 1 mM benzamidine, 4 µg/ml leupeptin, 0.1 % β-mercaptoethanol, 20 mM Tris / Ac pH 7, 1 mM EDTA, 1 mM EGTA, 10 mM Na β-glycerophosphate pH 7.4, 1 mM Na-orthovanadate, 5 % glycerol, 1 % Triton X-100, 0.27 M sucrose</td>
</tr>
</tbody>
</table>

115
2.2.15. Preparation of samples for immunoprecipitation

Cells were maintained in 24 well tissue culture plates, experimentally treated and then whole cell lysates were prepared for analysis by immunoprecipitation followed by SDS-PAGE and Western blotting. Briefly, culture medium was removed and the cells were scraped into 15 μl of ice cold RIPA buffer. This was pooled from twelve 13 mm coverslips to give approximately 180 μl of sample. RIPA buffer contains detergents which are able to specifically solubilise cytoskeletal proteins (McDonald et al., 1997) and a mixture of protease inhibitors to prevent protein breakdown. The mixture was incubated on ice for 3 hr, then the nuclei and membranes were pelleted by centrifugation at 13,000 g for 10 min. The supernatant, which contained the solubilised proteins, were subsequently assayed for protein.

Table 2.2.15. Solutions for immunoprecipitation cell lysis

| RIPA buffer | 1 mM benzamidine, 4 μg/ml leupeptin, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris (pH 7.5) |

2.2.16. Bradford protein assay

The protein concentration in cell lysate samples was determined by the technique of Bradford (1976). A standard curve of BSA (2.5 - 15 μg / ml protein) was constructed and 1 μl of sample cell lysate was added to the tubes in duplicate. Bradford reagent (1 ml) was pipetted into the tubes and the solutions were vortexed. After 5 min at room temperature the absorbance was measured at 595 nm against a Bradford reagent blank. Protein concentrations were calculated from the BSA standard curve.
Figure 2.2.16. Representative standard curve of protein concentration against optical density. 1 ml Bradford reagent was added to varying concentrations of BSA and the optical density at 595 nm measured.

2.2.17. Immunoprecipitation

RIPA buffer lysates were precleared by incubation with 20 μl of resuspended volume of Protein A/G-Agarose for 30 min at 4 °C. Samples were centrifuged for 1 min at 10,000 x g and the supernatant was transferred into a fresh tube. To approximately 100 - 500 μg of total cellular protein, 10 μg of primary antibody was added and incubated for 1 hour at 4 °C with end-over-end rotation. Subsequently 20 μl of resuspended volume of Protein A/G-Agarose was added and incubation at 4 °C was continued overnight with rotation. The samples were pelleted by centrifugation at 10,000 x g for 1 min and the pellet was washed 4 times with fresh RIPA buffer, each time repeating the centrifugation step. After the final wash the supernatant was aspirated and discarded and the pellet was resuspended in Laemelli sample buffer. Samples were then processed as under standard SDS-PAGE and Western blotting.
Table 2.2.17. Primary antibody specificities and source used for immunoprecipitation

<table>
<thead>
<tr>
<th>Antibody details</th>
<th>source</th>
<th>catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>goat anti-delta1 polyclonal, clone H-20</td>
<td>Autogen BioClear</td>
<td>Sc-12531</td>
</tr>
<tr>
<td>rabbit anti-cleaved notch 1 polyclonal, clone Val1744</td>
<td>New England BioLabs UK</td>
<td>2421</td>
</tr>
</tbody>
</table>

2.2.18. Denaturing polyacrylamide gel electrophoresis: SDS-PAGE

Proteins in whole cell lysates were separated using SDS-PAGE in a BioRad Mini-Protean II electrophoresis cells. Pre-cast gels (BioRad), as determined appropriate for the molecular weights of the proteins to be separated, were placed into the running apparatus and 45 μg protein was added to Laemmli sample buffer (Laemmli, 1970) and loaded into the wells. Coloured molecular weight markers (Sigma, UK), of an appropriate molecular weight range, were added to Laemmli buffer and run in one lane per gel. The inner and outer chambers were filled with running buffer and the gel was electrophoresed at a constant voltage of 180 V until the dye front had reached the bottom of the gel. The gel was then removed and prepared for Western blotting.

Table 2.2.18. SDS-PAGE Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laemmli sample buffer</td>
<td>2% SDS, 10% glycerol, 2.5% mercaptoethanol, 125 mM Tris / HCl pH 6.8, a few crystals bromophenol blue</td>
</tr>
<tr>
<td>Running buffer</td>
<td>125 mM Tris, pH 8.3, 1 M glycine, 0.01% SDS</td>
</tr>
</tbody>
</table>

2.2.19. Native polyacrylamide gel electrophoresis: PAGE

Conditioned-media samples were separated using native PAGE in a BioRad Mini-Protean II electrophoresis cell. Pre-cast gels (BioRad), as determined appropriate for the molecular weights of the proteins to be separated, were loaded as previously
described. Essentially the same basic protocol as SDS-PAGE was performed, however SDS containing buffers were replaced with non-SDS containing buffers.

<table>
<thead>
<tr>
<th>Table 2.2.19. Native-PAGE solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laemmli sample buffer (native)</td>
</tr>
<tr>
<td>Running buffer</td>
</tr>
</tbody>
</table>

2.2.20. Western blotting

2.2.20.1. Protein transfer

Proteins separated by SDS-PAGE were transferred to PVDF membrane using a BioRad Mini Trans-Blot electrophoretic transfer cell.

The gel was first equilibrated in chilled transfer buffer for 20 min. Meanwhile, the PVDF membrane was activated in methanol for 1 min, washed in distilled H$_2$O for 5 min and incubated in chilled transfer buffer for 15 min. A transfer ‘sandwich’ was constructed by laying a fibre pad on the inside of the transfer cassette, followed by two pieces of Whatman 3MM paper and the gel was laid on top. The activated PVDF membrane was lowered on top of this ensuring no air bubbles were present. This was covered by a further two pieces of Whatman 3MM paper and another fibre pad. The cassette was closed and placed into the holder in the transfer tank. The tank was filled with chilled transfer buffer and an ice pack was inserted to prevent overheating which may affect protein transfer. The samples were then electrophoretically transferred at 80 V for 2 hr or at 22 V overnight.
The sandwich was then disassembled and both the gel and PVDF membrane were washed in Tween 20-Tris buffered saline (TTBS) to remove any residual transfer buffer. The efficiency of the transfer could be assessed by staining the gel with either Coomassie or Ponceau S. The membrane was now ready for immunoblotting.

### Table 2.2.20.1. Solutions for protein transfer

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer buffer</td>
<td>25 mM Tris, 192 mM glycine, 0.01 % SDS, 20 % (v/v) methanol</td>
</tr>
<tr>
<td>TTBS</td>
<td>0.05 % Tween-20, 10 mM Tris / HCl pH 7.4, 150 mM NaCl</td>
</tr>
<tr>
<td>Coomassie</td>
<td>40 % acetic acid, 20 % methanol, 0.004 % coomassie brilliant blue</td>
</tr>
<tr>
<td>Coomassie Destain</td>
<td>50 % methanol, 43 % distilled H₂O, 7 % acetic acid</td>
</tr>
<tr>
<td>Ponceau S</td>
<td>0.5 % Ponceau-S red, 1 % acetic acid</td>
</tr>
<tr>
<td>Ponceau-S Destain</td>
<td>distilled H₂O</td>
</tr>
</tbody>
</table>

### 2.2.20.2. Immunoblotting

The membrane was incubated with blocking buffer for 2 hr at room temperature with constant agitation to minimise any non-specific antibody interactions. This was then replaced with primary antibody diluted to a suitable concentration in blocking buffer for either 2 hr at room temperature or overnight at 4 °C, followed by three 10 min washes in TTBS. An appropriate horse radish peroxidase (HRP)-conjugated secondary antibody was then added to the membrane, again this was diluted to an appropriate concentration in blocking buffer, for 2 hr at room temperature. The immunoblotting procedure was completed by three final 10 min TTBS washes.

The interaction between the primary and the HRP-conjugated secondary antibody was detected using ECL substrate solution. Firstly excess TTBS was removed from the edges of the PVDF membrane with absorbent paper, then the membrane was incubated with 2 ml mixed ECL reagent (1 ml of each reagent) for 60 sec. The membrane was
dried by blotting between two pieces of Whatman 3MM paper and enclosed in Saran wrap. The blot was then affixed in an X-ray film cassette with tape at the corners and exposed to X-ray film for between 1 min - overnight depending on the intensity of the signal.

If membranes needed to be re-probed with another antibody, any previously bound antibodies were removed by incubation in a commercially available stripping reagent (Chemicon International Ltd) for 15 min at room temperature with constant agitation. Following thorough washing in TTBS the membrane was ready to be re-probed.

Table 2.2.20.2.a. Solutions for Immunoblotting

<table>
<thead>
<tr>
<th>Solution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>TTBS with 5 % w/v low fat skimmed milk powder</td>
</tr>
<tr>
<td>ECL reagent 1</td>
<td>2.26 mM luminol, 0.4 % coumaric acid solution (90 mM stock in 100% DMSO) in 0.1 M Tris (pH 8.5) containing 1 % DMSO</td>
</tr>
<tr>
<td>ECL reagent 2</td>
<td>0.06 % H₂O₂ in 0.1 M Tris (pH 8.5)</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>Re-Blot Plus - mild (Chemicon International Ltd)</td>
</tr>
</tbody>
</table>

Table 2.2.20.2.b. Secondary antibody specificities and source used for Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>goat Anti-mouse IgG HRP</td>
<td>Autogen BioClear</td>
<td>Sc-2005</td>
</tr>
<tr>
<td>donkey anti-goat IgG HRP</td>
<td>Autogen BioClear</td>
<td>Sc-2020</td>
</tr>
<tr>
<td>goat anti-rabbit IgG HRP</td>
<td>Autogen BioClear</td>
<td>Sc-2004</td>
</tr>
<tr>
<td>Antibody Details</td>
<td>Source</td>
<td>Catalogue Number</td>
</tr>
<tr>
<td>------------------</td>
<td>--------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Mouse anti-β-actin monoclonal, clone AC-15</td>
<td>Sigma</td>
<td>A5441</td>
</tr>
<tr>
<td>Rabbit anti-Akt polyclonal</td>
<td>New England BioLabs UK</td>
<td>9272</td>
</tr>
<tr>
<td>Rabbit anti-p-Akt polyclonal</td>
<td>New England BioLabs UK</td>
<td>9271S</td>
</tr>
<tr>
<td>Goat anti-delta1* polyclonal, clone (H-20)</td>
<td>Autogen BioClear</td>
<td>Sc-12531</td>
</tr>
<tr>
<td>Mouse anti-p-ERK monoclonal, clone E-4</td>
<td>Autogen BioClear</td>
<td>Sc-7383</td>
</tr>
<tr>
<td>Mouse anti-GAP43 monoclonal, clone GAP-7B10</td>
<td>Sigma</td>
<td>G9264</td>
</tr>
<tr>
<td>Rabbit anti-iNOS polyclonal</td>
<td>BD PharMingen</td>
<td>610333</td>
</tr>
<tr>
<td>Mouse anti-rat Ki-67 monoclonal, clone MIB-5</td>
<td>DAKO, UK</td>
<td>M7248</td>
</tr>
<tr>
<td>Rabbit anti-p44/42 MAP kinase polyclonal</td>
<td>New England BioLabs UK</td>
<td>9102</td>
</tr>
<tr>
<td>Mouse anti-MAP2 monoclonal, clone 5F9</td>
<td>Upstate Biotechnology</td>
<td>05-346</td>
</tr>
<tr>
<td>Mouse anti-rat nestin monoclonal, clone rat 401</td>
<td>BD PharMingen</td>
<td>60051A</td>
</tr>
<tr>
<td>Rabbit anti-nNOS polyclonal</td>
<td>BD PharMingen</td>
<td>N31030</td>
</tr>
<tr>
<td>Rabbit anti-cleaved Notch 1* polyclonal</td>
<td>New England BioLabs UK</td>
<td>2421</td>
</tr>
<tr>
<td>Rabbit anti-TrkA polyclonal</td>
<td>New England BioLabs UK</td>
<td>9142</td>
</tr>
<tr>
<td>Rabbit anti-Trk B polyclonal, clone H-181</td>
<td>Autogen BioClear</td>
<td>Sc-8316</td>
</tr>
<tr>
<td>Rabbit anti-TrkC polyclonal, clone 798</td>
<td>Autogen BioClear</td>
<td>Sc-117</td>
</tr>
<tr>
<td>Mouse anti-β-tubulin monoclonal, clone TUB 2.1</td>
<td>Sigma</td>
<td>T4026</td>
</tr>
</tbody>
</table>

Note: * lysate samples first purified by immunoprecipitation and subsequently separated by SDS-PAGE.
2.2.21. Two dimensional electrophoresis

Conditioned-media samples and control media samples were separated by 2D electrophoresis. Initially proteins were separated in the first dimension by isoelectric focusing (IEF) using immobilized pH gradient (IPG) gels. IEF provides a measurement of the isoelectric point (pI) of the proteins, i.e. the pH at which the protein sample will not migrate in an electric field therefore the net charge of the protein is zero. Protein were subsequently separated in the second dimension using polyacrylamide gel electrophoresis (PAGE) to provide a measurement of protein size.

2.2.21.1. Sample preparation

Media samples and microglial conditioned media samples were dialysed using 3500 K dialysis tubing overnight to reduce the salt content present in the samples. High salt concentrations can interfere with the integrity of the IPG gel by resulting in zones of dehydration which result in a loss of current within the gel. The dialysed samples were then concentrated under vacuum using a Savan SpeedVac Plus AR. The concentrated samples were reconstituted in lysis buffer and the protein concentrations were determined according to the method of Bradford (1976; section 2.2.14.). Samples were adjusted accordingly to give an end concentration of 100-150 μg protein in 350 μl including 2 % ampholytes, of appropriate range, (Pharmacia Biotech Ab, Sweden), 3 % 2D molecular weight standards and 0.05 % bromophenol. Ampholytes are a mixture of amphoteric species with a range of pI values within the range of the IPG strips to be used. Pre cast IPG pH 3-10, 18 cm dry strips (Amersham Pharmacia, Immoboline Dry Strips) were reswelled overnight, at room temperature, using the prepared sample.
### Table 2.2.21.1. Lysis buffer for 2D electrophoresis

| Lysis buffer | 8 M urea, 2 M thiourea, 4 % Chaps, 0.5 % NP-40, 65 mM DTT in distilled H₂O |

### 2.2.21.2. First dimension: IEF Focusing

Once reswelled the proteins on the IPG strips were separated according to pH by step gradient electrophoresis using an Amersham Biosciences MULTPHOR II electrophoresis unit, at a constant temperature of 18 °C according to manufacturers instructions.

#### Table 2.2.21.2.a. First dimension electrophoresis protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Current</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-300 V</td>
<td>5 mA</td>
</tr>
<tr>
<td>2</td>
<td>300 V</td>
<td>5 mA</td>
</tr>
<tr>
<td>3</td>
<td>300-3500 V</td>
<td>5 mA</td>
</tr>
<tr>
<td>4</td>
<td>3500 V</td>
<td>5 mA</td>
</tr>
<tr>
<td>5</td>
<td>300 V</td>
<td>5 mA</td>
</tr>
</tbody>
</table>

Following isoelectric focusing the strips were removed and placed into equilibration buffer and rehydrated for 15 mins at room temperature with constant gentle agitation.

#### Table 2.2.21.2.b. Equilibration buffer composition

| Equilibration buffer | 6 M urea, 69 mM SDS, 30 % glycerol (v/v), 1 % DTT (w/v) in 5 mM Tris pH 6.8 |
2.2.21.3. Casting gradient PAGE gels

Gradient polyacrylamide gels, either 9-20 % or 12-20%, were poured using a BioRad gradient former through glass plates, which had been thoroughly cleaned with ethanol, housed in a BioRad PROTEAN II multi gel casting chamber. Approximately 2 ml of distilled H$_2$O was overlaid onto the top of each gel in order to obtain a smooth, flat gel top surface. Once poured the gels were left to polymerize for at least 3 hours at room temperature before being stored overnight at 4 °C.

<table>
<thead>
<tr>
<th></th>
<th>9 %</th>
<th>12 %</th>
<th>20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>51.7</td>
<td>51.7</td>
<td>51.7</td>
</tr>
<tr>
<td>30 % acrylamide solution</td>
<td>58.2</td>
<td>79.6</td>
<td>129.3</td>
</tr>
<tr>
<td>Distilled H$_2$O</td>
<td>84.2</td>
<td>62.7</td>
<td>13</td>
</tr>
<tr>
<td>Temed</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>10 % APS solution (w/v)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

If the separated proteins were to be assessed using the fluorescent stain SYPRO® Ruby (Molecular Probes) the glass plates were first bonded, prior to gel casting as detailed above. The front plate (20 cm x 20 cm) was coated with approximately 2 mls BindSilane solution, excess solution was removed using lint free wipes, and allowed to air dry for 1.5 hrs at room temperature. The rear plate (22 cm x 20 cm) was coated with approximately 5 mls Repel-Silane ES and allowed to air dry for a minimum of 15 mins at room temperature after which the plates were rinsed with ethanol and water to remove excess Repel-Silane ES, and dried using lint free wipes. Bind-Silane (γ-methacryloxy-propyl-trimethoxysilane) was used to covalently attach the polyacrylamide gel to the glass surface, thus ensuring the gel stayed firmly attached during staining and subsequent analysis procedures. Repel-Silane ES, a 2 % solution
of dimethyldichlorosilane dissolved in the solvent octamethyl cyclo-octasilane, was used to prevent the polyacrylamide gels sticking to the glass plates and so facilitating the removal of the rear plate.

Table 2.2.21.3.b. Composition of glass plate bonding solutions

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bind-Silane solution</strong></td>
<td>0.0008 % Bind-Silane (Amersham Biosciences, UK. Catalogue 17-1330-01); in 0.02 % acetic acid, 80 % ethanol in distilled H₂O</td>
</tr>
<tr>
<td><strong>Repel-Silane ES</strong></td>
<td>as supplied (Amersham Biosciences, UK. Catalogue 17-1332-01)</td>
</tr>
</tbody>
</table>

2.2.21.4. Second dimension: 2D SDS-PAGE

The equilibrated IPG strips were placed onto the interface of the pre-prepared polyacrylamide gradient gels and overlaid with approximately 5 ml hot agarose solution. The agarose was left to set for approximately 30 mins. The gel cassettes were inserted into the electrophoresis apparatus and the internal reservoir was filled with running buffer. The samples were initially electrophoressed at 40 mA per gel until the dye front had migrated out of the IPG strip and entered the polyacrylamide gel, after which the current was reduced to 10 mA per gel and the proteins were separated overnight. Following protein migration to the bottom of the gel, the gels were treated as appropriate for the subsequent method of visualisation.

Table 2.2.21.4. Solutions used for 2D SDS-PAGE

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agarose solution</strong></td>
<td>0.5 % agarose, 0.05 % bromophenol blue in dH₂O</td>
</tr>
<tr>
<td><strong>Running buffer</strong></td>
<td>25 mM Tris, 192 mM glycine, 3.5 mM SDS in dH₂O</td>
</tr>
</tbody>
</table>
2.2.21.5. Visualisation

2.2.21.5.1. Silver staining

Gels were ammoniacal silver stained according to the method of Hochstrasser (Hochstrasser and Merril, 1988; Shevchenko et al., 1996). Briefly once the dye front had migrated to the bottom of the gel, the gels were removed into distilled H$_2$O and rinsed for 2-5 min. Proteins were fixed for 1 hr at room temperature and subsequently transferred to storage fix for 3 hrs - 3 days as appropriate. Following fixing, stored gels were rehydrated in distilled H$_2$O for 5 min and subsequently soaked in 2.5 % glutaraldehyde solution for 30 min at room temperature. Following extensive washing, 3 x 10 mins and 4 x 30 mins, gels were stained for 10 mins with silver nitrate solution. Gels were washed, 3 x 5 min in distilled H$_2$O and incubated with the developer until the desired level of staining was visualised. The reaction was halted by removal of the developer and replacement with the stop solution for a minimum of 15 min. Stained gels were kept at room temperature in storage solution for further analysis as appropriate.

Table 2.2.21.5.1. Solutions used for silver staining

<table>
<thead>
<tr>
<th></th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fix</td>
<td>40 % ethanol, 10 % acetic acid in dH$_2$O</td>
</tr>
<tr>
<td>Storage fix</td>
<td>5 % ethanol, 5 % acetic acid in dH$_2$O</td>
</tr>
<tr>
<td>Silver nitrate</td>
<td>6 g AgNO$_3$, 10 ml NH$_4$OH, 1.5 ml 10 M NaOH in 750 ml dH$_2$O</td>
</tr>
<tr>
<td>Developer</td>
<td>0.1 g Citric acid, 1 ml formaldehyde in 1 L dH$_2$O</td>
</tr>
<tr>
<td>Stop</td>
<td>5 % acetic acid in dH$_2$O</td>
</tr>
<tr>
<td>Storage solution</td>
<td>7 % glycerol, 10 % ethanol in dH$_2$O</td>
</tr>
</tbody>
</table>
2.2.21.5.2 SYPRO® Ruby fluorescent staining

Gels were stained with SYPRO® Ruby (Amersham Biosciences, Bucks, UK) according to manufacturers instructions. Briefly once the dye front had migrated off the bottom of the gel, the plates were separated and the bonded gels were fixed for at least 3 hours at room temperature with mild agitation. Once fixing was complete the bonded gels were immersed in SYPRO® Ruby stain overnight at room temperature with gentle agitation. The containers were wrapped in aluminium foil to avoid light exposure. Prior to scanning bonded gels were briefly washed with distilled H₂O. Gels were scanned at 100 μM resolution, with side illumination, using a 2920-2D Master Multiwavelength Fluoroimager system (AmershamPharmaciaBiotech, UK). The excitation wavelength used was 480 nm while the emission wavelength was 618 nm emission. Images were captured using 2D master 1.9.5. software (PixCell) and inverted into black spots on a white background to allow analysis. Subsequent computer analysis was performed using Melanie III (GeneBio, Geneva, Switzerland). Excessive background fluorescent staining was reduced by immersing the bonded gels in destain for 5-30 minutes, as deemed necessary. Bonded gels were stored in sealed bags containing minimal storage solution.

Table 2.2.21.5.2. Solutions used for SYPRO® Ruby staining

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fix</td>
<td>40 % ethanol, 10 % acetic acid in distilled H₂O</td>
</tr>
<tr>
<td>Destain</td>
<td>10 % ethanol, 7 % acetic acid in distilled H₂O</td>
</tr>
<tr>
<td>Storage solution</td>
<td>5 % acetic acid in distilled H₂O</td>
</tr>
</tbody>
</table>
2.2.22. Statistical analysis

Cell counts were taken from 5 - 10 independent fields, on at least 2 coverslips, from 3 separate cell culture preparations. Analysis of supernatants was performed on supernatant collected from at least 2 treated coverslips, from 3 separate cell culture experiments. All experiments were repeated on at least three separate cell preparations. To compare a single treatment with control cultures, a two sided, unpaired t-test was used. Comparison of two or more independent treatments with control cultures was made using a one way analysis of variance (ANOVA). When significant effects were observed, values were compared pair wise using the Dunnett's post test. Effects of agonists and antagonists on control and conditioned medium treated cells was determined using two-way analysis of variance (ANOVA). When significant effects were observed, treatments were compared using Bonferroni's post test. Levels of significance are p<0.05 *, p<0.01 **, p<0.001 *** compared with control at that time point unless otherwise indicated.
3. **Microglia Release Soluble Factors Which Enhance Neuronal Survival and Proliferation**
3.1. INTRODUCTION

Microglia, as the resident macrophage of the CNS, play an important part in the processes of development and survival (Banati and Graeber, 1994). Several studies have shown that a variety of compounds can be secreted by microglia which can be toxic to injured neurones (Piani et al., 1991; Giulian et al., 1993; Théry et al., 1991; Chao et al., 1992; Boje and Arora, 1992; McMillian et al., 1995; Moore and Thanos, 1996).

However in the developing brain, microglia have been demonstrated to secrete compounds which enhance neuronal survival and differentiation. Protective compounds include cytokines e.g. interleukin-6 (IL-6) (Frei et al., 1989), interleukin-1 (IL-1) (Giulian et al., 1986), growth factors e.g. transforming growth factor-β (TGF-β) (Kreutzberg, 1996), basic fibroblast growth factor (bFGF) (Shimojo et al., 1991), hepatocyte growth factor (HGF) (Hamanoue et al., 1996), nerve growth factor (NGF) (Mallat et al., 1989; Lindholm et al., 1992) and extracellular glycoproteins (Baenziger et al., 1972; Lawler, 1986) which can stimulate neuronal differentiation and regeneration in vitro (Neugebauer et al., 1991; O'Shea et al., 1991; Osterhout et al., 1992; Chamak et al., 1994).

It would therefore seem possible that the actions of microglia, be they neurotrophic or neurotoxic, depend on the developmental stage of the neurones in their microenvironment. Furthermore, through their phagocytic activity (Perry et al., 1993), microglia are able to eliminate cellular debris formed when neurones apoptose after making inappropriate synapses or after failure to form a functional synapse. Microglia may also migrate to the cell bodies of injured neurones where they interrupt synaptic
contacts, a process called ‘synaptic stripping’. This may be the basis of a mechanism by which neurones are able to recover from injury and regenerate (Kreutzberg, 1996).

Many studies have primarily been carried out using neurones cultured in enriched primary cultures where neurones predominate. However this does not mimic the in vivo situation where a more complex interaction between neurones and glia exists. Recent studies have indicated that survival of neurones in long term cell culture requires the presence of microglial cells (Nagata et al., 1993; Zhang and Fedoroff, 1996; Cunningham et al., 1998; Zietlow et al., 1999). Furthermore microglial conditioned medium has been shown to have a neuroprotective effect against the induction of apoptosis caused by altering the neuronal culture conditions (Polazzi et al., 2001) and against neuronal damage induced by glutamate (Watanabe et al., 2000). The results presented in this chapter further explore the effects of microglial conditioned media on the growth and survival of developing CGCs, in vitro.
3.2. SUMMARY OF RESULTS

The effect of the addition of microglial-conditioned media to cultured cerebellar granule neurones was investigated in the research presented in this chapter. The survival and purity of the microglial cultures was ascertained, as were the conditions required to selectively deplete the number of microglia whilst leaving contaminating cells unaltered through the use of leucine methyl ester (LME). It was also established that the microglial cells were phenotypically distinguishable from other cell types of the macrophage lineage present in the CNS or peritoneum, as they exhibited down regulated expression of cell surface markers, with marginally increased expression during the course of 7 days in culture. The addition of the microglial conditioned medium to neurones which were aged in culture (day in vitro 7) resulted in an enhanced survival level as examined over an ensuing 7 day period. Addition of conditioned media collected from microglial-depleted cultures (LME-CM) resulted in the loss of the survival enhancing effect provided by the MG-CM, suggesting that microglial-derived diffusible factors are able to directly effect the survival of neurones in culture.

Furthermore, it was identified that the addition of MG-CM resulted in enhanced neuronal staining of the nuclear antigen Ki-67, suggesting an increased level of neuronal proliferation may be leading to the enhanced numbers of neurones observed following treatment with the MG-CM. This effect was confirmed using a colorimetric assay which demonstrated that only MG-CM and not LME-CM could enhance proliferation of the neuronal cultures above that observed in control cultures. It would seem possible that the MG-CM is able to promote the mitosis of the GCPs present at this developmental stage.
3.3 MICROGLIAL SURVIVAL IN CULTURE

Microglia were isolated as described and cultured under standard conditions. Microglial survival determined with the live-dead double stain assay (3.3.1.), whilst the purity of the cultures was investigated using immunocytochemical staining (3.3.2.). The lineage and reactivity of microglial cells was investigated by comparing them with peritoneal macrophages using fluorescence activated cell sorting (FACS) (3.3.3.). The concentration of metabolite levels in the microglial supernatants were appraised for nitric oxide breakdown products and glutamate (3.3.4.). Furthermore the conditions for the selective depletion of microglia were investigated (3.3.5.), as was the culturing of microglia in serum free medium (3.3.6.).

3.3.1. Assessment of microglial viability in culture

Following isolation the microglial cells were cultured under standard conditions. The cells developed a number of fine processes, which are indicative of a resting state (figure 3.3.1. A.). The survival of cultured microglia was assessed over the course of 7 days in vitro with the live-dead double stain assay using propidium iodide (PI) to stain dead cells and fluorescein diacetate (FDA) to label live cells.

The number of live microglia decreased over the course of 7 days from 28.6 ± 1.4 live microglia per field to 12.3 ± 1.2 live microglia per field (figure 3.3.1. Bi.). This represents a decrease in the number of viable cells of 57 % over the time course. The number of dead cells observed dropped from 1.8 ± 0.3 dead microglia per field to 1.2 ± 0.6 dead microglia per field. The number of dead microglia thus did not rise as the number of live microglia decreased this may be because as the microglia died they became detached from the coverslip, or were phagocytosed by the remaining
microglia. The ratio of live to dead cells was not observed to vary statistically over the course of the week (figure 3.3.1. Bi.). These results demonstrate that isolated microglia, maintained under standard conditions, survive at least 7 days in culture.

Figure 3.3.1. Isolated microglia can survive up to 7 days in culture. Following isolation cells were maintained under standard culture conditions. (A) Over the course of 7 days the microglial cells developed fine processes indicative of a resting state. The viability of the cultures was assessed over 7 days using the live dead-assay. (B) The number of live microglial cells diminished over the course of 7 days (i), however the number of live microglia expressed as a percentage of the total population did not vary (ii). Values are expressed as mean number of cells per fields of view ± sem. Levels of significance are compared with number of microglia at day 1.
3.3.2. Immunocytochemical staining of microglial cultures

The purity of the microglial cultures was assessed by immunocytochemistry. Antibodies against specific cell surface markers were used to study the activation state of the microglia and also to assess the number of contaminating astrocytes in the preparation. OX-42 was used as a microglial marker, ED-1 as a specific marker for activated microglia and glial fibrillary acidic protein (GFAP) as an astrocyte marker. The cultures were assessed at day in vitro 1 and 7 (table 3.3.2.a.).

Table 3.3.2.a. Immunoreactivity of microglial cultures. Following isolation, microglial cultures were assessed for immunoreactivity to OX-42, ED1 and GFAP after 1 and 7 days in vitro. Values are expressed as mean number of cells per field of view ± sem. Levels of significance (one-way anova) compared with 1 DIV microglia.

<table>
<thead>
<tr>
<th></th>
<th>Immunoreactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OX42</td>
</tr>
<tr>
<td>1 DIV unstimulated microglial culture</td>
<td>27.7 ± 4.2</td>
</tr>
<tr>
<td>7 DIV unstimulated microglial culture</td>
<td>13.5 ± 2.1 *</td>
</tr>
</tbody>
</table>

The microglial cultures represent essentially pure cultures of microglia. It was observed that whilst there was a decrease in the overall number of microglia (OX-42 positive) during the 7 days in vitro, the number of ED-1-positive microglia and GFAP-positive astrocytes remained low and did not vary statistically over the 7 days. This indicates that the purity of the microglial cultures was 90.5 % ± 2.6 at day in vitro 1 and 81.6 % ± 5.2 at day in vitro 7.

To further characterise the microglial cultures the reactivity to vimentin was assessed. Vimentin is a major component of the intermediate filaments in the cytoskeleton of
cells and is involved in the motility of cells (Clark et al., 1999). Vimentin is expressed in immature astrocytes (Kindy et al., 1992; Buchheim et al., 1994) and reactive microglia but not in resting microglia (Slepko and Levi, 1996). In astrocytes, as the cells mature, vimentin is slowly replaced by GFAP (Ha et al., 2000), however in response to activating signals vimentin can be re-expressed (Menet et al., 2001). Therefore the use of this antibody would determine whether the number of astrocytes had been previously underestimated using GFAP staining. The cultures were also labelled with a *Bandeiraea simplicifolia* isolectin, B₄, which is an established marker for microglia regardless of their phenotype (Streit and Kreutzberg, 1987; Boya et al., 1991). Finally the cultures were counterstained with DAPI to quantify total cell number (table 3.3.2.b.).

Table 3.3.2.b. Further immunoreactivity of microglial cultures. Following isolation, microglial cultures were assessed for immunoreactivity to vimentin and isolectin B₄ after 1 and 7 days in vitro. Cultures were counterstained using DAPI. Values are expressed as mean number of cells per field of view ± sem.

<table>
<thead>
<tr>
<th>Immunoreactivity</th>
<th>vimentin positive</th>
<th>vimentin negative</th>
<th>lectin B₄</th>
<th>DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DIV unstimulated microglial culture</td>
<td>17.6 ± 0.8</td>
<td>12.9 ± 0.8</td>
<td>28.5 ± 0.5</td>
<td>30.5 ± 0.8</td>
</tr>
<tr>
<td>7 DIV unstimulated microglial culture</td>
<td>13.6 ± 0.6</td>
<td>4.4 ± 0.4</td>
<td>15.9 ± 1.3</td>
<td>18.4 ± 0.9</td>
</tr>
</tbody>
</table>

These results indicate that the microglial cultures are approximately 58 % and 74 % vimentin positive at day 1 and day 7 respectively. All vimentin negative cells counted had a phenotype possessing fine processes leading from the cell body, which would be indicative of non-reactive microglia, furthermore these cells were also found to be lectin positive, confirming them as microglia. As discussed vimentin staining can be
used to identify both astrocytes and reactive microglia, therefore, from the above results, it can be estimated that approximately two vimentin positive cells are likely to represent astrocytes at both 1 and 7 day \textit{in vitro}. This implies that approximately 50\% of the microglia are vimentin positive which indicates a higher level of reactivity than observed using ED1 staining (table 3.3.2.a.), this is likely to be because vimentin expression is upregulated prior to the expression of ED1 (Slepko and Levi, 1996). This data confirms that the cultures are pure single cell microglial cultures, with 94.2\% ± 2.0 and 86.2\% ± 2.6 microglia at 1 and 7 days \textit{in vitro} respectively.

\subsection*{3.3.3. FACS analysis of microglial culture}

To further assess the characteristics of the neonatal microglial cultures, they were evaluated using flow cytometric phenotyping. As the origins of microglia are commonly ascribed to a bone marrow-derived monocyte/macrophage lineage, with entry into the CNS during the course of development (Ling and Wong, 1993), the cultures were compared with peritoneal macrophages, to demonstrate that the microglial cells, though bearing a resemblance to peripheral macrophages, are in fact a distinct cellular population. As discussed microglia and systemic monocytes/macrophages possess similar phenotypic and functional properties however it has been demonstrated that microglia can be distinguished from other leukocytes present in the CNS, including other CNS macrophages, on the basis of the lower than normal expression of CD45 on microglia versus other macrophages (Ford et al., 1995). Both isolated cell populations were assessed for immunoreactivity to a number of macrophage/monocyte cell surface antigens, namely CD11b/c (MRC OX-42), CD45 and ED1.
In the first instance the forward and side scatter of the cell populations were compared (figure 3.3.3.a.), forward scatter describes the size of the cell being analysed whilst side scatter assesses the granularity of the cell. This analysis revealed that the microglia were comparable in size to the peritoneal macrophages (figure 3.3.3.a. A.), but were less granular (figure 3.3.3.a. B.).

Both populations, microglia and peritoneal macrophages, were found to be reactive to CD45-FITC antibody (figure 3.3.3.b.), with the peritoneal macrophages exhibiting the greatest reactivity (figure 3.3.3.b. C.), with 94.0 % of cells fluorescing above basal, with a mean fluorescence index (MFI) of 166.2 units. The reactivity of the microglia was found to increase over time (figure 3.3.3.b. A and B), with 21.2 % microglia being positive at day 1 increasing to 75.6 % at day 7. The level of intensity of staining also increased from a MFI value of 37.0 units at day 1 to 41.3 units at day 7.

A comparable pattern was also observed for reactivity to CD11b-RPE antibody (figure 3.3.3.c.), where again the peritoneal macrophages were found to exhibit the greatest reactivity (figure 3.3.3.c. C.), with 96.1 % of cells fluorescing above basal levels, with a MFI of 260.8 units. Again the reactivity of the microglial cultures was found to increase over time (figure 3.3.3.c. A and B), with 80.2 % microglia being positive at day 1 increasing to 88.6 % at day 7. The level of intensity of staining also increased from a MFI value of 19.3 units at day 1 to 37.5 units at day 7.

Finally the reactivity of the cultures to ED1 was also assessed (figure 3.3.3.d). ED1 is expressed predominantly on the lysosomal membranes of myeloid cells although weak cell surface expression also occurs. To account for this ED1 reactivity was assessed in
Figure 3.3.3.a. Comparison of microglial and peritoneal macrophage size parameters. Microglia and peritoneal macrophages were isolated and cultured under standard conditions for 24 hours. The cells were subsequently harvested and analysed by flow cytometry. \((A)\) Comparison of microglial and peritoneal macrophage forward scatter (FSC), indicating overall cell size. \((B)\) Comparison of microglial and peritoneal macrophage side scatter (SSC), indicating overall internal granularity and complexity. Microglial cultures are represented by the line population and peritoneal macrophage cultures are the shaded population. Values represent the mean FSC and SSC values for each population.
standardly treated cells and cells which had been permeabilised prior to antibody application, by the addition of 0.1 % triton.

The peritoneal macrophages expressed high levels of ED1 reactivity, with 94.5 % of cells fluorescing above basal, with a MFI of 122.5 units under standard conditions (figure 3.3.3.d. Ci). Following permeabilisation this rose to 99.3 % of cells fluorescing above basal, with a MFI of 343.7 units (figure 3.3.3.d. Cii). The microglial cultures had in contrast a comparatively low reactivity to ED1 with little change over the course of 7 days, with 47.0 % microglia being positive at day 1 (figure 3.3.3.d. Ai) and 13.0 % at day 7 (figure 3.3.3.d. Bi) under standard conditions. Following cell permeabilisation this increased to 63.1 % microglia being positive at day 1 (figure 3.3.3.d. Aii) and 63.5 % at day 7 (figure 3.3.3.d. Bii). The level of intensity of staining did not vary statistically from a MFI value of 30.3 units at day 1 (figure 3.3.3.d. Aii) to 24.0 units at day 7 (figure 3.3.3.d. Bii). These results indicate that the microglia are expressing low levels of ED1 indicative of a down regulated phenotype.

To ascertain whether the reactivity to ED1 could be enhanced, thus indicating whether the cells could be further activated, cultures were treated with LPS (1mg/ml) for 24 hours prior to cell harvesting. ED1 reactivity was subsequently assessed on permeabilised cell populations. LPS had no effect on peritoneal macrophage ED1 reactivity (figure 3.3.3.d. Ciii), however the microglial reactivity was upregulated (figure 3.3.3.d. Aiii and Biii), with 84.1 % microglia becoming positive in 1 day \textit{in vitro} cultures and 88.3 % in 7 day \textit{in vitro} cultures. This result indicates that the microglia in culture have downregulated ED1 expression which can be stimulated via cellular activation with LPS.
Figure 3.3.3.b. Culture reactivity to CD45. Microglia and peritoneal macrophages were isolated and cultured under standard conditions. The cells were subsequently harvested and analysed for reactivity to CD45 cell surface marker expression using FACS. (A) 1 day in vitro microglia. (B) 7 day in vitro microglia. (C) 1 day in vitro peritoneal macrophage. Values represent the percentage of cells in the population, and the mean fluorescent index (MFI) in the relative M1 and M2 regions as depicted.

<table>
<thead>
<tr>
<th></th>
<th>1 DIV microglia</th>
<th>7 DIV microglia</th>
<th>1 DIV peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>basal</td>
<td>CD45</td>
<td>basal</td>
</tr>
<tr>
<td></td>
<td>% cells</td>
<td>MFI</td>
<td>% cells</td>
</tr>
<tr>
<td>M1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(A) 1 day in vitro microglia.

(B) 7 day in vitro microglia.

(C) 1 day in vitro peritoneal macrophage.
Figure 3.3.3.c. Culture reactivity to CD11b. Microglia and peritoneal macrophages were isolated and cultured under standard conditions. The cells were subsequently harvested and analysed for reactivity to CD11b cell surface marker expression using FACS. (A) 1 day in vitro microglia. (B) 7 day in vitro microglia. (C) 1 day in vitro peritoneal macrophage. Values represent the percentage of cells in the population, and the mean fluorescent index (MFI) in the relative M1 and M2 regions as depicted.

<table>
<thead>
<tr>
<th></th>
<th>1 DIV microglia</th>
<th>7 DIV microglia</th>
<th>1 DIV peritoneal macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% cells</td>
<td>MFI</td>
<td>% cells</td>
</tr>
<tr>
<td>M1</td>
<td>96.3</td>
<td>3.4</td>
<td>98.4</td>
</tr>
<tr>
<td>M2</td>
<td>3.7</td>
<td>8.4</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Values represent the percentage of cells in the population, and the mean fluorescent index (MFI) in the relative M1 and M2 regions as depicted.
Figure 3.3.3.d. Culture reactivity to ED1. Microglia and peritoneal macrophages were isolated and cultured under standard conditions. The cells were subsequently harvested and analysed for ED1 cell surface marker expression on (i) permeabilised and (ii) non-permeabilised cells using FACS. Cultures were also activated using (iii) LPS (1 mg/ml) for the final 24 hours of incubation in specified cultures. (A) 1 DIV microglia (B) 7 DIV microglia (C) 1 DIV peritoneal macrophages. Values represent the percentage of cells in the population, and the mean fluorescent index (MFI) in the relative M1 and M2 regions as depicted.
### 7 DIV microglia

<table>
<thead>
<tr>
<th>% cells</th>
<th>MFI</th>
<th>% cells</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>98.8</td>
<td>29.0</td>
<td>87.0</td>
</tr>
<tr>
<td>M2</td>
<td>1.2</td>
<td>19.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

### 7 DIV microglia

<table>
<thead>
<tr>
<th>% cells</th>
<th>MFI</th>
<th>% cells</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>96.4</td>
<td>2.8</td>
<td>36.5</td>
</tr>
<tr>
<td>M2</td>
<td>3.6</td>
<td>14.5</td>
<td>63.5</td>
</tr>
</tbody>
</table>

### 7 DIV microglia + LPS

<table>
<thead>
<tr>
<th>% cells</th>
<th>MFI</th>
<th>% cells</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>97.9</td>
<td>2.9</td>
<td>11.7</td>
</tr>
<tr>
<td>M2</td>
<td>2.1</td>
<td>17.0</td>
<td>88.3</td>
</tr>
</tbody>
</table>
### 1 DIV macrophages

<table>
<thead>
<tr>
<th></th>
<th>basal</th>
<th>ED1 permeabilised</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells</td>
<td>MFI</td>
<td>% cells</td>
</tr>
<tr>
<td>M1</td>
<td>99.6</td>
<td>2.1</td>
</tr>
<tr>
<td>M2</td>
<td>0.4</td>
<td>11.6</td>
</tr>
</tbody>
</table>

### 1 DIV macrophages + LPS

<table>
<thead>
<tr>
<th></th>
<th>basal</th>
<th>ED1</th>
</tr>
</thead>
<tbody>
<tr>
<td>% cells</td>
<td>MFI</td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>99.9</td>
<td>2.1</td>
</tr>
<tr>
<td>M2</td>
<td>0.1</td>
<td>28.1</td>
</tr>
</tbody>
</table>

**Table**: Measurement of fluorescence intensity (log) for different conditions.

**Diagram**: Flow cytometry histograms showing fluorescence intensity for M1 and M2 macrophages under various conditions.

### Notes
- **Ci**: Counts for fluorescence intensity (log).
- **M1** and **M2** represent different macrophage subtypes.
- **ED1**: An antibody used for permeabilization.
- **LPS**: Lipopolysaccharide, a component of bacterial cell walls.
3.3.4. Measurement of metabolite levels in microglial supernatant

The supernatant was collected from microglial cultures over the course of 7 days and assayed to measure the nitrite and glutamate concentrations, metabolites which are known to be released by microglia. The nitrite and total nitrite, i.e. nitrate and nitrite combined, levels increased during the 7 days in culture by 300 % and 60 % respectively (figure 3.3.4.a. A.), however only the increase in total nitrite was found to be significant compared to 1 DIV supernatant and that represented only a marginal increase (p<0.05). The glutamate levels increased by approximately 200 % (figure 3.3.4.a. B.), however this increase was not found to be statistically significant.

Figure 3.3.4.a. Metabolite levels in the retained microglial supernatant. Supernatant collected from microglial cultures was appraised for (A) nitrite and total nitrite concentrations and (B) glutamate concentrations. Values are expressed as the mean concentration (μM) ± sem. Levels of significance (one way anova) are compared with concentrations in 1 DIV microglial supernatant.

Microglial cell lysates were also appraised for the expression of iNOS, the enzyme which catalyses the synthesis of nitric oxide (Corradin et al., 1993). This revealed that unstimulated microglial cultures only showed a low level of iNOS expression (figure
3.3.4.b). Activation of the microglia, by the addition of LPS, demonstrated that the microglia could upregulate iNOS expression (figure 3.3.4.b.), suggesting that the unstimulated microglial cultures are down-regulated but responsive to external stimuli.

**Figure 3.3.4.b. Unstimulated microglia express low levels of iNOS.** Cell lysates were collected from 1 and 7 day in vitro microglial cultures, and from cultures activated by the addition of LPS (1 mg/ml) for 24 hrs. Proteins were subjected to Western blot analysis to assess iNOS expression. Protein loading was appraised by β-actin expression.

![Western blot analysis of iNOS and β-actin expression in microglial cultures.](image)

Under standard conditions unstimulated microglial cultures expressed low levels of iNOS and released low levels of both glutamate and nitric oxide (NO), in the form of nitrite and nitrate. As discussed previously these compounds have the capacity to be neurotrophic or neurotoxic both *in vitro* and *in vivo*.

The conditioned supernatant, which is referred to as the microglial-conditioned medium (MG-CM), was collected and pooled, centrifuged, and frozen at −20 °C for further use. This was carried out on 1 DIV cultures and 7 DIV cultures.

### 3.3.5. Selective depletion of microglia using L-LME

Medium was also collected from preparations where the number of microglia had been selectively depleted, thereby allowing any effects observed to be directly attributed to the MG. It has been reported that L-leucine methyl ester (LME) is able to selectively deplete MG (Brown et al., 1996; Giulian et al., 1986; Kingham and Pocock, 2000), due
to its intracellular conversion to membranolytic metabolites by the acyl transferase activity of dipeptidyl peptidase I (DPPI) which is enriched in these cells (Thiele et al., 1983; Thiele and Lipsky, 1990, 1992). However the conditions used varied between investigators, therefore in the first instance conditions were optimised.

Primary microglial cultures were treated with 5 mM and 10 mM LME following the 1 hr cell adherence step. Cells were incubated with LME for 1, 2, 8, 16 and 24 hours after which time the cells were washed and replenished with fresh media. Survival was assessed 24 hours later using the live-dead assay (figure 3.3.5.a.).

Addition of either 5 or 10 mM LME significantly reduced the number of microglia following 1 hr treatment. Following 24 hrs of treatment 10 mM LME reduced the number of microglia by 60 % whereas 5 mM LME reduced the number by 50 %. This indicated that the optimum conditions to deplete the number of microglia was to incubate the microglia with 10 mM LME for 24 hours.

**Figure 3.3.5.a. Effect of adding 5 and 10 mM LME to microglial cultures.** Following the 1 hr adherence step, microglial cultures were treated with 5 mM (circle) or 10 mM (square) LME for between 1 - 24 hr, following washing and media replenishment the number of live microglia was assessed after a further 24 hr. Values are expressed as the mean number of live microglia per field ± sem. Levels of significance (one way anova) are compared with number of microglia at day 1.
The survival and cellular composition of microglial cultures over the course of 7 days in culture following the 24 hr treatment of cultures with 10 mM LME was appraised using the live-dead assay (figure 3.3.5.b. A) and immunocytochemical staining with OX-42, ED-1 and GFAP (figure 3.3.5.b. B).

**Figure 3.3.5.b. Survival of microglial cultures with and without LME treatment.** Following 24 hr treatment with 10 mM LME microglial cultures were maintained under standard conditions for up to 7 days. (A) Culture survival was assessed at day 1 and day 7 using the live-dead assay. (B) Cultures were further appraised for immunoreactivity to OX-42, ED-1 and GFAP at day 1 and day 7. Values are expressed as mean number of cells per field of view ± sem. Levels of significance (one-way anova) compared with either 1 or 7 DIV untreated microglial cultures.

![Graph showing survival of microglial cultures](image)

<table>
<thead>
<tr>
<th></th>
<th>OX42</th>
<th>ED1</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DIV unstimilated MG cultures</td>
<td>27.7 ± 4.2</td>
<td>5.3 ± 0.3</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>1 DIV LME treated MG cultures</td>
<td>8.2 ± 0.8 **</td>
<td>5.8 ± 1.4</td>
<td>2.0 ± 0.6</td>
</tr>
<tr>
<td>7 DIV unstimilated MG cultures</td>
<td>13.5 ± 2.1</td>
<td>6.7 ± 2.8</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>7 DIV LME treated MG cultures</td>
<td>5.5 ± 0.7 **</td>
<td>4.8 ± 1.6</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

The LME-treatment significantly depleted the number of live microglia cells. After 1 day *in vitro* the number of microglia decreased from 26 ± 1.3 live microglial cells per
field in controls to 7.1 ± 0.8 live MG cells per field with LME-treatment (figure 3.3.5.b. A). After 7 days the number decreased from 15 ± 1.7 live MG cells per field in control to 5.7 ± 1.1 live MG cells per field following LME-treatment.

The number of OX-42 positive cells was significantly (p < 0.01) reduced following LME treatment compared with control cultures (figure 3.3.5.b. B), indicating that the number of microglia had been reduced. The majority of the microglia remaining were ED-1 positive suggesting increased activation. There was no alteration in the number of astrocytes present in the cultures following LME treatment.

The conditioned supernatant, referred to as the LME treated-conditioned medium (LME-CM), was collected and pooled, centrifuged and frozen at -20 °C for further use.

### 3.3.6. Culture of microglia in serum free medium

Microglial cultures were routinely maintained in medium containing foetal calf serum. This standard serum supplementation represents the significant addition of undefined biological response modifiers including proteins, electrolytes, non protein nitrogenous substances, lipids, carbohydrates, attachment factors, hormones, binding and carrier proteins, enzymes and other miscellaneous undefined components. Therefore to establish the involvement of these factors microglia were also cultured in serum-free media, as described, which provides a complete nutrient formulation capable of supporting cultured cells without the need for serum supplementation. The survival of microglia was assessed over the course of a week under standard culture conditions using the live-dead assay.
Figure 3.3.6. Microglial survival in serum free media. Once isolated microglial were plated and maintained using serum free media. (A) Cultures were appraised for survival at day 1 and day 7. (B) Number of live microglia expressed as a percentage of the total number of cells per field over the course of 7 days. Values are expressed as mean number of cells per fields of view ± sem. Levels of significance (one-way anova) are compared with number of microglia at day 1.

The number of live microglia decreased over the course of 7 days from 26.7 ± 2.3 live microglia per field to 15.3 ± 1.5 live microglia per field (figure 3.3.6. A), representing a 50 % decrease in the number of viable cells over the time course. The number of dead cells observed dropped from 5.0 ± 0.3 dead microglia per field to 3.9 ± 2.4 dead microglia per field. The ratio of live to dead cells was not observed to vary statistically over the course of the week (figure 3.3.6. B). These results demonstrate that isolated microglia, maintained under standard conditions, survive at least 7 days in culture.
3.4. EFFECTS OF CONDITIONED MEDIA ON NEURONAL SURVIVAL

To investigate the effects of non-stimulated microglia on cultured cerebellar granule neurones (CGCs), conditioned media was collected from differently treated microglial cultures and added to 7 day in vitro CGC cultures as described. Media was collected from standard microglial cultures (3.4.1), cultures maintained in serum-free medium (3.4.2.), L-leucine methyl ester depleted cultures (3.4.3.) and from cultures plated at varying microglial densities (3.4.4.). The survival of the CGCs was assayed over a further 7 days in culture and the concentration of metabolite levels in the CGC supernatants, from both control and conditioned medium treated cultures, was appraised for nitric oxide breakdown products and glutamate. The effect of adding peritoneal macrophage conditioned medium (PM-CM) was also appraised (3.4.5.).

3.4.1. Addition of microglial-conditioned medium

Addition of MG-CM significantly increased the number of live CGCs over the 7 days investigated (figure 3.4.1.a. A). 7 DIV MG-CM provided a greater level of neuronal cell survival than 1 DIV MG-CM. 7 DIV MG-CM enhanced the CGC survival from 38.6 % ± 4.1 above control at CGC DIV 8 to 80.4 % ± 7.3 above control at CGC day in vitro 14. The 1 DIV MG-CM increased the number of surviving neuronal cells from 12.2 % ± 3.4 above control at CGC day in vitro 8 to 20.4 % ± 4.3 above control at CGC DIV 14. Addition of the unconditioned microglial medium resulted in a comparable level of neuronal survival as the CGC control. In control cultures 70 - 100 live neurones were counted per field, whilst in 1 DIV MG-CM treated cultures approximately 90 - 110 live neurones were counted per field, and in excess of 110 live neurones were routinely counted in 7 DIV MG-CM treated cultures.
Figure 3.4.1.a. Addition of MG-CM enhances neuronal survival. Neurones were cultured for 7 days, after which 250 ml of media was removed, replaced with either fresh microglial media or MG-CM and cultures were maintained and assessed over the ensuing 7 days. (A) Neuronal survival was assessed using the live-dead assay, values are the number of live CGCs per field expressed as a percentage difference from CGC control culture values ± sem, where n=12, over 4 culture preparations. (B) Neuronal apoptosis was assessed using the Hoechst apoptosis assay, values are the number of apoptotic CGCs per field expressed as a percentage of the total cell number ± sem where n=9, over 3 culture preparations.

The percentage of apoptotic neurones at CGC day 8 decreased from 18.6 % ± 2.1 neurones in control cultures to 11.3 % ± 0.5 neurones in 7 DIV MG-CM treated cultures (figure 3.4.1.a. B). By CGC DIV 14 the number of apoptotic neurones in control cultures was 16.8 % ± 1.5 neurones, whilst in 7 DIV MG-CM treated cultures there were 8.0 % ± 1.0 apoptotic neurones. A similar effect was observed with addition of 1 DIV MG-CM, at CGC DIV 8 the number of apoptotic neurones was decreased to 12.3 % ± 1.6 neurones, and at CGC DIV 14 to 8.6 % ± 1.2 apoptotic neurones. Addition of fresh microglial medium resulted in levels of apoptosis comparable with control. This indicates that a diffusible factor present in the MG-CM
is able to decrease the number of apoptotic neurones and enhance the number of live neurones present in the MG-CM treated cultures. In control cultures between 10 - 20 apoptotic neurones were counted per field, whilst in 1 DIV MG-CM treated cultures less than 15 apoptotic neurones were counted per field, and less than 10 apoptotic neurones were routinely visible in 7 DIV MG-CM treated cultures.

The supernatant from MG-CM treated CGCs and control CGCs were collected over the course of a week and assayed to measure the nitrite, total nitrite and glutamate concentrations. In addition neuronal whole cell protein lysates were collected and subject to Western blot analysis to appraise nNOS and iNOS expression.

The expression of both nNOS and iNOS was not observed to vary greatly between treatments or indeed over time using Western blot analysis (figure 3.4.1.b. A). Furthermore the addition of the 7 DIV MG-CM was found to increase nitrite levels in the CGC supernatant to 30 % above control supernatant levels at CGC day 8, and 15 % above control by CGC day 14 (figure 3.4.1.b. Bii). The total nitrite concentration was increased by 6.7 % above control supernatants at CGC day 8 and by CGC day 14 had increased to 15 % above control levels (figure 3.4.1.b Biii), however these increases were not statistically significant. Addition of 1 DIV MG-CM also produced no statistically significant increase or decrease compared with control CGC supernatants.

A similar effect was also observed in the measured levels of glutamate in the treated CGC supernatant (figure 3.4.1.b Bi), where no statistically significant increase or decrease in concentrations was observed compared with control CGC supernatants.
Figure 3.4.1.b Neuronal cultures treated with MG-CM do not show enhanced nitrite and glutamate production. Neurones were cultured for 7 days, after which 250 ml of media was removed, replaced with either fresh microglial media or MG-CM and cultures were maintained and assessed over the ensuing 7 days. (A) Neuronal whole cell protein lysates, collected at CGC day 8 and 14, were separated by electrophoresis and expression of nNOS and iNOS was assessed by Western blotting. (B) Supernatants were collected from control and MG-CM treated cultures and appraised for (i) glutamate, (ii) nitrite, and (iii) total nitrite, values are expressed as the mean concentration (µM) ± sem, where n=8. All levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point.
3.4.2. Addition of microglial-conditioned serum free medium

To investigate the effects exerted by undefined components in serum conditioned media, media collected from microglial cultures maintained in serum free media (SF-CM) was added to the neurones as previously described. Addition of fresh serum free media (SFM) to neurones resulted in survival levels comparable to that in control. However addition of SF-CM, resulted in enhanced survival levels, comparable to those achieved following the addition of MG-CM. The 7 DIV SF-CM enhanced the CGC survival by 37.2 % ± 2.3 above control at CGC DIV 8 and by 25.8 % ± 5.1 above control at CGC day in vitro 14.

Figure 3.4.2. Addition of SF-CM enhances neuronal survival. Neurones were cultured for 7 days, after which 250 µl of media was removed, replaced with either fresh serum-free media or SF-CM and cultures were maintained and assessed over the ensuing 7 days. Neuronal survival was assessed using the live-dead assay, values are the number of live CGCs per field expressed as a percentage above CGC control culture values ± sem, where n=6 over 3 culture preparations. Levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point.
3.4.3. Addition of LME treated microglial-conditioned medium

Addition of both 1 DIV LME-CM and 7 DIV LME-CM resulted in neuronal survival levels comparable with untreated control CGC cultures at each time point investigated (figure 3.4.3. A). At CGC DIV 8 addition of 1 DIV MG-CM resulted in enhanced survival levels to 12.4 % ± 3.4 above untreated control cultures, whilst the addition of 1 DIV LME-CM resulted in levels of -14.3 % ± 3.7 below control CGC cultures. At CGC DIV 10 the difference was more pronounced with the 1 DIV MG-CM resulting in survival levels 22.8 % ± 5.8 above untreated control and the 1 DIV LME-CM resulting in survival levels of -7.6 % ± 7.3 below untreated control cultures. This trend continued at CGC DIV 14, with the survival of 1 DIV MG-CM treated cultures being 20.4 % ± 4.3 above control and 1 DIV LME-CM treated cultures having survival levels of -8.2 % ± 8.6 below control.

Whilst the addition of 7 DIV MG-CM enhanced neuronal survival 38.6 % ± 4.1 above control, 7 DIV LME-CM diminished neuronal survival to -4.6 % ± 6.9 below control CGC cultures at CGC DIV 8. The trend continued at both CGC DIV 10 and 14 with the 7 DIV MG-CM resulting in levels enhanced by 69.1 % ± 6.2 above control and 80.4 % ± 7.4 respectively, whilst the 7 DIV LME-CM resulted in survival levels -10.9 % ± 6.7 below control and -7.8 % ± 6.3 below control respectively.
Figure 3.4.3. Addition of LME-CM is not able to promote CGC survival. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either MG-CM or LME-CM. (A) Neuronal survival was assessed using the live-dead assay over a further 7 days in culture. Values are the mean number of live CGC per field expressed as a percentage of CGC control cultures ± sem, where n=9 over 3 culture preparations. (B) Supernatants collected from LME-CM treated CGC cultures were appraised for (i) nitrite, (ii) total nitrite and (iii) glutamate. Values are expressed as the mean concentration (μM) ± sem. All levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point.

A

100
75
50
25
0
-25

live CGC % above control

1 DIV MG-CM
7 DIV MG-CM

1 DIV LME-CM
7 DIV LME-CM

day in vitro

Bi

[i] nitrite [μM]

5.0
2.5
0.0

[total nitrite] [μM]

30
20
10
0

1 DIV LME-CM
7 DIV LME-CM

day in vitro

iii

[glutamate] [μM]

10
5
0

1 DIV LME-CM
7 DIV LME-CM

day in vitro

159
The difference between the MG-CM and related LME-CM was statistically significant (p>0.001) at each time point investigated, whilst the difference between the controls and the LME-CM was not found to be significant, demonstrating that the MG-CM did have a positive effect on neuronal survival. This suggests that the enhancement of survival effect exerted by MG-CM on neurones had been diminished by the depletion of microglial cells prior to collection of the conditioned media. This indicates therefore that MG-CM contains a microglial-derived diffusible factor with an inherent ability to effect the number of neurones surviving during the culture period.

The supernatant from LME-CM treated CGCs and control CGCs were collected over the course of a week and assayed to measure the nitrite, total nitrite and glutamate concentrations. The metabolite levels in the retained LME-CM treated CGC supernatant were not statistically different to concentrations in control, untreated CGC supernatants (compare figure 3.4.1.b. B with figure 3.4.3. B), indicating the production of these metabolites had not been upregulated.

3.4.4. Addition of MG-CM from varying microglial plating densities
At CGC DIV 8, the addition of low density 1 DIV MG-CM was marginally detrimental to neuronal survival (figure 3.4.4. Ai), resulting in a survival level of -1.6 % ± 5.2 below control CGC cultures, and by day 10 and 14 the low density MG-CM was enhancing survival to 1.0 % ± 6.4 and 16.2 % ± 12.5 above control CGC cultures respectively. Addition of low density 7 DIV MG-CM enhanced neuronal survival levels to 5.1 % ± 5.4, 28.4 % ± 11.0 and 57.2 % ± 14.2 above untreated control CGC cultures at CGC DIV 8, 10 and 14 respectively (figure 3.4.4. Aii).
Figure 3.4.4. Plating density of the microglial cultures affects the efficacy of the conditioned media. Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with conditioned media retrieved from microglia cultures plated at low (0.15 x 10^6 cells/ml), medium (0.6 x 10^6 cells/ml) and high (1.2 x 10^6 cells/ml) cell densities. (A) Neuronal survival was assessed following treatment with (i) 1 DIV MG-CM or (ii) 7 DIV MG-CM using the live-dead assay over a further 7 days in culture. Values are the mean number of live CGC per field, expressed as a percentage of CGC control cultures ± sem, where n=9 over 3 culture preparations. (B) Supernatants collected from treated CGC cultures were appraised for (i) nitrite, (ii) total nitrite and (iii) glutamate. Values are expressed as the mean concentration (µM) ± sem. All levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point.
Addition of high density 1 DIV MG-CM (figure 3.4.4. Ai) enhanced neuronal survival levels to 11.5 % ± 5.0, 28.0 % ± 11.1 and 30.9 % ± 13.0 above untreated control CGC cultures at CGC DIV 8, 10 and 14 respectively. Whilst the addition of high density 7 DIV MG-CM (figure 3.4.4. Aii) enhanced neuronal survival levels to 33.8 % ± 7.3, 58.3 % ± 12.3 and 89.1 % ± 10.4 above untreated control CGC cultures at CGC DIV 8, 10 and 14 respectively.

The supernatant from the treated CGCs and control CGCs were once again collected over the course of a week and assayed to measure the nitrite, total nitrite and glutamate concentrations. The metabolite levels in the retained treated CGC supernatant were again found to not be statistically different to concentrations in control, untreated CGC supernatants (compare figure 3.4.1.b B with figure 3.4.4. B).

3.4.5. Addition of peritoneal macrophage-conditioned medium

To investigate whether the survival enhancing effects observed with the MG-CM were unique or could be mimicked by the addition of medium conditioned by peritoneal macrophages (PM-CM), a related cell type, PM-CM was added to the neurones as previously described (figure 3.4.5.).

Addition of 7 DIV PM-CM resulted in enhanced survival levels compared with control (figure 3.4.5.). The level attained was less than that achieved following the addition of 7 DIV MG-CM but was comparable to the levels attained following the addition of 1 DIV MG-CM (figure 3.4.5.).
These results indicate that cells of the same lineage can enhance neuronal survival, but that the factors derived from the microglia are more efficacious than those released by the peritoneal macrophages.

**Figure 3.4.5. PM-CM enhances neuronal survival but with a lower efficacy than MG-CM.** Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with conditioned media retrieved from 7 day *in vitro* peritoneal macrophage cultures. Neuronal survival was assessed over the ensuing 7 days *in vitro*. Values are the mean number of live CGCs per field expressed as a percentage of CGC control cultures ± sem, where n=6 over 3 culture preparations. All levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point.
3.5. **Assessment of developmental stages of the CGC culture**

The neuronal cultures were further assessed by investigating the developmental stages of the cells present in both control and MG-CM treated cultures. Antibodies against specific developmental markers were then used to assess protein levels in neuronal lysates and to immunocytochemically assess the cultures (figure 3.5.a.). Antibodies used were; (1) anti-microtubule associated protein type 2 (MAP2), which plays a crucial role in the development and structure of nerve cells as these proteins are important for the assembly and stability of microtubules during neurite outgrowth (Shelanski et al., 1973). MAP2 expression is associated with differentiating and differentiated neurones; (2) anti-nestin, an intermediate filament protein which is expressed in neural progenitors and immature neurones but is absent from nearly all mature central nervous cells (Tohyama et al., 1993); and (3) anti-growth associated protein-43 (GAP43), which is an axonal membrane protein that is involved in neuronal outgrowth and synaptic plasticity of developing and regenerating neurones (Mahalik et al., 1992; Frey et al., 2000; Arnold et al., 2001). Expression of these proteins would indicate the existence of immature post mitotic neurones undergoing synaptogenesis. The expression patterns were investigated using immunocytochemistry and Western blotting.

Immunocytochemistry of cultures incubated with and without MG-CM revealed high levels of MAP2, nestin and GAP43 expression (figure 3.5.a. A). MAP2 expression was clearly discernable in the neurites and cell bodies, as was GAP43 expression. Nestin reactivity was predominantly observed in the astrocytes present in the cultures, however a proportion of neurones also displayed nestin expression. Western blotting for MAP2, nestin and GAP43 (figure 3.5.a. B) revealed that at both day 8 and day 14
Figure 3.5.a. CGCs cultures contain cells which represent neurones in all stages of development. Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with conditioned media retrieved from either 1 or 7 day in vitro microglial cultures as indicated. The expression of MAP2, nestin and GAP43 was subsequently assessed by (A) immunocytochemistry 24 hours after addition and (B) Western blot analysis of protein lysates, collected after 1 and 7 day incubation, resolved by SDS-PAGE. Blots shown are representative of three repeats performed for each antibody.
there was high expression of nestin, MAP2 and GAP43 in controls, MG-CM and LME-CM treated cultures. There was no discernible differences between the culture conditions when compared with β-actin, which was used to verify equal protein loading, suggesting that all the CGC cultures contains cells that represent all the major developmental stages of the cerebellum, namely progenitors and immature neurones, differentiating and differentiated neurones and neurones undergoing synaptogenesis.

During the development of the cerebellum it has been reported that β-actin expression decreases with age (Micheva et al., 1998). Therefore to clarify the use of β-actin to ascertain equal protein loading, blots were further appraised for expression of β-tubulin. β-tubulin expression has been shown to reach a maximal level in dissociated cerebellar granule neuronal cultures by day 7 in vitro (Przyborski and Cambray-Deakin, 1997) and would therefore highlight any differences in β-actin expression. Previous blots were reprobed and it was observed that the expression levels of β-tubulin correlated to the expression of β-actin and that there were no discernible differences over time (figure 3.5.b.). This confirms the use of β-actin as a method of ensuring protein loading in the experimental system employed within this thesis.

Figure 3.5.b. **Expression of β-actin and β-tubulin remains constant during neuronal period in culture.** Previous blots were stripped, reprobed and assessed by Western blot analysis, as described, for β-tubulin expression and compared with matched β-actin blots.
3.6. **EFFECTS OF CONDITIONED MEDIA ON PROLIFERATION**

It is known that granule cell precursors are present in the cerebella of rats during the first two postnatal weeks (Miyazawa et al., 2000), after which time they become fully differentiated. Furthermore, as discussed in section 3.5, the neuronal cultures were found to contain cells at all stages of development. It was therefore postulated that the MG-CM may be able to actively recruit these precursor cells and cause them to continue to proliferate and so increase the number of surviving neurones.

To test this hypothesis two different methodologies were used to quantify proliferation, (1) Ki-67 nuclear antigen expression and (2) a commercially available colorimetric kit, Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Firstly, the expression of the nuclear antigen Ki-67 was appraised by immunocytochemistry. Ki-67 is only expressed in proliferating cells during late G1, S, M and G2 phases of the cell cycle, cells in the resting state, G0, do not express the protein (Gerdes et al., 1991; Schluter et al., 1993) (3.5.1.). Secondly the ‘CellTiter’ kit results in the formation of a coloured formazan end product in the presence of dividing cells, thus allowing proliferation to be quantified.

**3.6.1. Neuronal Ki-67 expression**

MG-CM was added to 7 day *in vitro* cerebellar granule neurones as previously, and incubated for up to 7 days. The neurones were subsequently fixed after a further 1 and 7 days incubation, and immunocytochemically stained as described using an anti-Ki-67 antibody. Controls were also carried out where the neurones were stained without addition of the primary antibody but with addition of the secondary antibody only, thus allowing any non-specific interactions of the primary antibody to be evaluated. Ki-67
positive neurones were subsequently scored under light microscopy (figure 3.6.1. A). Neuronal whole cell lysates were also collected and proteins were separated using precast 5 % polyacrylamide gels (BioRad). The proteins, once transferred to PVDF membrane, were assessed by Western blotting for levels of Ki-67 expression (figure 3.6.1. B).

**Figure 3.6.1. Neuronal Ki-67 expression is enhanced following addition of MG-CM.** Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with conditioned media retrieved from 7 day *in vitro* microglial cultures. Ki-67 expression was subsequently assessed after 1 and 7 days incubation. (A) Immunocytochemically stained cultures were scored for number of Ki-67 positive neurones. Positive neurones displayed punctate staining around the nucleus. Values are the mean number of Ki-67 CGCs per field ± sem, where n=6 over 3 culture preparations. All levels of significance (one-way anova) are compared with values from untreated CGC controls at that time point. (B) Whole neuronal lysates were collected from MG-CM treated neuronal cultures and assessed by Western blotting for Ki-67 expression. Blots were re-probed with antibodies recognising β-actin to ensure equal protein loading. Blots shown are representative of three repeats performed.

![Graph](image1.png)  
**A**

![Graph](image2.png)  
**B**

<table>
<thead>
<tr>
<th>Day in vitro</th>
<th>Ki-67 positive neurones per field</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>14</td>
<td>40 ± 3</td>
</tr>
</tbody>
</table>

![Image](image3.png)

<table>
<thead>
<tr>
<th>Protein</th>
<th>CGC DIV 8</th>
<th>CGC DIV 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>fresh media</td>
<td>1 DIV MG-CM</td>
</tr>
<tr>
<td>β-actin</td>
<td>fresh media</td>
<td>1 DIV MG-CM</td>
</tr>
</tbody>
</table>

345 kDa  
42 kDa
The number of Ki-67 positives neurones was observed to be higher in 7 DIV MG-CM treated CGC cultures (figure 3.6.1. A). This could be observed after 24 hr incubation with the conditioned medium but was only found to be significantly higher following 7 day incubation. The Western blot analysis further confirmed the presence of elevated levels of Ki-67 in the cultures treated with both 1 DIV MG-CM and 7 DIV MG-CM (figure 3.6.1. B), observable with lysates collected at both CGC day in vitro 8 and 14. Again 7 DIV MG-CM appeared more efficacious in enhancing Ki-67 levels compared with 1 DIV MG-CM. The level of Ki-67 expression following the addition of 7 DIV LME-CM was comparable with that observed in control cultures.

3.6.2. ‘CellTiter’ proliferation assay

The Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay was a colorimetric assay measured at absorbance 492nm. The coloured end product, the formazan, has been shown to be directly proportional to the number of cells present (Mosmann, 1983; Cory et al., 1991). The solution was added to the conditioned media treated cells as described (section 2.2.11.).

Both the fresh microglial medium and fresh serum free medium showed a level of absorbance comparable with control (figure 3.6.2.), therefore indicating a low level of proliferation in control CGC cultures. Addition of MG-CM resulted in significantly higher absorbancies compared with control indicating an increased number of cells and hence increased proliferation. Furthermore, treatment with 7 DIV MG-CM and 7 DIV SF-CM enhanced the level of proliferation more than addition of 1 DIV MG-CM.
Figure 3.6.2. Neuronal proliferation is enhanced following MG-CM treatment. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either MG-CM, LME-CM or SF-CM and cultured for 24 hours. Neuronal proliferation was subsequently assessed using the 'CellTiter' kit. Values are the mean proliferation absorbance expressed as a percentage of CGC control cultures ± sem, where n=6 over 3 culture preparations. Levels of significance (one-way anova) are compared with values from untreated CGC controls.

To check that the effect was due to a microglial-derived factor the experiment was repeated using LME-CM instead of MG-CM. This showed that the level of proliferation was comparable with control levels at all timepoints (figure 3.6.2.). This suggests that diffusible factors in the MG-CM are of microglial origin and are able to enhance the proliferation of granule cells.
3.7. DISCUSSION

3.7.1. Survival of microglia in culture

It has been demonstrated that non-stimulated primary microglial cells can survive up to 7 days in cultures (figure 3.3.1.). It was also found that microglia could be cultured in serum-free medium (figure 3.3.6.). The composition of the microglial cultures was shown to be predominately microglia, 90.5 % ± 2.6 microglia at day 1 and 81.6 % ± 5.2 microglia at day 7, with only a low level contamination with astrocytes (table 3.3.2.a.). The number of ED-1 positive cells did not alter significantly over the 7 days in culture (table 3.3.2.a.). The number of GFAP-positive cells also remained low throughout the 7 days suggesting very little contamination of the preparation with astrocytes. However it is possible that any astrocytes present in the preparation may not have become GFAP-positive therefore the staining with vimentin was utilised to stain immature astrocytes (table 3.3.2.b.). Reactive microglia are also positive for vimentin therefore a double stain using isolectin B4, which is an established microglial marker (Streit and Kreutzberg, 1987; Boya et al., 1991), was also performed. Using this method it was established that the microglial cultures were pure cell cultures, containing 94.2 % ± 2.0 and 86.2 % ± 2.6 microglia at day in vitro 1 and 7 respectively (table 3.3.2.b.). A number of alternative markers for the identification of immature astrocytes could also have been employed including CD44 (Alfei et al., 1999), RC-2 (Thomas et al., 1996), RC-1 (Uesugi et al., 1996), 1D11 (Horie et al., 2000) or S100 alpha (Kahn et al., 1997) but using the stains described and the close corroboration of results achieved the microglial cultures used are pure cell cultures.

Through the use of flow cytometry (3.3.3.), it was shown that microglial cells possess a comparable reactivity to CD11b as peritoneal macrophages (figure 3.3.3.c.), thus
indicating that the cells are of the same lineage. However the microglial population could be distinguished from peritoneal macrophages by expression of CD45. The microglia exhibited low CD45 expression compared with peritoneal macrophages (figure 3.3.3.b.). This is in accordance to the expression profile observed by other investigators (Williams et al., 1992; Ford et al., 1995). It was also demonstrated that the microglia expressed downregulated levels of ED1 (figure 3.3.3.d.), which is indicative of less activated phenotype. Furthermore ED1 expression could be upregulated by activating the microglial cultures with LPS. The level of ED1 was found to increase during time in culture but could still be further upregulated by the action of LPS. This demonstrates that the unstimulated microglial cultures used in this study are a downregulated phenotype.

It was also shown that the microglia released NO\(^-\) into the culture medium, which was assessed by measuring the concentrations of the stable end products, nitrite and total nitrite (indicative of concentration of nitrate), in the culture medium (figure 3.3.4.a.). Over the course of a week in culture the amount of both nitrite and total nitrite released by the microglia increased but only the levels of total nitrite were found to be significant. However the expression of iNOS by Western blot was found to be low, though inducible by activation of the microglial cells with LPS (figure 3.3.4.b.). This is also indicative of a downregulated microglial phenotype. Furthermore whilst the microglia were seen to release glutamate, there was no significant increase in the amount released during the time in culture (figure 3.3.4.a.), and the amount released was less than that normally considered to be neurotoxic (Matthews et al., 2000; Montoliu et al., 2000; Plaitakis and Shashidharan, 2000). These results add further credence to the assignment of the microglial cultures as being downregulated, non-
reactive cell cultures as activated microglia have been previously shown to release high levels of both NO and glutamate (Kingham et al., 1999).

It has further been demonstrated that treatment of the microglial cultures with 10 mM L-leucine methyl ester for 24 hours is able to selectively deplete the number of microglia, whilst retaining approximately the same level of astrocyte contamination (figure 3.3.5.a, figure 3.3.5.b A and B). Therefore through the use of LME-CM any affects of the MG-CM could be positively attributed to the microglia and not to any contaminating cell types present in the preparation, such as astrocytes.

3.7.2. Addition of conditioned media

Results presented have shown that the addition of MG-CM to the primary CGCs is able to increase the number of live neurones, with the 7 DIV MG-CM being more efficacious that the 1 DIV MG-CM (figure 3.4.1.a. A). This suggests that there is a diffusible factor present in the MG-CM, which accumulates potency or concentration with time. A comparable result was observed using the Hoechst apoptosis assay, where the number of apoptotic CGCs decreased following treatment with the MG-CM, again the 7 DIV MG-CM was more effective than the 1 DIV MG-CM (figure 3.4.1.a. B).

Following the addition of the MG-CM there was no significant increase in the levels of nNOS and iNOS expression (figure 3.4.1.b. A) or the concentrations of nitrite, total nitrite and glutamate present in the retained CGC supernatant (figure 3.4.1.b. B). It seems likely that nitrite and nitrate represent the stable end products of NO which do not exert any neurotoxicity damage in this system. The glutamate concentrations
observed were comparable with normal physiological levels, which is less than 20 μM (Matthews et al., 2000). As glutamate is normally only neurotoxic at elevated levels in excess of 100 μM (Matthews et al., 2000; Montoliu et al., 2000; Plaitakis and Shashidharan, 2000), it is therefore not surprising that under these conditions the presence of the glutamate is not detrimental to neuronal survival. It may be possible that the NO and glutamate released by the microglia is inducing the release of a soluble factor from the microglia themselves and that it is this which is able to enhance neuronal survival.

Conditioned media collected from the microglial preparation maintained in serum-free media were also able to enhance neuronal survival (figure 3.4.2.) which demonstrates that the active factor(s) are not derived from the foetal calf serum routinely used in the medium, and are as a result of the presence of the microglia.

It has been further demonstrated that the addition of conditioned media collected from LME depleted microglial cultures results in survival levels comparable with untreated CGC controls (figure 3.4.3. A). This indicates that the soluble factor responsible for enhancing neuronal survival present in the MG-CM, but not in the LME-CM, is attributable to the microglia. The presence of low level contaminating cells in the primary microglia cultures have no observable effect on the survival of the CGCs. Again no effect was observed on metabolite concentrations in the retained supernatants (figure 3.4.3. B).

The dependency of the MG-CM potency on the cell plating density of the microglial cultures from which it was collected was also investigated (figure 3.4.4.). Results
indicated that if the plating density was reduced by a factor of 4 then survival levels were reduced to near control levels. However doubling the plating density did not significantly further increase the survival levels above that of the standard, medium density plating, this suggests that a saturation point had been achieved, where no greater benefit could be observed. It was therefore determined that the optimal plating concentration lies around $0.6 \times 10^6$ cells/ml. This concentration gives an approximate neurone to microglia ratio of 10:1. Such a ratio is also physiologically relevant as the neurone to microglia ratio of the whole brain is taken to be approximately 10:1 (Leuba and Garey, 1989; Feldman et al., 1997), and in the cerebellum in particular is approximately 25:1 (Braitenberg, 1967; Lawson et al., 1990). This shows that the effect observed in this model is in the correct order of magnitude compared with the in vivo situation.

It was also found that the addition of PM-CM could also enhance neuronal survival but to a lesser extent than MG-CM (figure 3.4.5.). This suggests that cells of the same lineage, i.e. microglia and peritoneal macrophages, can enhance neuronal survival but that factors released by the microglia are more effective.

In addition it was hypothesised that the increased survival was as a result of an increased number of neurones due to neuronal proliferation. Analysis, by immunocytochemistry and Western blotting, showed the presence of neurones in all stages of development (figure 3.5.a.) including nestin positive cells which is a common marker used for precursor cells. Therefore assessment of neuronal culture proliferation, in both control and conditioned medium treated cultures was performed. The results presented demonstrate that the addition of MG-CM leads to enhanced
proliferation above that observed in the control cultures (figure 3.6.1, and figure 3.6.2.). It is accepted that granule precursor cells are still present in the cerebellum at the time the neurones are isolated, thus it is possible that the proliferating cells represent these precursor cells. Under control conditions, where the CGCs had been untreated, there was a basal level of neuronal proliferation, as shown by Ki-67 expression, as measured by immunocytochemistry and Western blotting (figure 3.6.1.), and by the ‘CellTiter’ assay (figure 3.6.3.). Following the addition of the MG-CM the level of proliferation was observed to significantly increase as measured by both assays. The 7 DIV MG-CM resulted in a level of enhancement above that of the 1 DIV MG-CM, which is consistent with earlier results showing an increased survival following treatment with the 7 DIV MG-CM compared with the 1 DIV MG-CM.

The proliferation observed appears to be insensitive to AraC. AraC is a pyrimidine anti-metabolite that prevents cell proliferation by inhibiting DNA synthesis and induces CGC apoptosis at high concentrations, eg 300 μM (Dessi et al., 1995; Ishitani et al., 1998). However, in our cultures, we use 20 μM Ara-C which, whilst sufficient to limit astrocytic proliferation, may not inhibit CGC proliferation and survival over the time frame of our experiments.

It can be postulated that more than merely enhancing the survival of the neurones, the MG-CM is able to actively promote the proliferation of the neurones. It is also possible that both of these effects are being carried out in conjunction with each other. Whether these effects are due to separate factors acting to enhance survival whilst others enhance proliferation, or whether the same factors enhance survival and proliferation has not been determined. Furthermore it is plausible that the enhanced
survival is as a result of enhanced proliferation and only one pathway is been manipulated.

**Figure 3.7.2. Schematic representing effects of MG-CM on cultured CGCs.** Diffusible factors released by microglia into culture media are able to effect neuronal survival and proliferation. The factors may be acting either (1) independently on survival and proliferation pathways, (2) directly able to affect both survival and proliferation or (3) the factors may be affecting proliferation which in turn enhances levels of survival.

Microglia have in the past been considered to be detrimental to the survival of neurones through the release of a plethora of cytotoxic agents (Giulian and Baker, 1986, Giulian et al., 1986; Colton, 1987). However, more recently microglia have been demonstrated to release an array of neurotrophic compounds (Mallat and Chamak, 1994). Furthermore a survival-enhancing effect of MG-CM has been reported by a number of investigators.
Microglial cells have been reported to be able to protect cerebellar granule neurones from apoptosis via a reciprocal neurone-microglia interaction (Polazzi et al., 2001). In this study the neurones were initially cultured in medium containing high K\(^+\) (25 mM), once differentiated apoptosis was initiated by exposure to medium containing low K\(^+\) (5 mM). If unstimulated microglia were also added at the time of the media switch, and co-cultured, then the level of neuronal apoptosis was reduced. Furthermore it was observed that this effect could be mimicked by conditioning the low K\(^+\), apoptosis-inducing medium by 48 hour exposure to unstimulated microglial cells. If the medium was conditioned for only 24 hours then the neuroprotective capability of the microglia was not conferred to the conditioned medium. Interestingly however if the medium was double-conditioned, first by exposure to apoptotic neurones and then to microglial cells, unknown signal(s) released by the apoptotic neurones, could confer to 24 hour conditioned medium a strong neuroprotective action, similar to that observed in the co-culture experiments. Here a comparable effect has been observed where the 7 DIV MG-CM was able to enhance the number of non-apoptotic neurones in the cultures with a greater efficacy than the 1 DIV MG-CM.

Microglia have been shown to release soluble factor(s) which support the survival of cerebral cortical neurones (Zhang and Fedoroff, 1996). It was observed that the MG-CM had a dose related effect on neuronal survival and above a certain threshold neuronal survival could not be further potentiated. Jonakait et al. (1996) have shown that MG-CM is able to promote cholinergic (Ch-AT) survival in the basal forebrain and Nakajima et al. (1989) reported that MG-CM had a neurotrophic effect on cultured rat neocortical neurones. These workers further demonstrated that the MG-CM could
not only also enhance mesencephalic neuronal survival and maturation but could furthermore promote neurite extension in culture (Nagata et al., 1993). They identified that the neurotrophic molecule in the MG-CM was heat labile, suggesting that it might be a protein factor and discounted the possibility that the effect was due to the presence of NGF, IL-1β, IL-6 or TNF-α.

As discussed some investigators have found MG-CM to be neurotoxic (Zietlow et al, 1999), including that collected from unstimulated microglia. This observation may have been due to a high level of basal activation in the microglial cultures used, as in this particular study no difference was observed between activated microglia and unstimulated microglia. Such discrepancies may serve to highlight the probability that the actions of microglia are tightly controlled in the brain and are able to perform different functions under different circumstances.
4. **Signal Transduction Pathways Regulated By Microglial-Conditioned Medium**
4.1 INTRODUCTION

During the past couple of decades there has been an explosive growth of knowledge about how neuronal survival is controlled (review Goldberg and Barres, 2000). These survival mechanisms commonly depend on extrinsic signalling by other neighbouring and target cell types (Burek and Oppenheim, 1996; Jacobson et al., 1997). These signals, which are commonly peptide ligands such as neurotrophins, growth factors, and cytokines, function by binding to, or affecting the activation of, transmembrane receptors which in turn activate one or more intracellular signalling cascade. Once activated these cascades can modulate the intrinsic apoptosis and proliferation pathways and mediate the fate of the neurone. (Raff, 1992; Raff et al., 1993; Burek and Oppenheim, 1996; Jacobson et al., 1997; Nunez and del Peso, 1998; Vaux and Korsmeyer, 1999). Indeed it has been shown that the very survival of neurones, both in vivo and in vitro, depends on this continuous inhibition of the intrinsic cell suicide machinery by trophic signals from neighbouring cells (Raff, 1992; Jacobson et al., 1997).

A number of intrinsic cell pathways are intimately involved in neuronal survival, the major two being the ras/raf/MAP kinase pathway and the PI-3 kinase/Akt pathway (Nunez and del Peso, 1998; Datta et al., 1999), both of these pathways function to modulate survival by adjusting both prenuclear mechanisms and nuclear transcription (Datta et al., 1999). Neuronal depolarisation is also able to significantly affect neuronal survival, indicating an important role for neurotransmitter receptors such as the ionotropic (iGlu) and metabotropic (mGlu) glutamate receptors (Barger, 1999; Blanc et al., 1999; Vaillant et al., 1999). Other pathways of interest include the Notch pathway which has the ability to directly modulate neuronal proliferation and mediate...
cell-to-cell communication (Artvanis-Tsakonas et al., 1999; Wechsler-Reya and Scott, 1999).

As preluded to, each of these pathways are extrinsically modulated, to varying levels, by tropic factors released not only by neighbouring neurones by also by surrounding glial cells. As microglia are ubiquitously found throughout the CNS (Lawson et al., 1990), and are known to release a plethora of growth factors (see table 1.2.5.2.), it would seem plausible to assume that these microglial-derived factors could assist neuronal survival. In the previous results chapter it was established that microglial factors were able to support and indeed enhance both neuronal survival and proliferation, an observation which is supported by the work of others (Nakajima et al., 1989; Jonakait et al., 1996; Zhang and Fedoroff, 1996; Wantanabe et al., 2000; Polazzi et al., 2001). Therefore the aim of this chapter was to explore the possible mechanisms by which the microglial-derived factors were functioning.
4.2. SUMMARY OF RESULTS

The mechanism by which microglial-conditioned media enhances neuronal survival and proliferation was investigated in the research presented in this chapter. It was found that a component of the effect exerted by the MG-CM involves modulation of Notch signalling as the inhibition of Notch, through the addition of neutralising antibodies had little significant effect on control culture survival and proliferation but reverted the survival and proliferation of MG-CM treated cultures back to levels associated with control conditions. It was further postulated that this effect may be due to chelation of extracellular calcium. In addition it was found that the MG-CM also is able to modulate pertussis toxin sensitive G-protein coupled receptors, an effect which was found to be negatively linked to adenylate cyclase. Consistent with this result, effects were also observed following the inhibition of metabotropic glutamate receptors, which are G-protein coupled receptors. Other effects were also observed following the blockade of ionotropic glutamate receptors and L-type calcium channels. The antagonism of the Trk receptors was also found to dissipate the enhancement of neuronal survival effect associated with MG-CM. Downstream targets would appear to include the MAPK cascade, along with the Akt pathway.

These results highlight the fact that an individual pathway does not act independently, instead each cascade is an integral member of an overall network of pathways which maintain the cell. It is therefore not surprising that the potentially wide number of factors released by the microglia are able to modulate a variety of pathways and indeed the overriding effect of the MG-CM on neuronal survival and proliferation may be due to a cohort of factors and not one specific factor.
4.3. **Modulation of signalling pathways**

As discussed in the introduction, a wide number of signalling pathways have implications for neuronal survival and proliferation (see section 1.4.). The effects of these pathways generally result in altered gene expression, which in turn alters protein synthesis affecting the development of the neurone. To determine if the active factors in MG-CM acted via the modulation of such signalling pathways a number of agonists and antagonists were added, to both control and microglial-conditioned medium treated neuronal cultures, and the effect on neuronal survival and proliferation was assessed. As the 7 DIV MG-CM had been found to be most effective at enhancing both neuronal survival and proliferation only the effect using the 7 DIV MG-CM were assessed and compared with matched control CGC cultures. The same experimental procedure was performed as described previously (section 2.2.8.), where neurones were cultured for 7 days, after which time 250 ml of media was removed and replenished with either (i) fresh CGC media or (ii) 7 DIV MG-CM. The experimental wells were also supplemented, as appropriate, with agonists and antagonists.

**4.3.1. Modulation of Notch signalling**

Notch signalling is an important pathway by which cell-cell contacts regulate whether a cell is maintained in the proliferative cycle or whether terminal differentiation is initiated. The activation of the Notch receptor by one of its ligands (expressed on a neighbouring cell) prevents a cell from differentiating and furthermore downregulates its own expression of the ligand thereby promoting lateral inhibition. The presence and nature of extracellular factors which are also involved in the regulation of this pathway remains to be determined, although growth factors and extracellular calcium depletion have been observed to exert effects (Rand et al., 2000; Faux et al., 2001). To
investigate whether MG-CM functions by altering the association of Notch and its ligands, a neutralizing antibody against the extracellular domain of delta1 (anti-delta1), a Notch receptor known to be expressed on CGCs (Solecki et al., 2001), was added to both control and conditioned-medium treated cultures. Furthermore, the effects of extracellular calcium depletion, via EGTA-calcium chelation, was also appraised.

Initially, to confirm the expression of the active intracellular domain (ICD) of the Notch receptor, and also the expression of one of its ligands (delta1), neuronal cell lysates were immunoprecipitated and subjected to Western blot analysis. Both control and 7 DIV MG-CM treated cultures expressed both the active Notch ICD and the ligand delta (figure 4.3.1.a.). It proved difficult to ascertain whether the relative levels of protein were significantly altered either between control and 7 DIV MG-CM cultures or over time. However the Western blots clearly demonstrate the likelihood that there is certainly the capacity for active Notch signalling in the neuronal cultures. A control lane, containing the agarose A/G beads, was also assessed to confirm that the observed bands were not an artefact due to the beads.

**Figure 4.3.1.a. Neuronal cultures express delta and active Notch ICD.** To confirm the expression of active Notch and its ligand, delta, neuronal cell lysates, collected at CGC day 8 and 14, were immunoprecipitated in the presence of rabbit anti-cleaved Notch1 and goat anti-delta1 combined with protein A/G agarose beads. The resultant pellet was electrophoresed and subjected to Western blot analysis.
To explore the influences of MG-CM on Notch signalling the antibody against deltal was initially added simultaneously to the change in media, either fresh medium or 7 DIV MG-CM. The survival and proliferation was subsequently assessed after 24 hour incubation. In conditioned-medium treated cultures the addition of anti-delta1 depleted survival in a concentration-dependent manner (figure 4.3.1.b Ai). Addition of between 0.01 and 0.02 μg/ml significantly reduced survival to control levels (one-way annova analysis, p<0.001), whilst higher concentrations resulted in levels below that observed in control cultures suggesting that the optimum concentration was in this range. In control cultures addition of anti-delta1 up to 0.06 μg/ml had no effect on survival compared with untreated control (figure 4.3.1.b. Ai). A similar effect was also observed on proliferation where the survival of cultures treated with MG-CM was depleted in a concentration-dependent manner whilst control cultures were comparatively unaffected (figure 4.3.1.b. Aii). Further statistical analysis of variation (two-way anova) indicated that addition of anti-delta1 resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.01).

To ascertain whether the primary effect was due to a modulation of sites on the neurones or due to modulation of factors present in the conditioned medium the anti-delta1 was used to pre-treat either the neurones or the MG-CM for 1 hour prior to assay set up. Antibody was added, either to neurones or MG-CM, to result in the same end concentration as indicated in the first experiments. If the neurones were pre-treated with anti-delta 1 prior to media replenishment then the subsequent addition of 7 DIV MG-CM resulted in depletion of neuronal survival in a concentration-dependent manner (figure 4.3.1.b. Bi). The level of proliferation was reduced to control levels regardless of antibody concentration (figure 4.3.1.b. Bii). If the 7 DIV MG-CM was
Figure 4.3.1.b. MG-CM modulates Notch signalling. To assess involvement of Notch signalling the concentration-response to addition of an antibody against delta1, a Notch ligand, to control and MG-CM treated cultures was assessed. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with appropriately treated media. (A) Anti-delta1 was added to the cells simultaneously with media replacement. (B) CGCs were pre-treated with anti-delta1 for 1 hr prior to media replacement. (C) MG-CM was pre-treated with anti-delta1 for 1 hr prior to addition to the cells. Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of anti-delta1 resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.01). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
pre-treated with the anti-delta1 for 1 hour and then added to untreated CGC cultures
then again neuronal survival was depleted in a concentration-dependent manner (figure
4.3.1.b. Ci) as was proliferation (figure 4.3.1.b. Cii).

To determine that the effects observed were a result of direct pathway modulation and
not due to non-specific interactions, the anti-delta1 was denatured by boiling before
adding to the neurones. To further clarify that the effects were also not as a result of
steric hindrance due to the presence of a protein, a separate antibody was also added
which has no known external receptor, anti-p53. The functions of this antibody were
also denatured by boiling prior to addition to the neurones.

**Figure 4.3.1.c.** **MG-CM modulation of Notch signalling is specific.** To confirm the
effects following addition of anti-delta1 were as a result of direct modulation of Notch
activation, and not due to non-specific interactions/steric hindrance, the antibody was heat
denatured and added to both control and MG-CM treated cultures. As a control a separate
antibody, against a protein with no known external receptor (p53) was added to both control
and MG-CM treated cultures. Neurones were cultured for 7 days, after which 250 μl of media
was removed and replaced with appropriately treated media. After 24 hour incubation the
level of (A) neuronal survival and (B) neuronal proliferation was assessed. Values are the
mean number of live neurones or proliferation absorbance, expressed as percentage
difference from CGC controls, ± sem, where n=6 over 3 culture preparations. All levels of
significance are compared with values from untreated CGC controls.

- no added antibody  
- anti-delta1 boiled  
- anti-p53  
- anti-p53 boiled
Following 24 hour incubation the level of neuronal survival (figure 4.3.1.c. A) and proliferation (figure 4.3.1.c. B) were assessed in both control and 7 DIV MG-CM treated cultures. Addition of boiled anti-delta1, anti-p53 and boiled anti-p53 had no effect on survival or proliferation in either control of MG-CM treated cultures indicating that the affects observed with the anti-delta1 were true effects.

As discussed it has been demonstrated that chelation of extracellular calcium is able to enhance the activation of the Notch pathway (Rand et al., 2000), therefore it was of interest to determine whether MG-CM may be functioning in such a manner, and indeed if the chelation of calcium in control cultures could result in survival levels comparable with those achieved in MG-CM treated cultures. The concentration response to the addition of EGTA, a calcium chelator was therefore appraised in both control and 7 DIV MG-CM treated cultures.

In control cultures the addition of EGTA enhanced both survival (figure 4.3.1.d. Ai) and proliferation (figure 4.3.1.d. Aii) in a concentration-dependent manner (p<0.05). The addition of >50 μM EGTA caused the neuronal survival levels to be comparable with those normally achieved with the microglial-conditioned medium. The levels of proliferation were not enhanced to the same level as those of 7 DIV MG-CM treated cultures but there was a discernible increase. In 7 DIV MG-CM treated cultures the addition of EGTA had relatively little effect on neuronal survival (figure 4.3.1.d. Bi) or proliferation (figure 4.3.1.d. Bii).

These results demonstrate that chelation of extracellular calcium can enhance the survival of control cultures but cannot further enhance the survival of MG-CM treated
cultures. This suggests that the factors present in the MG-CM have enhanced neuronal survival to a maximal level and they may be functioning, at least in part via a mechanism that enhances Notch receptor activity possibly by chelating calcium or via modulation of the receptor-ligand interaction.

Figure 4.3.1.d. Chelation of extracellular calcium enhances neuronal survival but has minimal influence on neuronal proliferation. To investigate whether the MG-CM exerts its effect by calcium chelation and whether the survival and proliferation of control CGCs cultures could be enhanced to a level in line with MG-CM treated cultures the concentration-response effect of the addition of a calcium chelator, EGTA, was assessed. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either (A) fresh media or 7 DIV MG-CM further supplemented with EGTA. Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage above CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of anti-delta1 resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.01). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
4.3.2. Modulation of cAMP/PKA and G-protein coupled receptor pathways

The survival and proliferation of precursor cells in the cerebellum can be inhibited by enhanced cAMP and PKA activity (Hynes et al., 1995; Epstein et al., 1996). Therefore to investigate the involvement of these signalling molecules in the current findings, cAMP/PKA mediated pathways were selectively activated and deactivated by the addition of forskolin and H89 respectively (Chijiwa et al., 1990). Since the production of such second messenger signalling molecules can be as a result of a number of upstream events including the activation of G-protein coupled receptors (GPCRs) (Luttrell et al., 1999), the effect of uncoupling GPCRs from their downstream pathways by the addition of pertussis toxin (PTX) was also investigated.

The addition of forskolin, to enhance cAMP production and, by inference, PKA activity, caused the survival levels of the MG-CM treated cultures to be reverted to control levels in a concentration-dependent manner (figure 4.3.2. Ai). Whilst addition of forskolin to control cultures had only a relatively minor effect on survival levels, indeed statistical analysis of variation (two-way anova) found that forskolin exert significantly different effects on control and 7 DIV MG-CM treated cultures (p<0.01). The addition of forskolin had no effect on the proliferation of control CGCs but significantly inhibited the MG-CM treated CGCs, reverting the proliferation to control (figure 4.3.2.Aii). This suggests that MG-CM may enhance pathways in the CGC cultures which suppress cAMP production.

If the enhancement of cAMP can inhibit the MG-CM promoted CGC survival and proliferation, we might expect that inhibition of PKA activity, which is downstream of
cAMP production, would have the opposite effect. The addition of H89, to inhibit PKA activity presumably without affecting cAMP levels, caused the survival of both MG-CM treated and control CGCs to be enhanced (figure 4.3.2. Bi) with the greatest effect being exerted on control cultures. This is thus the reverse of enhancing cAMP production with forskolin (figure 4.3.2. Ai). Inhibition of PKA had no effect on the proliferation of control cultures (figure 4.3.2. Bii). However at high concentrations of H89 the proliferation level of MG-CM treated cultures was reduced to control levels (figure 4.3.2. Bii). This would appear to be the opposite of what might be expected given that forskolin (and thus enhanced cAMP production and presumably enhanced PKA activity) also inhibited MG-CM induced CGC proliferation (figure 4.3.2. Aii). However since adenylate cyclase can be modulated by different pathways to produce an overall decrease or increase in cAMP production it may be that survival is modulated by one pathway whilst proliferation is regulated by another.

Since the production of cAMP can be activated or inhibited by the activation of plasma membrane receptors coupled to G-proteins, it was determined whether pertussis-toxin sensitive G-proteins were involved in the MG-CM promotion of CGC survival and proliferation. Uncoupling of PTX sensitive G proteins from their downstream pathways by a 24 hour treatment with PTX inhibited MG-CM induced CGC survival and proliferation to control levels, with controls being unaffected (figure 4.3.2. Ci and Cii). This would seem to suggest that the enhanced survival and proliferation observed following the treatment with the MG-CM may be modulated via PTX sensitive GPCRs which are negatively coupled to adenylate cyclase since enhanced cAMP with forskolin is not permissive for cell survival and proliferation (figure 4.3.2. A).
Figure 4.3.2. MG-CM acts via PTX sensitive G-protein coupled receptor pathways and affects cAMP/PKA signalling. To investigate whether MG-CM acts via a G-protein coupled receptor and results in modulation of cAMP/PKA levels, the concentration-response to specific agonists and antagonists was assessed. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh media or 7 DIV MG-CM, further supplemented with either (A) forskolin (cAMP/PKA agonist); (B) H89 (cAMP/PKA) antagonist or (C) 24 hr treatment with pertussis toxin (PTX; uncouples pertussis toxin sensitive GPCR). Following 24 hr incubation the level of neuronal (i) survival and (ii) proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of forskolin, H89 or PTX resulted in a significantly different interaction on survival and proliferation in control and 7 DIV MG-CM cultures (p<0.05). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
4.3.3. **Modulation of glutamate receptors and calcium channels**

Glutamate receptors, which are highly responsive to the major neurotransmitter of the brain, namely glutamate, are known to play an important function in neuronal development. Stimulation of these receptors can result in altered survival and proliferation via a number of signal transduction pathways. As neurones express both ionotropic (iGlu) and metabotropic (mGlu) glutamate receptors the effect of modulating these receptors was investigated to ascertain whether the MG-CM may be functioning to either enhanced or inhibit their action. Also cerebellar granule neurones express a number of different voltage operated calcium channels (VOCC) including L-, N-, P/Q- and R type (Bossu et al., 1994; Graham and Burgoyne, 1995; Randall and Tsien, 1995). The expression profile of these channels changes during development both *in vivo* and *in vitro* (Rossi et al., 1994; Parri and Lansman 1996; Harold et al., 1997), suggesting specific functional roles for each subtype. In the early stages of development, around the time scale investigated in the course of this thesis, L-type calcium channels are known to predominate (Rossi et al., 1994; Pocock et al., 1995), therefore the modulation of L-type calcium channels was investigated. Furthermore the different glutamate receptors families are known to have interactions with L-type channels (Chavis et al., 1995; Kimura et al., 1999; Rajadhyaksha et al., 1999; Yatsushiro et al., 2000; Linn and Gafka, 2001).

4.3.3.1. **Ionotropic glutamate receptors and L-Type calcium channels**

In the first instance the modulation of the iGlu receptor family subtypes, (both NMDA and AMPA receptors), and L-type VOCC was investigated by the addition of selective antagonists (10 μM MK801, 20 μM CNQX and 500 nM nifedipine respectively). Cultures, treated with 1 DIV MG-CM, 7 DIV MG-CM or fresh medium, were
supplemented with the addition of the appropriate antagonist. Neuronal survival was subsequently assessed at CGC day in vitro 7 and 14 (figure 4.3.3.1.a).

In control cultures, where fresh medium was added, inhibition of both NMDA receptors and AMPA receptors had no significant effect on survival at either day 8 (figure 4.3.3.1.a. A) or day 14 (figure 4.3.3.1. B). Inhibition of L-type VOCC was severely detrimental to neuronal survival demonstrating the vital role of these channels (figure 4.3.3.1. A and B). In both 1 DIV MG-CM and 7 DIV MG-CM treated cultures blockade of NMDA receptors also had no effect on the enhanced level of neuronal survival associated with the addition of the MG-CM, however inhibition of AMPA receptors and L-type VOCC resulted in survival levels below that in control cultures (figure 4.3.3.1. A and B).

These results suggest that the MG-CM may be functioning via modulation of AMPA receptors and L-type VOCC. To further investigate the roles of these the concentration-response effect of the addition of CNQX and nifedipine was appraised. Cultures were either treated with fresh medium or 7 DIV MG-CM supplemented with the appropriate antagonist. Neuronal survival and proliferation was subsequently assessed after 24 hours (figure 4.3.3.1.b.).

The blockade of AMPA receptor in MG-CM treated cultures depleted the neuronal survival in a concentration response manner (figure 4.3.3.1.b Ai). Indeed, addition of 2.5-10 μM CNQX was sufficient to reduce neuronal survival of MG-CM treated cultures to control levels, whilst having minimal effect on control cultures. At
concentrations above 10 μM the survival of both control and MG-CM treated cultures was hampered.

**Figure 4.3.3.1.a. Modulation of AMPA receptor and L-Type VOCCs affects the potency of MG-CM, but NMDA receptor modulation does not.** The involvement of ionotropic glutamate (iGlu) receptor and L-type voltage operated calcium channels (VOCC) stimulation under control conditions and following MG-CM treatment was assessed by the addition of specific antagonists. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh CGC media, 1 DIV MG-CM or 7 DIV MG-CM which had been further supplemented the appropriate antagonist; 10 μM MK801 (NMDA iGlu receptor antagonist), 20 μM CNQX (AMPA iGlu receptor antagonist), 500 nM nifedipine (nif) (L-type VOCC antagonist). The level of neuronal survival was assessed at CGC day in vitro (A) 8 and (B) 14. Values are the mean number of live neurones, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. One-way anova analysis was performed and all levels of significance are compared with values from untreated CGC at that time point.

![Graph A](image1)

![Graph B](image2)
A comparable effect was observed on proliferation where again addition of 2.5-10 μM CNQX inhibited neuronal proliferation of MG-CM treated cultures back to control whilst having no significant effect on control cultures (figure 4.3.3.1.b. Aii).

The inhibition of L-type VOCC also depleted neuronal survival and proliferation of MG-CM treated cultures in a concentration-specific manner (figure 4.3.3.1.b. Bi and Bii). Addition of between 10-50 nM was sufficient to deplete MG-CM survival levels to control, whilst not affecting the control survival levels (figure 4.3.3.1.b. Ai). Whilst addition of 1-500 nM nifedipine depleted the proliferation of MG-CM treated cultures to control whilst having no significant effect on control culture proliferation (figure 4.3.3.1.b. Aii).

These results confirm that the MG-CM exerts some function via both the AMPA receptor and the L-type VOCC. It has been shown that AMPA receptors can modulate the activity of L-type VOCC (Rajadhyaksha et al., 1999) so the effect observed here may be linked to this phenomenon.
Figure 4.3.3.1.b. Further modulation of AMPA receptor and L-Type VOCCs following treatment with MG-CM. The involvement of AMPA iGlu receptors and L-type VOCC stimulation was further investigated by observing the concentration-response to addition of specific antagonists in control and MG-CM treated cultures. Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with either fresh CGC media or 7 DIV MG-CM which had been further supplemented the appropriate antagonist, (A) 0-50 µM CNQX (AMPA iGlu receptor antagonist) or (B) 0-1000 nM nifedipine (NIF; L-type VOCC antagonist). Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of CNQX or nifedipine resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

□ control ■ 7 DIV MG-CM
4.3.3.2. Metabotropic glutamate receptors

To investigate the effect of mGlu receptor modulation on neuronal survival and proliferation in control and 7 DIV MG-CM treated cultures a number of specific agonists and antagonists were added. As CGCs express all groups of mGlu receptors (Catania et al., 1994; Prezeau et al., 1994), specific activators and inhibitors of group I, II and III were added, namely ADA and AIDA, DCGIV and MCCG, AP4 and MAP4 respectively (Schoepp et al., 1999). Cultures, treated with 7 DIV MG-CM or fresh medium, were supplemented with the addition of the appropriate agonist or antagonist. Neuronal survival and proliferation was subsequently assessed after 24 hours (figure 4.3.3.2.a.).

Modulation of mGlu receptors which the different agonists and antagonists had no significant effect in control cultures on neuronal survival (figure 4.3.3.2.a. Ai), although modulation of group II and group III receptors reduced the level of proliferation in control cultures (figure 4.3.3.2.a. Aii). In 7 DIV MG-CM treated cultures addition of group I and group III receptor agonist or antagonists resulted in survival levels comparable to untreated control cultures, as was the case with inhibition of group II with MCCG (figure 4.3.3.2.a. Bi). In contrast activation of group II mGlu receptors with DCG IV did not alter the enhanced-survival normally associated with MG-CM treatment (figure 4.3.3.2.a. Bi). Modulation of all mGlu receptor groups reduced proliferation in 7 DIV MG-CM, reverting the level to control (figure 4.3.3.2.a. Bii).
Figure 4.3.3.2.a. Modulation of mGlu receptors alters the effect of MG-CM on both neuronal survival and proliferation. The involvement of metabotropic glutamate (mGlu) receptor stimulation in control and MG-CM treated cultures was assessed by the addition of specific agonists and antagonists. (A) Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh CGC media or 7 DIV MG-CM which had been further supplemented with mGlu receptor subclass agonists and antagonists; 250 μM ADA, 250 μM AIDA (group I mGlu receptor agonist and antagonist), 500 nM DCGIV, 500 μM MCCG (group II mGlu receptor agonist and antagonist), 100 μM AP4 or 500 μM MAP4 (group III mGlu receptor agonist and antagonist). Following 24 hr incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of mGlu agonists and antagonists resulted in significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
These results show that in MG-CM treated cultures the activation or inhibition of the different mGlu receptor groups exerts a potent effect on neuronal survival. The activation of group I mGlu receptor with ADA reduced neuronal survival more effectively than the addition of the antagonist AIDA. This suggests that modulation of group I is detrimental to survival and that in particular the neurones are most sensitive to over stimulation. With group III mGlu receptor modulation, both stimulation and inhibition, with AP4 and MAP4 respectively, decreased neuronal survival levels comparable with that of control. This suggests that the neurones are equally sensitive to both stimulation and inhibition of group III mGlu receptors. With group II mGlu receptor modulation only inhibition had any effect. If the receptor was stimulated through the addition of DCGIV then the normal enhanced level of survival was still observed following MG-CM treatment, however if the receptor was antagonised with MCCG then the level of survival was depleted to control, suggesting that the MG-CM treated neurones are only sensitive to group II inhibition.

A number of these results were not as would have been predicted. Previous experiments, using forskolin and H89 to increase and decrease cAMP levels respectively, had shown that enhanced cAMP resulted in decreased survival, whilst decreased camp caused increased survival. Upon this premise it would seem reasonable hypothesise that activation of group II and group III mGlu receptors, which would result in decreased levels of PKA and so cause an enhancement of survival, especially in control cultures. However DCGIV and AP4, group II and group III agonist respectively, had no significant effect on control cultures, and furthermore the addition of AP4 actively reduced the survival in 7 DIV MG-CM treated cultures. Conversely it would have been assumed that the addition of antagonists to group II and
group III mGlu receptors, MCCG and MAP4 respectively, would result in enhanced cAMP levels and so be detrimental to survival. Such an effect was observed in conditioned medium treated cultures but no effect was found on control cultures. These results would therefore seem to imply that the effect of the 7 DIV MG-CM was not mediated via group II or group III mGlu receptors.

To further investigate the effect of mGlu receptor modulation antibodies against specific receptor subtypes were used to block their function. The antibodies utilised were against group I mGlu receptors, anti-mGluR1 and anti-mGluR5; group II mGlu receptors, anti-mGluR2/3; and group III mGlu receptors, anti-mGluR4a. Cultures, treated with 7 DIV MG-CM or fresh medium, were supplemented with the addition of the appropriate mGlu receptor antibody, at either 0.02 or 0.2 µg/ml. Neuronal survival and proliferation was subsequently assessed after 24 hours (figure 4.3.3.2.b.).

In control cultures blocking the group I and group II mGlu receptors with the antibodies had no significant effect on survival levels, however the blocking of group III mGlu receptor, mGluR4a, was detrimental to neuronal survival (figure 4.3.3.2.b. Ai). Proliferation levels of control cultures were impeded following the addition of high concentrations of group I and group II antibodies, also following inhibition of group III mGlu receptors (figure 4.3.3.2.b. Aii). In 7 DIV MG-CM treated cultures only the blocking of group III receptors severely decreased the neuronal survival, indeed to a level below control, the blockade of the other receptor subtypes was only marginally harmful to survival levels (figure 4.3.3.2.b. Bi). Addition of all the antibodies was detrimental to proliferation in the MG-CM treated cultures, again with the high concentration being most pernicious.
Figure 4.3.3.2.b. Addition of neutralising mGlu receptor antibodies has minimal effect on the enhancement of neuronal survival caused by the addition of MG-CM, however neuronal proliferation is severely hindered. The involvement of mGlu receptor stimulation was further investigated by observing the concentration-response to addition of mGlu receptor antibodies, to block the external receptor sites, in both control MG-CM treated cultures. (A) Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh CGC media or 7 DIV MG-CM which had been further supplemented with mGlu receptor subclass antibodies; group I mGlu receptor antibodies: rabbit anti-mGlu1, rabbit anti-mGlu5; group II mGlu receptor antibodies: rabbit anti-mGlu2/3; group III mGlu receptor antibodies: rabbit anti-mGlu4a. Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of mGlu receptor antibodies resulted in significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

![Graph A](image)

![Graph B](image)
4.3.4 Modulation of intracellular calcium stores

One function of the endoplasmic reticulum (ER) is to sequester calcium from the cytosol, so maintaining the low cytosolic Ca\(^{2+}\) concentration (<10\(^{-7}\) M) whilst maintaining its own high intracellular calcium concentration (~ 10\(^{-3}\) M). The ability of the ER to release calcium and its subsequent reuptake of calcium allows the ER to mediate many rapid responses (Alberts et al., 1994), by the action of a Ca\(^{2+}\)-ATPase pump. Extracellular stimuli can cause the release of Ca\(^{2+}\) from the ER and result in levels of cytosolic calcium rising to approximately 10\(^{-6}\) M. Such a stimulus functions via G-protein coupled receptors which activate phospholipase C, resulting in the formation of two intracellular messaging products, diacylglycerol (DAG) and inositol triphosphate (IP\(_3\)). IP\(_3\) in turn actives specific Ca\(^{2+}\)-ATPases present on the ER and results in the release of Ca\(^{2+}\). The addition of thapsigargin promotes the release of Ca\(^{2+}\) from these intracellular stores by specifically inhibiting the Ca\(^{2+}\)-ATPase pump and preventing the reuptake of released Ca\(^{2+}\). As Ca\(^{2+}\) exerts many regulatory effects on neuronal proliferation it was of interest to establish whether the microglial-conditioned medium was altering intracellular Ca\(^{2+}\) concentrations. Cultures, treated with 7 DIV MG-CM or fresh medium, were supplemented with thapsigargin, and neuronal survival and proliferation was assessed after 24 hours (figure 4.3.4.).

As anticipated inhibition of the Ca\(^{2+}\)-ATPase using high concentration (> 10 nM) thapsigargin was detrimental to neuronal survival (figure 4.3.4. Ai), however proliferation was less sensitive and an excess of 50 nM was required to affect proliferation levels (figure 4.3.4. Aii). In MG-CM treated cultures addition of low concentrations of thapsigargin (1-10 nM) reduced neuronal survival levels comparable with control (figure 4.3.4. Bi) and proliferation was reduced to control following the
addition of 1-50 nM thapsigargin (figure 4.3.4. Bii). These results suggest that the MG-CM is unlikely to be functioning via the release of Ca\(^{2+}\) from the ER as addition of thapsigargin, even at low concentration, is detrimental to neuronal survival in both control and MG-CM treated cultures.

**Figure 4.3.4. Thapsigargin-sensitive release of intracellular Ca\(^{2+}\) is detrimental to both neuronal survival and proliferation.** To investigate the effect of the release of calcium from intracellular stores the concentration-response effect of the addition of thapsigargin, which inhibits the Ca\(^{2+}\)-ATPase of the endoplasmic reticulum, was appraised. (A) Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with either fresh media or 7 DIV MG-CM further supplemented with thapsigargin. Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of thapsigargin resulted in significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
4.3.5 Modulation of Trk receptors and their signalling cascades

The tyrosine kinase (Trk) family of receptors play an important function in neuronal survival and proliferation as they form the extracellular receptor for a number of growth factors. Stimulation of these receptors leads to altered survival and proliferation via a number of signal transduction pathways, mainly via MAPK stimulation and PI3K modulation. To investigate whether MG-CM functions via these receptors specific antibodies against the extracellular domains of the different subclasses of Trk receptors, TrkA, TrkB and TrkC, were added to control and 7 DIV MG-CM treated cultures. Following 24 hour incubation neuronal survival was assessed. As the Trk can function by affecting both MAPK signalling and PI3K, signalling specific inhibitors of these pathways were also added to control and 7 DIV MG-CM treated cultures. Again, following 24 hour incubation the level of neuronal survival and proliferation was assessed.

Figure 4.3.5.a. Neuronal cultures express TrkA, -B and -C. To confirm the expression of Trk receptors, neuronal cell protein lysates, collected at CGC day 8 and 14, were separated by electrophoresis and expression of TrkA, -B and -C was assessed by Western blotting. Blots were reprobed with β-actin to assess protein loading. Blots shown are representative of three repeats for each antibody.
In the first instance to confirm the presence of the different subtypes of Trk receptor neuronal cell lysates were electrophoresed and subjected to Western blot analysis. This demonstrated that the cerebellar granule neurones expressed all the subfamily receptors, TrkA, TrkB and TrkC (figure 4.3.5.a.). There were no clearly discernable differences in level of expression in treated or control cultures for TrkA and TrkB, though Trk C. Overall this suggests that the addition of MG-CM causes minimal, if any, alteration in the expression of the Trk receptors. To assess the effect of blocking the receptor and so preventing its external stimulation and activation, specific antibodies were added at the same time as the medium change.

**Figure 4.3.5.b.** Blocking Trk receptors abates the effect of MG-CM on neuronal survival. To investigate whether MG-CM enhanced neuronal survival was due to Trk receptor stimulation by neurotrophins, the concentration-response effect of the addition of antibodies against different Trk receptors, Trk A, Trk B and Trk C, was observed. Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with either (A) fresh media or (B) 7 DIV MG-CM containing the Trk antibody as specified. Following 24 hour incubation the level of neuronal survival was assessed. Values are the number of live CGCs per field expressed as a percentage difference from CGC control culture values ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of Trk receptor antibodies resulted in significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.
In control cultures the addition of antibodies to TrkA, TrkB and TrkC had no significant effect on the level of neuronal survival observed (figure 4.3.5.b. A). However in MG-CM treated cultures the level of neuronal survival was abated to control levels following the addition of the blocking antibodies (figure 4.3.5.b. B). The blocking of TrkB had the most prominent effect followed by TrkA and then TrkC.

To investigate whether the MG-CM was affecting the downstream signal transduction pathways of Trk receptors, specific inhibitors of MAPK and PI3K signalling cascades were added to control and MG-CM treated cultures. In the first instance neuronal cell lysates were appraised using Western blotting to ascertain whether components of MAPK signalling pathway and PI3K cascade were expressed in CGC cultures, specifically the profile of ERK1/2, phospho-ERK1/2, Akt and phospho-Akt were assessed. Whilst ERK1/2 demonstrates that the components of the MAPK cascade are present, the phosphorylated ERK1/2 demonstrates the activation and function of the MAPK cascade. Similarly Akt demonstrates that the components of the PI3K cascade are present, and the phosphorylated Akt demonstrates its activation and function.

It was observed that both neuronal control cultures and those exposed to conditioned-medium expressed high levels of ERK 1/2 and also phospho-ERK 1/2 (figure 4.3.5.c.) indicating that the MAPK cascade is expressed and functioning in all treatments, though no differences could be discerned. However the expression of p-Akt was found to be upregulated following the addition of MG-CM (Figure 4.3.5.c.).
Figure 4.3.5.c. Neuronal cultures express total ERK 1/2, p-ERK 1/2, Akt and p-Akt. To confirm the expression and function of MAPK signalling, neuronal cell protein lysates, collected at CGC day 8 and 14, were separated by electrophoresis and expression of ERK1/2, p-ERK1/2, Akt and p-Akt was assessed by Western blotting. Blots were reprobed with β-actin to assess protein loading.

Following the addition of specific antagonists it was observed that in control cultures inhibition of MAPK signalling, by addition of U0126 or PD98059, had no effect on either survival (figure 4.3.5.d. Ai and Bi) or proliferation (figure 4.3.5.d. Aii and Bii) at any of the concentrations assessed. However in MG-CM treated cultures the addition of these inhibitors resulted in diminished levels of survival (figure 4.3.5.d. Ai and Bi) and proliferation (figure 4.3.5.d. Aii and Bii) in a concentration-dependent manner, resulting in levels comparable to control.

In control cultures the inhibition of PI3K signalling through the addition of Wortmannin had no significant effect at low concentration on either survival (figure 4.3.5.d. Ci) or proliferation (figure 4.3.5.d. Cii), however at high concentrations, i.e. > 10 μM, Wortmannin proved to be detrimental to control survival. In MG-CM treated cultures the addition of Wortmannin depleted levels of survival (figure 4.3.5.d. Ci) and proliferation (figure 4.3.5.d. Cii) to control in a concentration-dependent manner.
Figure 4.3.5.d  Inhibition of PI3K signalling diminishes the effect of MG-CM on both neuronal survival and proliferation to a greater extent than MAPK signalling modulation. To establish whether MG-CM exerts its effect via PI3K or MAPK signalling, the concentration-response to specific signalling inhibitors was investigated. Neurones, were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh media or 7 DIV MG-CM, further supplemented with either (A) U0126, MAPK inhibitor, (B) PD98059, MEK inhibitor or (C) Wortmannin, PI3K inhibitor. Following 24 hour incubation the level of (i) neuronal survival and (ii) neuronal proliferation was assessed. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage above CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of all inhibitors resulted in significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

- Control
- 7 DIV MG-CM

**Figure Images**

- **Ai**
  - **i**
    - Live cells
    - % difference from CGC control
  - **ii**
    - Proline absorption
    - % difference from CGC control

- **Bi**
  - **i**
    - Live cells
    - % difference from CGC control
  - **ii**
    - Proline absorption
    - % difference from CGC control

- **Ci**
  - **i**
    - Live cells
    - % difference from CGC control
  - **ii**
    - Proline absorption
    - % difference from CGC control
4.4. DISCUSSION

4.4.1. Modulation of Notch signalling

Recently it has been demonstrated that cerebellar granule neurones express genes which are components of the Notch signalling cascade including those for the receptor itself, a number of possible ligands, e.g. delta and Jagged, and a number of downstream targets e.g. HES and CBF1 (Solecki et al., 2001). In agreement with these findings this study has confirmed the expression of the protein for both the active receptor (cleaved Notch) and ligand (delta1) using Western blotting (figure 4.3.1.a.), however it proved difficult to ascertain whether levels of expression were altered following treatment with MG-CM compared with control.

Further investigations employed an antibody against the Notch ligand delta1 (Hicks et al., 2000) to inhibit the activity of the ligand delta1 and repress its interaction with the Notch receptor. Disturbance of delta-Notch signalling by this method was able to completely abrogate both the proliferative and survival effects of MG-CM following the addition of low concentrations (0.004 - 0.006 µg/ml) of anti-delta1 (figure 4.3.1.b. Ai and ii). Comparably treated control cultures were unaffected at these low concentration of antibody, indeed a 10 fold increase in antibody concentration was required to elicit a significant alteration in control survival or proliferation (figure 4.3.1.b. Ai and ii). These results suggest that MG-CM treated cultures were more sensitive to Notch dysregulation and that the elevated levels of both neuronal survival and proliferation are due, at least in part, to Notch signal transduction as addition of the anti-delta1 antibody reverts levels back to those associated with control cultures. Notch signalling is associated with the maintenance of a precursor state and the inhibition of differentiation (Austin et al., 1995; Dorsky et al., 1995). Normally, as
development proceeds, the level of ligand binding decreases and so Notch becomes increasingly deactivated, allowing the precursors to begin to differentiate into neurones (de la Pompa et al., 1997). This study has revealed that MG-CM treated cultures show elevated levels of neuronal proliferation compared with control cultures, and following neutralisation by the addition of anti-delta1 antibody the levels of proliferation return to control suggesting that at this stage, the MG-CM is enhancing the activation of this pathway and that the activation of Notch by endogenous delta1 is directing the cells to remain as precursors (de la Pompa et al., 1997; Sestan et al., 1999; Verdi et al., 1999).

To ascertain that the effects observed following antibody neutralisation were ‘real’ effects, and not due to steric hinderance or indeed non-specific interactions, the antibody was denatured by boiling and subsequently added to the neurones. Under these conditions the antibody exerted no effect on either control or MG-CM treated cultures (figure 4.3.1.c.), suggesting that the afore mentioned results were veritable effects.

Pre-treatment of the neurones or the MG-CM with the anti-delta1 antibody prior to media replacement revealed that the most prominent effects were elicited via modulation of sites on the neurones themselves (figure 4.3.1.b. B). However modulation of factors present in the MG-CM also effected, though to a much less extent, both survival and proliferation levels (figure 4.3.1.b. C), indicating that the microglia may perhaps produce and release delta1. Recent evidence has shown that soluble delta1 is able to activate the Notch receptor (Han et al., 2000) so it is plausible that the MG-CM does contain delta1 and that this is able to activate Notch signalling.
The extracellular factors which regulate this pathway remain to be fully determined, however the chelation of extracellular calcium has been found to upregulate Notch activity by dissociating the Notch receptor and causing the formation of the active intracellular domain (Rand et al., 2000). To ascertain whether increased activation of Notch, via calcium chelation, could enhance the survival and proliferation of control cultures to levels associated with MG-CM treatment the concentration-dependancy of addition of EGTA was investigated. Chelation of extracellular calcium was indeed found to enhance neuronal survival and proliferation in control cultures but had little or no effect on MG-CM treated cultures (figure 4.3.1.d.). This suggests that the MG-CM treated cultures had attained a level of survival and proliferation that could not be surpassed, whilst the addition of EGTA to control cultures exerted a concentration-dependent effect on neuronal survival and proliferation.

The concentrations of EGTA added were consistent with those used in other studies (Rand et al., 2000). It is of interest that in further investigations where L-type voltage operated calcium channels were inhibited it was found that neuronal survival was severely impeded (see section 4.3.3.1.). This would therefore seem to suggest that the concentrations of EGTA added, whilst sufficient to cause upregulation of Notch signalling, still resulted in extracellular calcium concentrations permissive for the maintenance of activation of other channels not related to the Notch cascade. From these observations it may be postulated that MG-CM may contain factors which promote calcium chelation though the nature of these factors has not been elucidated.
4.4.2. Modulation of cAMP/PKA and G-protein coupled receptor pathways

Previously it has been shown that the survival and proliferation of precursor cells in the cerebellum can be inhibited by enhanced cAMP/PKA activity (Hynes et al., 1995; Epstein et al., 1996), via a mechanism that involves the antagonism of Sonic hedgehog signalling (Epstein et al., 1996). Furthermore this mechanism was found to be mediated via G-protein coupled receptors acting upstream of cAMP/PKA (Klein et al., 2001). Therefore to investigate whether MG-CM treatment of neuronal cultures was enhancing neuronal survival by a comparable mechanism, cAMP and PKA mediated pathways were selectively activated and deactivated by the addition of the pharmacological reagents forskolin and H89 respectively (Chijiwa et al., 1990), and the effect of GPCR uncoupling, by the addition of pertussis toxin (Luttrell et al., 1999), was also assessed.

The enhancement of cAMP production, and by inference PKA activity, by the addition of forskolin resulted in the survival of MG-CM treated neuronal cultures to be reverted to control levels in a concentration dependent manner (figure 4.3.2. Ai), whilst having little significant effect on the survival of control cultures. A comparable effect was observed on proliferation where addition of forskolin completely abated proliferation in MG-CM treated cultures whilst exerting no effect on control cultures (figure 4.3.2. Aii).

Conversely the addition of H89 to inhibit PKA activity, whilst not directly affecting cAMP production (as PKA activation is downstream of cAMP production), resulted in an enhancement of survival in control cultures to levels associated with the addition of
MG-CM (figure 4.3.2. Bi). The survival of MG-CM treated cultures was marginally increased but appeared to plateau following the addition of 0.5 - 2 μM H89 (figure 4.3.2. Bi), suggesting a saturation point had been attained where no further enhancement could be achieved.

Interestingly H89 had the opposite effect to what would have been expected on proliferation. It would have seemed justifiable to assume that as addition of forskolin to enhance cAMP/PKA resulted in decreased proliferation, then addition of H89 to inhibit PKA would result in an increase in proliferation. However the level of proliferation in control cultures was unaltered following the addition of H89 (figure 4.3.2. Bii), whilst the levels of proliferation observed in MG-CM treated cultures were returned to control following addition of high concentration H89 (1 - 2 μM). This result may indicate that adenylate cyclase, the upstream producer of cAMP, can be modulated via a number of different pathways to produce an overall decrease or increase in cAMP production and it may well be that survival is modulated by one pathway whilst proliferation is regulated by another.

The uncoupling of PTX sensitive G-proteins from their downstream pathways by 24 hour treatment with PTX significantly inhibited MG-CM induced CGC survival and proliferation to control levels (figure 4.3.2. Ci and ii) whilst exerting no effect on control neuronal cultures. Such a dramatic effect would suggest that the MG-CM is acting via a mechanism involving $G_{ai}$ G-protein an effect analogous to that observed by other investigators (Klein et al., 2001). The involvement of this sub-type of G-protein would also correlate with the result that enhanced cAMP is not permissive for cell survival and proliferation, furthermore $G_{ai}$ G-proteins are generally associated
with being negatively coupled to adenylate cyclase (Fields and Casey, 1997). Overall from these experiments it can be postulated that MG-CM enhances neuronal survival and possibly a component of proliferation, via a $G_{ai}$-protein coupled receptor that is negatively coupled to adenylate cyclase.

4.4.3. Modulation of glutamate receptors and calcium channels

The activation of glutamate receptors and voltage operated calcium channels (VOCC) have well established effects on neuronal survival and proliferation (Barger, 1999; Blanc et al., 1999; Vaillant et al., 1999). As such it was of interest to determine whether the active factor(s) present in MG-CM were functioning via modulation of these. In the first instance the modulation of the ionotropic glutamate (iGlu) receptors and L-type VOCC was investigated (4.3.3.1.). It was found that antagonism of AMPA iGlu receptors and L-type VOCC abated the effects of MG-CM on neuronal survival, whilst the inhibition of NMDA iGlu receptors had no significant effect (figure 4.3.3.1.a.). The impairment of the MG-CM mediated effect, by blockade of AMPA iGlu receptors or L-type VOCC through the addition of CNQX and nifedipine respectively, was found to be concentration dependent (figure 4.3.3.1.b.).

Previous investigators have identified a strong link between AMPA iGlu receptors and L-type VOCCs (Leski et al., 1999; Rajadhyaksha et al., 1999; LoPachin et al., 2001), and it is established that such calcium entry has an important affect on neuronal growth (Catsicas et al., 2001), learning, memory, and neuronal loss in a number of neuropathologies (Perkinton et al., 1999). Indeed it has been suggested that over activation of ionotropic glutamate receptors is involved in the loss of neuronal osmoregulation and ion homeostasis (LoPachin et al., 2001), which can lead to
excitotoxic cell death through an increase in intracellular calcium concentration (Allcorn et al., 1996), whilst positive modulation of AMPA receptors has been found to increase neurotrophin expression (Lauterbom et al., 2000). The results presented in this thesis have demonstrated that blocking AMPA iGlu receptors reverts survival of MG-CM treated cultures to control levels, suggesting that factors present in MG-CM may be stimulating the actions of AMPA iGlu receptors. AMPA iGlu receptors may then be acting by a consecutive pathway to affect L-type Ca\(^{2+}\) channels as investigations in this thesis also found that blocking L-type Ca\(^{2+}\) channels reverted the survival of MG-CM treated cultures to control levels. Such a link between AMPA iGlu receptors and L-type Ca\(^{2+}\) channels has been previously found to be able to alter gene expression (Rajadhyaksha et al., 1999), possibly via a mechanism involving the modulation of PI3K and MAPK, which in turn would have the capacity to affect neuronal development and plasticity (Perkinton et al., 1999).

Next, the modulation of the different classes of metabotropic glutamate (mGlu) receptors was investigated (4.3.3.2.). Previously it has been shown that modulation of mGlu receptors has the capacity to offer neuroprotection (Pizzi et al., 2000), therefore it was of interest to see if factors in MG-CM were functioning via such a route. It was observed that activation or inhibition of the different classes of mGlu receptors in MG-CM treated cultures, through the addition of selective chemical agonists and antagonists, exerted potent effects on levels of neuronal survival and proliferation (figure 4.3.3.2.a.). Activation of group I mGlu receptors abated MG-CM driven survival, suggesting that over stimulation of group I mGlu receptors is detrimental to neuronal survival. Antagonism of group I mGlu receptors also depleted the survival enhancing effects of MG-CM though to a lesser extent. Such findings are in
accordance with previously reported findings where modulation of group I mGlu receptors was also found to be detrimental to neuronal survival (Copani et al., 1998; Catania et al., 2001). It was also established that antagonism of group II mGlu receptors could also dissipate the neuronal survival normally associated with MG-CM treatment, whilst overstimulation did not alter the level of survival in MG-CM treated cultures. However, no significant effect was observed in control cultures.

Finally it was observed that both over stimulation and inhibition of group III mGlu receptors resulted in depleted levels of survival in MG-CM treated cultures, whilst exerted no effect in control cultures. However it has been reported that activation of group III mGlu receptors can enhance the survival of CGCs (Graham and Burgoyne, 1994), no such effect was observed during this investigation.

The effect of the addition of neutralising antibodies, against extracellular regions of mGlu receptors was also investigated. The receptors were blocked prior to the addition of MG-CM. This study showed that blockade of group III mGlu receptor was most detrimental to the effects of MG-CM (figure 4.3.2.b). Blockade of group I or group II mGlu receptors in this manner had less of an effect, however a significant decrease in potency of MG-CM was observed (figure 4.3.2.b.).

4.4.4. Modulation of Trk receptors and their signalling cascades

A wide number of extracellular signals are transmitted into the cell via an interaction with the tyrosine kinase (Trk) receptors and it is well established that Trk receptor signalling is of vital importance to neuronal survival (Huang and Reichardt, 2001). The Trk receptors in turn modulate a number of important pathways integral to
neuronal survival and proliferation (Miller and Kaplan, 2001). It was therefore of interest to determine if factors present in MG-CM functioned via these receptors. Furthermore it was investigated whether the factors had an effect on the downstream signal transduction cascades of MAPK and PI3K/Akt.

There are three different subtypes of the Trk receptor, namely Trk-A, -B and -C, therefore in the first instance the expression of these in the treated neuronal cultures was appraised by Western blotting (figure 4.3.5.a.). All subtypes were found to be present in both control and MG-CM treated cultures. The levels of expression of Trk-A and Trk-B did not appear to vary between conditions, however the expression of Trk-C did appear slightly higher following MG-CM treatment compared with control cultures. The effect of blocking the Trk receptors by adding neutralising antibodies against each receptor subtype was also investigated. Such blockade had no significant effect on control cultures however the survival levels of MG-CM treated cultures were reduced to near control (figure 4.3.5.b.). All were affected to comparable levels suggesting that the MG-CM is functioning through these receptors.

Next, the expression of components of the MAPK and PI3K/Akt signalling pathways was investigated in control and MG-CM treated cultures. Firstly the expression of ERK 1/2, and functioning p-ERK, was appraised. All culture conditions showed high levels of expression suggesting that the MAPK cascade was functioning. However there were no discernable differences between the different culture conditions (figure 4.3.5.c.). Subsequently the expression of Akt, and functioning p-Akt was appraised. Again all cultures showed p-Akt activity, however the MG-CM treated cultures showed enhanced p-Akt expression suggesting that MG-CM was causing and
enhancement in the activity of the PI3K/Akt signal transduction pathway. The PI3K/Akt pathway is known to be integral in promoting neuronal survival and proliferation (Brazil and Hemmings, 2001; Katso et al., 2001) so such a result would appear to correlate with this.

To further investigate the effects of MAPK and PI3K modulation, cultures were treated with the addition of specific antagonists (figure 4.3.5.d.). The antagonists, on the whole, exerted minimal effects on control cultures at the concentrations tested, however the survival-enhancing effect of MG-CM was abated following treatment with both MAPK and PI3K/Akt antagonists. The functioning of PI3K/Akt was deemed to be of the most importance as control cultures did show decreased survival at the highest concentration added. Such a result would appear to correlate with the Western blot findings where the expression of p-Akt was enhanced in conditioned medium treated cultures. Other investigators have reported recently that factors of microglial origin are able to affect neuronal survival through activation of the protein kinase Akt, which was in turn promotes the nuclear translocation of NF-kappaB (Meucci et al., 2000), therefore the unidentified factors described in this study could well be exerting their effect via such a mechanism.
5. CHARACTERISATION
OF DIFFUSIBLE FACTORS
PRESENT IN
MICROGLIAL-CONDITIONED MEDIUM
5.1 INTRODUCTION

A number of methods can be used to characterise and identify unknown compounds. Firstly the effect of chemical modification, i.e. the effect of heat, proteases and acid on the compounds, is routinely used to determine whether a compound of interest is likely to be a protein, though inevitably yields no information about the identity. Fractionation studies can also give details about approximate molecular weights. However to further characterise and identify a factor more complicated studies need to be performed. One approach is to sequentially remove likely factors from the solution of interest, generally done by immunoprecipitating out factors by the addition of primary antibodies directed against specific factors. Conversely, receptors present on the cell surface can be blocked by the addition of neutralising antibodies raised against specific receptor types.

In more recent time, the advances in proteomic analysis has facilitated the identification of proteins. Proteins can be separated, in 2 dimensions, according to their pI and molecular weight. Once separated, the proteins can be visualised by a number of methods including ammonical silver staining and the use of fluorescent dyes. The positions of the revealed proteins can then be determined, and the values can be cross referenced in established databases of protein characteristics. If a protein has already been reported with comparable characteristics then this information is returned. In addition, once separated, these proteins can also be further processed and assessed for identification using mass spectroscopy. Again the information yielded can be used to cross reference the proteomic databases to establish whether possible proteins have been reported or whether novel proteins have been identified.
5.2. SUMMARY

The characteristics and identity of the diffusible factors present in the microglial conditioned medium responsible for the enhancement of neuronal survival and proliferation were investigated in the research presented in this chapter. It was found that the factors were sensitive to chemical modification by heat, acid and protease treatment suggesting that the factor(s) are proteins. By fractionating the MG-CM it was established that the active component had a molecular weight of $< 30$ kD. Other factors were also identified by this method with agonistic and antagonistic effects on both neuronal survival and proliferation but the $< 30$ kD fraction had the most striking effect. Furthermore it was found that the MG-CM enhanced neuronal survival by upregulating neuronal protein synthesis since addition of cyclohexamide, a chemical which prevents protein synthesis, abated the effect of MG-CM. To establish whether the factor, or factors, were likely to be a growth factor, a number of likely factors were immunoprecipitated from MG-CM and the resultant immunodepleted MG-CM was added to the neurones. Removal of PDGF was found to completely abate the effect of MG-CM whilst EGF, bFGF and TGF-β1 also exerted effects but to lesser extents. Blockade of growth factor receptors on the neurones confirmed the effect of PDGF.

In an attempt to identify the protein samples of SF-CM were separated using 2 dimensional electrophoresis, and the pI and molecular weights of the proteins were assessed. This analysis revealed the presence of in excess of 100 proteins in the microglial-conditioned medium. A search of the SWISS-PROT protein database yielded a large number of possible identities for the proteins assessed, including a number of growth factors and interleukins.
5.3. CHARACTERISATION OF MG-CM

Previous chapters have described that medium conditioned in the presence of unstimulated microglia contains diffusible factors which can enhance neuronal survival and proliferation. It has been determined that the factor is of microglial origin and accumulates its potency over the course of 7 days, such that 7 DIV MG-CM exerts a more pronounced effect than 1 DIV MG-CM. Investigation into signalling pathway modulation has revealed that the active factors are able to function via a number of routes including: (1) enhancement of Notch signalling; (2) functioning via a PTX-sensitive GPCR which is negatively couple to adenylate cyclase; (3) also a function via AMPA iGlu receptors; (4) and finally through Trk receptor stimulation. As a number of pathways are affected it would appear to suggest that a number of factors are acting synergistically to enhance neuronal survival and proliferation. It is of interest to characterise these diffusible factors present in the microglial conditioned medium. To ascertain the nature of the diffusible factors a number of studies were carried out. Initially the MG-CM was fractionated to establish the approximate molecular weight of the active factors, MG-CM was also subjected to heat, protease and chemical modification. The conditioned medium was also separated by 1 dimension native gel electrophoresis and also by 2D gel electrophoresis.

5.3.1. Modification of MG-CM

To further characterise the properties of the microglial-conditioned medium, sensitivity to heat, acidification and protease treatment was assessed. Samples were either (1) heated to boiling for 10 mins, (2) subjected to trypsin protease digestion (2.5 μg/ml) for 1 hr at 37 °C followed by addition of SBTI (100μg/ml) to stop further processing by the trypsin or (3) subjected to 30 min HCl (1 M) exposure followed by
rneutralization with NaOH (1 M). This was performed on control, serum containing medium, 1 DIV MG-CM, 7 DIV MG-CM, control SFM and 7 DIV SF-CM. The treated conditioned medium was subsequently added to 7 day *in vitro* CGCs as previously described (section 2.2.8.). Neuronal survival was assessed after 24 hour incubation (figure 5.3.1.).

Heat and protease modification of control, serum containing, medium, 1 DIV MG-CM and 7 DIV MG-CM resulted in comparable levels of neuronal survival approximately 40 % below that normally associated with control cultures (figure 5.3.1. A). Acid modification of these media also severely affected neuronal survival, resulting in levels approximately 90 % below that normally associated with control (figure 5.3.1. A).

These results show that modification of MG-CM results in neuronal survival levels which are depleted to the same level as modified control medium. It would seem possible that these results could be artefactual, and may be due to a survival inhibiting effect of the denaturation of other proteins present in the serum in these media samples. To ascertain whether these modifications affected the diffusible factors released by the microglia into the conditioned media, the experiments were repeated using SFM and 7 DIV SF-CM, as these do not contain all the other unknown proteins, and thus any effect could be attributed to modification of microglial derived diffusible factors.

Heat and protease treatment of control SFM had no effect on the level of neuronal survival observed in the treated cultures (figure 5.3.1. B), however both of these treatments reduced the survival of 7 DIV MG-CM treated cultures back to control
levels (figure 5.3.1. B). Again acidification was detrimental to both control SFM and 7 DIV SF-CM, reducing survival to 90% below normally associated with control (figure 5.3.1. B), suggesting that the effects may be non-specific. These results demonstrate that the diffusible factors, derived from the microglia, which are responsible for enhancing neuronal survival, are sensitive to both heat and protease modification.

**Figure 5.3.1.** The active factor in MG-CM is heat-, protease- and acid-sensitive. The properties of MG-CM were analysed by subjecting samples to chemical modification and comparing them to matched control media samples. Neurones were cultured for 7 days, after which 250 µl of media was removed and replaced with media that had either been boiled, protease treated or acid treated. (A) Control media, 1 DIV MG-CM and 7 DIV MG-CM were assessed, as was (B) control SFM and 7 DIV SF-CM. Following 24 hour incubation the level of neuronal survival assessed. Values are the mean number of live neurones, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. All levels of significance are compared with values from untreated CGC controls at that time point.
5.3.2. Fractionation of MG-CM

To determine the molecular weights of the active factor(s) in conditioned medium, medium samples were fractionated using Pall Gelman microconcentrators, into fractions with approximate molecular weight cutoffs of > 300 kDa, 100 - 300 kDa, 30 - 100 kDa and < 30 kDa. This was performed on both 1 DIV MG-CM and 7 DIV MG-CM samples. The fractionated conditioned medium was subsequently added to 7 DIV CGCs as previously described (section 2.2.8.). Neuronal survival was assessed at CGC day in vitro 8 and 14, and neuronal proliferation was assessed after 24 hour incubation (figure 5.3.2.).

Fractionation of microglial conditioned medium revealed the presence of a number of different factors which either agonised or antagonised neuronal survival or proliferation. In both 1 DIV MG-CM and 7 DIV MG-CM the > 300 kD fraction resulted in a significantly lower level of neuronal survival (figure 5.3.2. Ai and Bi) and proliferation (figure 5.3.2. Aii and Bii), compared with the addition of whole MG-CM, however levels were still above that observed in control cultures. The 100 - 300 kD and 30 - 100 kD fractions were both found to exert an adverse effect on neuronal survival (figure 5.3.2. Ai and Bi) and proliferation (figure 5.3.2. Aii and Bii). The < 30 kD fraction propagated an elevated level of both neuronal survival (figure 5.3.2. Ai and Bi) and proliferation (figure 5.3.2. Aii and Bii), in some instances even above that observed following the addition of whole MG-CM.

These results indicate that the active factor(s) present in MG-CM responsible for the enhancement of neuronal and proliferation has a molecular weight < 30 kD, such a molecular weight would suggest the possibility that the factor could be a growth factor.
as these commonly have a molecular weight of 15 - 30 kD (Hill et al., 1992). The action of these factors is evidently able to override the inhibiting effects of compounds present in other fractions as it has been demonstrated that the whole conditioned medium is beneficial to enhanced survival and proliferation.

**Figure 5.3.2.** The active component of MG-CM has a molecular weight of < 30 kD. To examine the factors present in MG-CM which were able to enhance neuronal survival MG-CM was fractionated into fractions with molecular weight cut-offs of > 300 kD, 100 - 300 kD, 30 - 100 kD and < 30 kD. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either (A) fractionated 1 DIV MG-CM or (B) fractionated 7 DIV MG-CM. The level of (i) neuronal survival was assessed at CGC day in vitro 8 and 14, whilst (ii) the level neuronal proliferation was assessed after 24 hrs incubation. Values are the mean number of live neurones or proliferation absorbance, expressed as a percentage difference from CGC controls, ± sem, where n=9 over 3 culture preparations. All levels of significance are compared with values from untreated CGC at that time point.
5.3.3. **MG-CM effects on neuronal protein synthesis**

To determine whether the addition of MG-CM directly affected the granule neurones by modulating *de novo* protein synthesis, cyclohexamide was added to specifically inhibit such protein synthesis. Cyclohexamide inhibits protein synthesis by interacting directly with the translocase enzyme and so interfering with the translocation step (Obrig et al., 1971). Cultures, treated with 7 DIV MG-CM or fresh medium, were supplemented with the addition of the cyclohexamide (0 - 100 μM). Neuronal survival was subsequently assessed after 24 hours incubation (figure 5.3.3.).

Figure 5.3.3. **The active factor in MG-CM functions by enhancing *de novo* protein synthesis in the neurones.** To determine if MG-CM enhanced *de novo* protein synthesis the dose-response effect of addition of cyclohexamide was appraised. Neurones were cultured for 7 days, after which 250 μl of media was removed and replaced with either fresh or 7 DIV MG-CM further supplemented with cyclohexamide. Following 24 hour incubation the level of neuronal survival assessed. Values are the mean number of live neurones, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the addition of cyclohexamide resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.001). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

Treatment with cyclohexamide for 24 hours had no significant effect on control levels of survival, however the survival of conditioned-medium treated cultures was
suppressed to control levels in a dose-dependent manner (figure 5.3.3.). This suggests that MG-CM functions by enhancing protein synthesis, and if this function is specifically blocked then survival is depleted to control levels.

5.3.4. Release of growth factors by microglia

As discussed (section 1.2.5.2.) a wide variety of factors are known to be released by microglia, and a number of these factors exert established effects on both neuronal survival and proliferation including bFGF, PDGF, EGF, and TGF-β1. Furthermore such factors have been shown to be able to selectively modulate neurotransmitter action (Miller et al., 1995). It was therefore of interest to determine the possible involvement of these factors in the enhancement of neuronal survival exerted by MG-CM as previously discussed in this thesis. Samples of MG-CM were immunodepleted of bFGF, EGF, PDGF and TGF-β1 by immunoprecipitation. Whole MG-CM and immunoprecipitated MG-CM samples were subsequently appraised using Western blot analysis for the presence of these growth factors. The results demonstrated the presence of each of the growth factors in the whole MG-CM, whilst the levels were significantly reduced following removal by immunoprecipitation (figure 5.3.4.a. A).

To determine the contribution of each individual growth factor to MG-CM-induced enhancement of neuronal survival, the immunoprecipitated MG-CM samples were added to the neurones and the level of neuronal survival was assessed. Controls were performed where samples of fresh unconditioned media, which had also been immunodepleted, were added to neurones and the ensuing neuronal survival was assessed. Immunodepletion of unconditioned media had little effect on survival levels
achieved in the neuronal cultures (figure 5.3.4.a. Bi), however significant effects were observed following the removal of the factors from MG-CM (figure 5.3.4.a. Bii).

**Figure 5.3.4.a. Immunodepletion of growth factors from MG-CM diminishes MG-CM mediated neuronal survival.** To investigate whether the neuronal enhancing effect of the MG-CM was mediated by either TGF-β1, PDGF, EGF or bFGF, samples were immunoprecipitated by addition of a specific antibody and protein A/G agarose. (A). The resulting depleted conditioned media was assessed by electrophoresis and Western blotting and compared with whole MG-CM. (B). Immunodepleted fresh unconditioned media (i) and immunodepleted MG-CM (ii) were subsequently added to 7 day *in vitro* neurones as previously. Following 24 hour incubation the level of neuronal survival assessed. Values are the mean number of live neurones, expressed as a percentage difference from CGC controls, ± sem, where n=6 over 3 culture preparations. Two-way anova established that the removal of the respective growth factor resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.01). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

**A.**

<table>
<thead>
<tr>
<th></th>
<th>whole MG-CM</th>
<th>immune-depleted</th>
<th>MW kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td></td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>PDGF</td>
<td></td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>bFGF</td>
<td></td>
<td></td>
<td>19</td>
</tr>
</tbody>
</table>

**Bi**

![Image of immunodepletion of growth factors from MG-CM](image)

**Bi i**

- control TGF-β1
- PDGF
- EGF
- bFGF

**Bi ii**

- 7 DIV MG-CM TGF-β1
- PDGF
- EGF
- bFGF

231
Immunodepletion of PDGF from MG-CM had the most marked effect (figure 5.3.4. Bii), reverting neuronal survival to levels comparable with control. Immunodepletion of EGF and bFGF from MG-CM had a significant effect and reduced the survival enhancement capacity of MG-CM to approximately 15% above control cultures. The depletion of TGF-β1 from MG-CM had the least effect, but neuronal survival was reduced.

As demonstrated the depletion of both PDGF and EGF from MG-CM were able to compromise the capacity of MG-CM to enhance neuronal survival. To further investigate this effect the cell surface neuronal receptors for these growth factors were blocked by the addition of neutralising antibodies. As previously performed, antibodies against PDGF and EGF were supplemented into the culture wells when the media were replaced with either fresh unconditioned medium or 7 DIV MG-CM at CGC day in vitro 7. Neuronal survival was subsequently assessed after 24 hours incubation.

The inhibition of the receptors by the addition of neutralising antibodies had no significant effect on control cultures (figure 5.3.4.b. A). However in MG-CM treated cultures, blockade of the PDGF receptor severely hampered the ability of MG-CM to enhance neuronal survival and resulted in survival levels comparable with control (figure 5.3.4.b.B). Inhibition of the EGF receptor had a smaller effect, however enhanced survival levels were still compromised to levels below that usually associated with MG-CM treatment (figure 5.3.4.b.B).
Figure 5.3.4.b. Inhibition of growth factor receptors blocks MG-CM mediated enhanced neuronal survival. To further investigate whether the neuronal enhancing effect of the MG-CM was mediated by growth factors antibodies against specific growth factor receptors, EGF-R and PDGF-Rb, were added to 7 day in vitro neurones prior to the addition of either (A) fresh medium or (B) 7 DIV MG-CM. Following 24 hour incubation the level of neuronal survival assessed. Values are the mean number of live neurones, expressed as a percentage difference from CGC controls, ± SEM where n=6 over 3 culture preparations. Two-way anova established that the removal of the respective growth factor resulted in a significantly different interaction with control and 7 DIV MG-CM culture (p<0.01). Post-test analysis was performed (Bonferroni) and levels of significance, comparing 7 DIV MG-CM treated cultures with control cultures, are indicated.

A.

B.

These results imply a major role for growth factors in the effects observed following MG-CM treatment on neuronal survival and most likely proliferation. Such results also correlate to the chemical modification findings, where the factor was found to be heat, acid and protease sensitive. Furthermore, the fractionation studies revealed a
active component with a molecular weight of < 30 kD which again correlates with the likelihood of a growth factor identity.

5.3.5. Analysis of conditioned medium by electrophoresis

To further characterise the nature of and to possibly identify the components of microglial-conditioned medium, the proteins were separated using native polyacrylamide gel electrophoresis (PAGE). In the first instance single dimension (1D) electrophoresis was performed to separate proteins according to their apparent molecular weight (5.3.5.1.). Proteins were also resolved using 2D-PAGE, where they are separated firstly according to their isoelectric point (pI) and subsequently according to molecular weight (MW) (5.3.5.2.).

5.3.5.1. 1D native PAGE separation of proteins

The protein composition of conditioned media samples was assessed using native PAGE. Samples, mixed with Lamelli sample buffer, were loaded onto pre-cast 12 % polyacrylamide gels and electrophoresessed at a constant voltage of 180 V until the dye front reached the bottom of the gel. The gel was then removed and protein bands were visualised using Coomassie blue stain. Molecular weight standards were also separated.

The staining of the gel revealed the presence of a large number of proteins in all samples, including the fresh medium only samples (figure 5.3.5.1.). It was difficult to discern significant differences between the different samples, however fractionation of the conditioned medium, resulting in the < 30 kD fraction, did indeed remove the vast majority of the proteins at higher molecular weights.
Figure 5.3.5.1. Separation of MG-CM by 1D native PAGE indicates varied protein levels in control and conditioned media. To establish whether there were differences between the protein composition of control and conditioned media, samples were separated by native polyacrylamide gel electrophoresis (PAGE). Maximal sample volumes were loaded onto pre-cast gels and electrophoresed. The protein bands were visualised using Coomassie blue stain.

This analysis clearly shows the presence of a wide number of proteins in the media and conditioned media samples. To further assess the protein content samples were separated using 2D PAGE.

5.3.5.2. 2D PAGE separation of proteins

To assess the protein composition of the conditioned media by 2D gel electrophoresis samples were processed as described (section 2.2.21.1.) and separated across a pH 3 - 10 gradient, to provide a measure of their pl. The proteins were subsequently further separated by SDS-PAGE, on a 9 - 20 % gradient gel, to provide a measure of their
molecular weight. Samples were electrophoresed until the dye front reached the bottom of the gel. The gel was then removed and protein bands were visualised using silver nitrate stain. Only whole serum-free medium samples were assessed, as the large number of other non-defined proteins present in the serum-containing media would have complicated the end analysis.

The control, unconditioned serum free media (SFM) showed very little protein content (figure 5.3.5.2.a. A). In contrast the media conditioned in presence of microglia (7 DIV SF-CM) showed the expression of a wide number of proteins across the whole spectrum of both pI and MW (figure 5.3.5.2.a. B).

Silver staining, though a powerful and widely used technique, has limitations in that its dynamic range is relatively low, 1-60 ng protein (Syrový and Hodny, 1991). Furthermore regions with high protein content become saturated and are subsequently negatively stained (Yan et al., 2000). These problems make quantitative gel analysis impossible and inaccurate, as intensities of gel spots do not necessarily reflect the true abundance of the different proteins. More recently methods of protein detection employ the use of fluorescent dyes (Steinberg et al., 1996; Berggren et al., 2000; Patton, 2000), in particular the use of SYPRO fluorescent dyes (Molecular Probes) such as SYPRO Ruby™. Though the structures for these dyes are proprietary, it has been reported that SYPRO Ruby™ dye is a transition metal organic complex that binds directly by electrostatic mechanisms (Berggren et al., 2000; Patton, 2000). The main advantage of the use of fluorescent dyes is that the binding of the dye to the protein is stoichiometric and fluorescence intensity is therefore related to the amount of dye binding, and the dynamic range spreads over three orders of magnitude,
Figure 5.3.5.2.a. Analysis of SF-CM using 2D electrophoresis and silver stain visualisation. Concentrated samples of (A) SFM and (B) 7 DIV SF-CM were initially separated according to protein pl values using isoelectric focusing. The proteins were further separated according to their molecular weight using SDS-PAGE. Once separation was completed the proteins were fixed and visualised using ammonical silver nitrate staining.
1-1000 ng protein (Yan et al., 2000). Therefore to further assess the protein composition of the 7 DIV SF-CM further gels were run and stained using SYPRO Ruby™. Control gels loaded with unconditioned SFM were also processed. A comparable pattern of proteins was observed, with minimal staining on control gels (figure 5.3.5.2.b. A), whilst the 7 DIV SF-CM gel contained many proteins (figure 5.3.5.2.b. B). Interestingly several regions were more distinct following SYPRO Ruby™ staining of MG-CM proteins (see marked regions of figure 5.3.5.2.b. B), and there were no areas of negative staining as seen following silver nitrate staining.

In order to be able to assign pi and MW values to the proteins visualised, further gels were run, though this time the sample was supplemented with the addition of specialised 2D gel protein markers (BioRad). The markers contain 7 proteins, 5 denatured and 2 native, which have a defined pi and MW (see table 5.3.5.2.a.). Control gels loaded with unconditioned SFM were also processed (figure 5.3.5.2.c.).

<table>
<thead>
<tr>
<th>Table 5.3.5.2.a.</th>
<th>Composition of standards used for 2D electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pl (pH)</td>
</tr>
<tr>
<td>hen egg white conalbumin type I</td>
<td>6.0, 6.3, 6.6</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>5.4, 5.6</td>
</tr>
<tr>
<td>bovine muscle actin</td>
<td>5.0, 5.1</td>
</tr>
<tr>
<td>rabbit muscle glyceraldehyde-3-phosphate dehydrogenase</td>
<td>8.3-8.5</td>
</tr>
<tr>
<td>bovine carbonic anhydrase</td>
<td>5.9, 6.0</td>
</tr>
<tr>
<td>soybean trypsin inhibitor</td>
<td>4.5</td>
</tr>
<tr>
<td>equine myoglobin</td>
<td>7.0</td>
</tr>
</tbody>
</table>
Figure 5.3.5.2.b. Analysis of SF-CM using 2D electrophoresis and SYPRO Ruby visualisation. Concentrated samples of (A) SFM and (B) 7 DIV SF-CM were initially separated according to protein pl values using isoelectric focusing. The proteins were further separated according to their molecular weight using SDS-PAGE. Once separation was completed the proteins were fixed and visualised using SYPRO Ruby fluorescent dye staining. Regions with more defined staining, compared with silver nitrate staining, have been indicated with black circles.
Figure 5.3.5.2.c. Further analysis of SF-CM using 2D electrophoresis and SYPRO Ruby visualisation. 2D standards only (A) and concentrated samples, supplemented with 2D standards, of SFM (B) and 7 DIV SF-CM (C) were initially separated according to protein pl values using isoelectric focusing. The proteins were further separated according to their molecular weight using SDS-PAGE. Once separation was completed the proteins were fixed and visualised using SYPRO Ruby fluorescent dye staining.

A. 2D standards only

B. SFM + 2D standards

C. 7 DIV SF-CM + 2D standards
The images collected from the samples supplemented with 2D standards were subsequently analysed using Melanie 3 (Swiss Institute of Bioinformatics, Geneva), a computer software package specifically designed for the analysis of 2D gels. Initially the location of the standards were identified (table 5.3.5.2.b.) and marked (figure 5.3.5.2.d.), thus allowing the gels to be calibrated.

Table 5.3.5.2.b. Identification of standards separated by 2D electrophoresis. The standards, and their designated isoforms, were located on the gels and marked for future reference.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Standard ID</th>
<th>pl</th>
<th>MW kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>albumin</td>
<td>A</td>
<td>6.60</td>
<td>76.00</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.30</td>
<td>76.00</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6.00</td>
<td>76.00</td>
</tr>
<tr>
<td>conalbumin</td>
<td>D</td>
<td>5.60</td>
<td>66.20</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>5.40</td>
<td>66.20</td>
</tr>
<tr>
<td>actin</td>
<td>F</td>
<td>5.10</td>
<td>43.00</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>5.00</td>
<td>43.00</td>
</tr>
<tr>
<td>GAPDH</td>
<td>H</td>
<td>8.50</td>
<td>36.00</td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>I</td>
<td>6.00</td>
<td>31.00</td>
</tr>
<tr>
<td>trypsin inhibitor</td>
<td>J</td>
<td>4.50</td>
<td>21.50</td>
</tr>
<tr>
<td>myoglobin</td>
<td>K</td>
<td>7.00</td>
<td>17.50</td>
</tr>
</tbody>
</table>

Following the identification of the standards, other proteins present on the gels were analysed. The gel loaded with unconditioned SFM did not express any other significant protein spots, therefore no further analysis was required. The gel loaded with 7 DIV SF-CM was found to contain 112 separate significant spots using the Melanie software (figure 5.3.5.2.e.). Each of these proteins were analysed and its pl and MW were calculated by comparison to the position of the defined standards on the gel (table 5.3.5.2.c). Furthermore, normalised values, % optical density (% OD) and % volume (% vol), both referring to amount of protein in a spot, were also retrieved.
Figure 5.3.5.2.d. **Identification of standards.** Images from SYPRO Ruby stained gels were analysed using Melanie software to locate the position of the 2D standards on (A) 2D standard only gel, (B) SFM containing 2D standards gel and (C) 7 DIV SF-CM containing 2D standards gel. Standards are highlighted and labelled A-K as described table 5.3.5.2.b.

A. 2D standard only  
B. SFM + 2D standard  
C. 7 DIV SF-CM + 2D standard
Figure 5.3.5.2.e. Analysis of proteins in 7 DIV SF-CM. The SYPRO Ruby stained 7 DIV SF-CM gel, containing 2D samples, was analysed using Melanie software to locate the position of all significant protein spots present. Each protein spot was assigned an ID, numbered 1-112, and its pI and MW were calculated by comparison to the position of the standards. The positions of the standards are marked for reference, but not included in the 112 proteins identified.
Table 5.3.5.2.c. Identification and analysis of protein spots in 7 DIV-SFCM 2D gels, visualised using SYPRO Ruby staining.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>% OD</th>
<th>% Vol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.70</td>
<td>76.00</td>
<td>0.66</td>
<td>0.24</td>
</tr>
<tr>
<td>2</td>
<td>5.66</td>
<td>76.00</td>
<td>0.63</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>5.66</td>
<td>76.00</td>
<td>0.68</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>5.70</td>
<td>76.00</td>
<td>0.71</td>
<td>0.23</td>
</tr>
<tr>
<td>5</td>
<td>5.26</td>
<td>76.00</td>
<td>0.72</td>
<td>1.40</td>
</tr>
<tr>
<td>6</td>
<td>4.86</td>
<td>76.00</td>
<td>0.80</td>
<td>0.09</td>
</tr>
<tr>
<td>7</td>
<td>5.70</td>
<td>76.00</td>
<td>0.76</td>
<td>0.40</td>
</tr>
<tr>
<td>8</td>
<td>5.66</td>
<td>76.00</td>
<td>0.70</td>
<td>0.28</td>
</tr>
<tr>
<td>9</td>
<td>4.55</td>
<td>76.00</td>
<td>0.73</td>
<td>0.77</td>
</tr>
<tr>
<td>10</td>
<td>5.70</td>
<td>76.00</td>
<td>0.67</td>
<td>0.49</td>
</tr>
<tr>
<td>11</td>
<td>5.65</td>
<td>76.00</td>
<td>0.68</td>
<td>0.36</td>
</tr>
<tr>
<td>12</td>
<td>5.24</td>
<td>76.00</td>
<td>0.74</td>
<td>0.82</td>
</tr>
<tr>
<td>13</td>
<td>4.92</td>
<td>76.00</td>
<td>0.70</td>
<td>0.13</td>
</tr>
<tr>
<td>14</td>
<td>6.83</td>
<td>90.80</td>
<td>0.64</td>
<td>0.13</td>
</tr>
<tr>
<td>15</td>
<td>5.60</td>
<td>83.56</td>
<td>1.01</td>
<td>0.39</td>
</tr>
<tr>
<td>16</td>
<td>5.37</td>
<td>84.56</td>
<td>0.90</td>
<td>0.58</td>
</tr>
<tr>
<td>17</td>
<td>5.65</td>
<td>82.88</td>
<td>1.01</td>
<td>0.88</td>
</tr>
<tr>
<td>18</td>
<td>5.00</td>
<td>82.58</td>
<td>0.70</td>
<td>0.19</td>
</tr>
<tr>
<td>19</td>
<td>5.83</td>
<td>81.61</td>
<td>1.00</td>
<td>0.70</td>
</tr>
<tr>
<td>20</td>
<td>5.95</td>
<td>76.00</td>
<td>1.74</td>
<td>3.76</td>
</tr>
<tr>
<td>21</td>
<td>8.55</td>
<td>79.69</td>
<td>0.53</td>
<td>0.21</td>
</tr>
<tr>
<td>22</td>
<td>6.53</td>
<td>76.91</td>
<td>1.57</td>
<td>1.44</td>
</tr>
<tr>
<td>23</td>
<td>6.20</td>
<td>76.00</td>
<td>1.91</td>
<td>2.90</td>
</tr>
<tr>
<td>24</td>
<td>4.67</td>
<td>76.00</td>
<td>1.01</td>
<td>0.82</td>
</tr>
<tr>
<td>25</td>
<td>4.39</td>
<td>66.20</td>
<td>1.54</td>
<td>8.23</td>
</tr>
<tr>
<td>26</td>
<td>5.90</td>
<td>70.93</td>
<td>1.47</td>
<td>0.59</td>
</tr>
<tr>
<td>27</td>
<td>5.85</td>
<td>70.93</td>
<td>1.33</td>
<td>0.66</td>
</tr>
<tr>
<td>28</td>
<td>5.35</td>
<td>72.58</td>
<td>1.25</td>
<td>0.56</td>
</tr>
</tbody>
</table>

... (continued with similar entries for other protein IDs)
The Melanie 3 software is a sophisticated 2Dgel image analysis software package that is able to automate the identification, quantification and comparison of 2D samples. Melanie is an acronym for medical electrophoresis analysis interactive expert, and was developed by the Swiss Institute of Bioinformatics (SIB) under the guidance of Professor Hochstrasser at the Geneva University Hospital’s Central Clinical Chemistry Laboratories. The software functions by implementing a number of algorithms (Appel et al., 1997b) to provide efficient spot detection. The package is also linked to proteome databases available on the World Wide Web thus allowing protein identification (Appel et al., 1997a). Unfortunately the licensed copy of this software used to analyse the 2D gels presented in this thesis, namely that at the Ludwig Institute University College London, had technical difficulties and as a result the automated identification of proteins could not be performed.

However a manual search of a single protein database was performed which resulted in a number of possible proteins being identified. This was carried out by generating a list of proteins close to a given molecular weight and pH by searching the SWISS-PROT database using the TagIdent Tool on the ExPASy homepage (www.expasy.ch/tools/tagident.html). As the results has previously shown that the active factor had a molecular weight of < 30 kDa the search was performed on only those proteins identified on the 2D gel with a molecular weight < 40 kDa. MW and pH values were entered and a search was performed which would return all proteins within the given range. The MW ranges investigated were MW ± 1 % whilst the pH ranges were ± 0.25 pH units. Only those proteins reported from the rat were chosen to be returned. As anticipated a large number of possible proteins were identified for each spot, a summary of which is provided (table 5.3.5.2.d.).
Table 5.3.5.2.d. Identification of proteins using SWISS-PROT database. Molecular weights and isoelectric points of proteins identified on the 2D gels using Melanie 3 software were searched in the SWISS-PROT database using the Tagldent. The scan produced a number of proteins within the specified ranges, examples of which are given. **Abbreviations:** bFGF, basic fibroblast growth factor; CRH-BP, corticotropin-releasing factor binding protein; DAD2, defender against cell death 2; DH, dehydrogenase; ECGF, endothelial cell growth factor; eIF-6, Eukaryotic translation initiation factor 6; GADD45, growth arrest and DNA-damage inducible protein; GDF-1, embryonic growth/differentiation factor 1; HSF, Heat Shock Factor; IL, interleukin; KCIP-1, protein kinase C inhibitor protein; KIP2, kinase interacting protein-2; NPDC, neural proliferation and control protein precursor; PDGF, platelet derived growth factor; PLA1, phospholipase A1; PNMTase, phenylethanolamine N-methyltransferase; PtdlnsTP, Phosphatidylinositol transfer protein; Rgs-1, regulator of g-protein signalling; Rho GDI-1, Rho GDP-dissociation inhibitor-1; TGF-β, transforming growth factor-beta; TNF, tumor necrosis factor, V-CAM, vascular adhesion protein; WT1-associated protein, Wilms tumor 1-associating protein.

<table>
<thead>
<tr>
<th>protein ID</th>
<th>number of proteins identified</th>
<th>examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>44</td>
<td>Lipocortin, HSF2, CD34, CD44, CD34, MHC class I antigen</td>
</tr>
<tr>
<td>58</td>
<td>39</td>
<td>PDGF, TNF-10</td>
</tr>
<tr>
<td>59</td>
<td>31</td>
<td>Lipocortin, TNF-10, IFN-response binding factor</td>
</tr>
<tr>
<td>60</td>
<td>31</td>
<td>CD1d2, lactate DH, malate DH</td>
</tr>
<tr>
<td>61</td>
<td>30</td>
<td>Lipocortin, TNF-10, pyruvate DH, growth arrest protein-1</td>
</tr>
<tr>
<td>62</td>
<td>34</td>
<td>Lipocortin, IL-12, INF-γ, estrogen sulfotransferase</td>
</tr>
<tr>
<td>63</td>
<td>28</td>
<td>presenilin 1, CRH-BP, TNF-9, HSF6</td>
</tr>
<tr>
<td>64</td>
<td>27</td>
<td>TNF-9, CRH-BP</td>
</tr>
<tr>
<td>65</td>
<td>22</td>
<td>CRH-BP, malate DH, myeloid differentiation protein</td>
</tr>
<tr>
<td>66</td>
<td>18</td>
<td>Cyclin D3, ketohexokinase</td>
</tr>
<tr>
<td>67</td>
<td>12</td>
<td>TNF-10, Complement C4 precursor</td>
</tr>
<tr>
<td>68</td>
<td>11</td>
<td>Cyclin D3, neurotrimin precursor, survival motor neuron protein</td>
</tr>
<tr>
<td>69</td>
<td>20</td>
<td>V-CAM 1 precursor</td>
</tr>
<tr>
<td>70</td>
<td>20</td>
<td>NPDC-1, TNF-13, survival motor neuron protein</td>
</tr>
<tr>
<td>71</td>
<td>20</td>
<td>NPDC-1, TNF-13, survival motor neuron protein</td>
</tr>
<tr>
<td>72</td>
<td>20</td>
<td>F-actin, survival motor neuron protein</td>
</tr>
<tr>
<td>73</td>
<td>24</td>
<td>NADH-cytochrome b5 reductase, esterase D, PtdlnsTP</td>
</tr>
<tr>
<td>74</td>
<td>13</td>
<td>NPDC-1, TNF-13, survival motor neuron protein</td>
</tr>
<tr>
<td>75</td>
<td>22</td>
<td>NPDC-1, TNF-13, survival motor neuron protein</td>
</tr>
<tr>
<td>76</td>
<td>17</td>
<td>PLA1, survival motor neuron protein</td>
</tr>
<tr>
<td>protein ID</td>
<td>number of proteins identified</td>
<td>examples</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>77</td>
<td>12</td>
<td>Carbonyl reductase, PNMTase</td>
</tr>
<tr>
<td>78</td>
<td>22</td>
<td>IL-2, phosphoglycerate mutase</td>
</tr>
<tr>
<td>79</td>
<td>22</td>
<td>Lipocortin precursor, stem cell factor</td>
</tr>
<tr>
<td>80</td>
<td>36</td>
<td>KCIP-1, cathepsin B, stem cell factor</td>
</tr>
<tr>
<td>81</td>
<td>22</td>
<td>Lipocortin precursor, cathepsin B, stem cell factor</td>
</tr>
<tr>
<td>82</td>
<td>19</td>
<td>Plasminogen activator, lipocortin precursor</td>
</tr>
<tr>
<td>83</td>
<td>16</td>
<td>Lipocortin precursor, trypase precursor</td>
</tr>
<tr>
<td>84</td>
<td>10</td>
<td>eIF-6</td>
</tr>
<tr>
<td>85</td>
<td>32</td>
<td>TNF, cathepsin B</td>
</tr>
<tr>
<td>86</td>
<td>66</td>
<td>Rgs-1, super oxide dismutase, trypsin precursor</td>
</tr>
<tr>
<td>87</td>
<td>53</td>
<td>HSF26, acetylcholine-binding protein precursor</td>
</tr>
<tr>
<td>88</td>
<td>24</td>
<td>Beta casein precursor Rho GDI-1</td>
</tr>
<tr>
<td>89</td>
<td>9</td>
<td>EGF precursor, brain acid soluble protein-1, KIP-2</td>
</tr>
<tr>
<td>90</td>
<td>20</td>
<td>IL-1α precursor, IL-18 precursor, WT1-associated protein</td>
</tr>
<tr>
<td>91</td>
<td>16</td>
<td>Bcl-2 interacting killer protein, GADD45</td>
</tr>
<tr>
<td>92</td>
<td>6</td>
<td>IL-1β precursor, IL-20 precursor, TNF-α precursor, bFGF</td>
</tr>
<tr>
<td>93</td>
<td>6</td>
<td>TNF-α precursor, IL-1β precursor, IL-20 precursor, ECGF</td>
</tr>
<tr>
<td>94</td>
<td>3</td>
<td>IL-3, transcription factor B3</td>
</tr>
<tr>
<td>95</td>
<td>17</td>
<td>TNF-α precursor, IL-1 precursor, caspase 2 precursor</td>
</tr>
<tr>
<td>96</td>
<td>8</td>
<td>Galactose-binding lectin precursor</td>
</tr>
<tr>
<td>97</td>
<td>15</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>98</td>
<td>14</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>99</td>
<td>16</td>
<td>TGF-β, NTF-2</td>
</tr>
<tr>
<td>100</td>
<td>18</td>
<td>Myoglobin, retinoic acid binding protein, super oxide dismutase</td>
</tr>
<tr>
<td>101</td>
<td>5</td>
<td>Fatty acid-binding protein</td>
</tr>
<tr>
<td>102</td>
<td>16</td>
<td>IL-2 precursor, lactophorin precursor, superoxide dismutase</td>
</tr>
<tr>
<td>103</td>
<td>14</td>
<td>IL-3 precursor, multipotent colony-stimulating factor</td>
</tr>
<tr>
<td>104</td>
<td>16</td>
<td>Chromogranin A precursor, beta-galactoside-binding lectin, PLA2</td>
</tr>
<tr>
<td>105</td>
<td>13</td>
<td>PLA2 precursor, astrocytic phosphoprotein</td>
</tr>
<tr>
<td>106</td>
<td>7</td>
<td>PLA2 precursor, neuronal protein</td>
</tr>
<tr>
<td>107</td>
<td>13</td>
<td>IL-15 precursor, PLA2 precursor</td>
</tr>
<tr>
<td>108</td>
<td>14</td>
<td>Cytokine C10, PLA2 precursor, IL-15 precursor</td>
</tr>
<tr>
<td>109</td>
<td>14</td>
<td>Gonadotropin precursor, PLA2 precursor</td>
</tr>
<tr>
<td>110</td>
<td>8</td>
<td>TGF-β2 precursor, PLA2 precursor</td>
</tr>
<tr>
<td>111</td>
<td>6</td>
<td>Gonadotropin precursor</td>
</tr>
<tr>
<td>112</td>
<td>13</td>
<td>TGF-β2 precursor, DAD2, TGF-β1 precursor, GDF-1 precursor</td>
</tr>
</tbody>
</table>
5.4. DISCUSSION

To determine the nature of the diffusible factors, released by microglia into culture medium, which were able to enhance neuronal survival and proliferation, the effects of heat, acidification and protease digestion were investigated. Each of these modifications reduced the capacity of the MG-CM to enhance neuronal survival (figure 5.3.1. A), however these conditions also prevent survival in cultures treated with unconditioned medium (figure 5.3.1. A). Such results indicate that the modifications were not only affecting those factors released by the microglia, but also other factors present in the medium. Therefore it was impossible to determine if modification of microglial-derived factors was able to impair their function. To circumvent this problem the same modifications were carried out on samples of serum-free medium (SFM) and microglial-conditioned serum-free medium (SF-CM). In this set of experiments it was observed that heat and protease digestion had no effect on the level of survival in unconditioned SFM treated cultures (figure 5.3.1. B). However in SF-CM treated cultures the survival was abated to levels comparable with control following both heat and protease digestion (figure 5.3.1. B). These results indicate that the microglial-derived factors are both heat and protease sensitive. Acidification of both SFM and SF-CM negated neuronal survival to comparable levels (figure 5.3.1. B), indicating that acidification was not permissible to survival. Taken together these results suggest that the factors released by microglia, as identified in this study, are likely to be protein.

To further explore the nature of the released proteins MG-CM samples were fractionated using microconcentrators with molecular weight cut-offs of > 300 kDa, 100 - 300 kDa, 30 - 100 kDa and < 30 kDa. This analysis revealed that 1 DIV MG-
CM and 7 DIV MG-CM contain a number of factors which exert agonising and antagonising effect on both neuronal survival and proliferation (figure 5.3.2.). In both cases, the < 30 kDa fraction was able to promote both neuronal survival and proliferation to levels associated with the related whole conditioned medium. These results indicate that the primary active factor(s) has a molecular weight of < 30 kDa. Such a molecular weight suggests that the factor(s) could be a growth factor or cytokine, as these are small molecular weight proteins. Such a hypothesis can be corroborated by the fact that it is well established that microglia release a plethora of growth factors and cytokines (see section 1.2.5.2.).

A number of the growth factors released by microglia have established effects on neuronal survival and proliferation therefore the effect of immunoprecipitating these growth factors out of the MG-CM was assessed. Primary antibodies against bFGF, EGF, PDGF and TGF-β1 were added to sample of 7 DIV MG-CM and fresh medium, in conjunction with protein A/G agarose beads. The samples were incubated overnight at 4°C with constant rotation. The samples were subsequently centrifuged to pellet the beads and the resulted depleted medium samples were added to neurones and their survival assessed. The removal of the growth factors had minimal effect on cultures treated with depleted fresh medium (figure 5.3.4.a. Bi), however their removal from 7 DIV MG-CM abated the effects normally associated with the conditioned-medium (figure 5.3.4.a. Bii). Removal of PDGF had the most marked effect, whilst removal of EGF and bFGF compromised survival levels to approximately 70 % below that normally observed. Removal of TGF-β1 has the least effect, but neuronal survival was still conceded to approximately 50 % below that normally observed.
To further assess the impact of growth factors, antibodies against the extracellular domains of growth factor receptors were added to inhibit their activity on the neurones. The neurones were subsequently treated with either fresh medium of 7 DIV MG-CM and neuronal survival was assessed. The inhibition of the growth factor receptors investigated had no effect on control cultures (figure 5.3.4.b. A), however in MG-CM treated cultures a dose response effect was observable (figure 5.3.4.b. B). The inhibition of the PDGF receptor had maximal effect, able to revert survival of MG-CM treated cultures back to control levels, even at a low dose, 0.02 μg/ml antibody. Blockade of the EGF receptor also caused survival levels to be depleted, though 10 times as much antibody was required to achieve this effect.

Taken together this data suggests that removal of growth factors from the microglial-conditioned medium, or the blockade of growth factor receptors present on the neurones, is able to remove the capacity of the conditioned medium to enhance neuronal survival. Other studies that have observed an effect of microglial-derived factors on neuronal survival have tried comparable investigations to determine the factor functioning under their specific experimental conditions. For example the microglial-derived neurotrophic factor described by Nagata et al (1993) was determined not to be NGF, IL-1β, IL-6 or TNF-α.

This experimental approach relies on prior knowledge of factors released by microglia. An alternative and complimentary method of identification was also employed in this thesis to try to determine the factors of interest, namely the use of 2D protein electrophoresis. The utilisation of this method allowed proteins to be separated not only according to their molecular weight (MW) but also according to their isoelectric
point (pI). Once determined, such data can be used to cross reference all described proteins present on proteomic databases. During the course of this thesis samples of whole 7 DIV SF-CM were concentrated to approximately 1/25 of its original volume and separated according to the described method (section 2.2.21.). Once completed the separated proteins were visualised with either ammonical silver nitrate (figure 5.3.5.2.a.), or the fluorescent dye, SYPRO Ruby (figure 5.3.5.2.b. - figure 5.3.5.2.e.). This methodology confirmed the presence of a large number of proteins, and analysis of images using the Melanie software was able to identify 112 separate proteins (table 5.3.5.2.c.). As total 7 DIV SF-CM was assessed, a number of the proteins identified had molecular weights greater than 30 kDa. Each of the proteins was ascribed an ID and its pI and MW were calculated. Although the automated cross referencing of these proteins using the Melanie 3 software could not be completed, a manual search of a single database (SWISS-PROT) was performed, which identified a large number of protein possibilities for each protein spot assessed. As previous results had shown that the active factor(s) responsible for the survival-enhancing effect of MG-CM had a molecular weight of $<30$ kDa, only those protein spots with an ascribed molecular weight of $<40$ kDa, to allow for any inaccuracies of the fractionation studies, were investigated in this manual database search.

These searches revealed a number of interesting possible identities for the proteins, in particular a number of growth factors were suggested. The immunodepletion studies revealed that removal of PDGF and to a lesser extent bFGF, EGF and TGF-β1, all of which have known effects on neuronal survival and proliferation (Shimojo et al., 1991; Nagata et al., 1993; Kreutzberg, 1996), resulted in a diminished capacity for the MG-CM to enhance neuronal survival, and each of these growth factors were found on the
database search results for protein ID 58, 92, 89 and 99 respectively. A number of interleukins were also identified, including IL-1 and -12, both of which have been shown to be released by microglia and have beneficial effects on neuronal survival (Giulian et al., 1986; Nagai et al., 2001).

These results imply that further assessment of the proteins in MG-CM by this method would be beneficial, and perhaps could concentrate on the use of fractionated conditioned-medium. Furthermore the extended assessment of the MW and pI values, using other database, may yield more possibilities. In addition the protein spots could be enzymatically excised and the resulting peptides analysed and sequenced by mass spectroscopy (Shevchenko et al., 1996). This data could in turn facilitate the identification of the proteins.
6. **GENERAL DISCUSSION**
6.1 EVALUATION OF TISSUE CULTURE

In this thesis, the effects of microglial-derived diffusible factors on neuronal survival and proliferation have been explored using a cell culture model. The main advantage of using this system is that it has permitted the responses of an individual neuronal type to be dissected, both under control conditions and following the addition of microglial-conditioned medium. This method has also been permissive to the study of the mechanisms of action by which the microglial-derived factors have affected neuronal survival and proliferation. Furthermore the use of cell culture has allowed these effects to be observed on live, developing neurones. An alternative approach would have been to use post mortem tissue to perhaps mimic the \textit{in vivo} situation more closely. Such an approach however would itself have had problems as it takes a considerable length of time to collect and prepare post mortem tissue during which time a number of cell changes can occur. For example, if medium had been conditioned by post mortem microglial then the factors released would most likely have been different, as enhanced microglial activation may have occurred.

A number of different methods can be employed to isolate cultures of single cells. Microglial primary cultures for example can be retrieved from mixed glial cultures, or by methods involving enzyme dissociation or homogenisation. The retrieval of microglial from mixed glial populations is a long established method, however this method involves extended period of time in culture, indeed such cultures are typically maintained for up to two weeks prior to microglial isolation by mild shaking and centrifugation (Giulian et al., 1986; Théry et al., 1991; Boje and Arora, 1992; Nagata et al., 1993; Minghetti et al., 1997; Polazzi et al., 2001). Such a method may not be optimal, as it has been established that microglia become increasingly activated during
time in culture (Slepko and Levi, 1996). Furthermore the interactions of microglia with other glial cells during the period in mixed culture may alter the properties and characteristics of the microglia, and therefore may differ from microglia freshly isolated from the brain or indeed in vivo microglia.

Alternatively microglia can be isolated by enzymatically dissociating them from other cell types (Ford et al., 1995; Slepko and Levi, 1996; Kingham et al., 1999; Zietlow et al., 1999). This method, however, raises concern as to whether the enzyme dissociation may in fact alter the membrane lipid structure of the microglia and thus affect the microglial properties (Havenith et al., 1998).

More recently microglial isolation from other cell types has been achieved using homogenisation of whole tissue followed by separation on a discontinuous percoll gradient (Kingham and Pocock, 2000 and 2001; Taylor et al., 2002). Although microglia isolated by this method are temporarily activated by the action of homogenisation, when left in culture they assume a more resting phenotype and indeed, as shown in this study, express downregulated levels of classical markers for activated microglia. Furthermore, these microglia behave more like those in vivo, as they have been shown to respond to known activators by altering their morphology and by the release of toxic factors (Kingham and Pocock, 2000; Taylor et al., 2002). This method also allows the isolation of microglia from adult as well as neonatal brains, and both respond similarly to stimuli, however the basal level of activation is lower in neonatal microglial cultures compared with adult microglial cultures. During the course of this thesis the characteristics of the microglial cultures were investigated to assess culture purity, and it was demonstrated, as had been previously shown.
(Kingham and Pocock, 2000), that the cultures had minimal contamination by other cell types.

The other major primary cell type utilised in this thesis was that of the cerebellar granule neurones (CGC). CGC cultures have been widely used to investigate neuronal development as their pattern of maturation is well characterised. Furthermore, CGC cultures are homogeneous neuronal cultures with minimal glial contamination. The cultures themselves are relatively easy to prepare and maintain in culture, where they migrate and exhibit a fully differentiated appearance after 5-7 days in culture (figure 6.1.; Kingsbury et al., 1985). For this reason neuronal cultures were only used after 7 days in this thesis.

**Figure 6.1. Development of cerebellar granule neurones in culture**

![Development of cerebellar granule neurones in culture](image)

Although cell culture models are widely used, there is still concern that they do not represent the true 3-dimensional cellular architecture of the brain with all its complexities of cell-cell interactions and plethora of diffusible factors. Additionally, though cell culture medium is required to support cell growth it does contain components, such as serum, which would normally be absent *in vivo*. As discussed serum contains hundreds of undefined components, therefore to compensate for this
experiments in this study were also performed using a specialised serum-free culture medium. Despite their limitations, cell culture models are essential and useful tools to explore cell signalling in ways that would not be possible in vivo as such cultures allow individual cell types to be investigated.

6.2. MICROGLIAL-NEURONAL INTERACTIONS

Microglia exert a number of effects on neurones; microglial release of soluble factors may influence neuronal signalling (Giulian et al., 1993), and also support neuronal survival (Nakajima et al., 1989; Nagata et al., 1993; Jonakait et al., 1996; Zhang and Fedoroff, 1996; Wantanabe et al, 2000; Polazzi et al., 2001) through the release of factors such as NGF, TGF-β1, PDGF, bFGF and IL-2, -6 and -12 (see Barron, 1995).

However once activated, the role of microglia alters and they release factors, including as NO (Chao et al., 1992; Boje et al., 1992), glutamate (Piani et al., 1991), quinolinic acid (Heyes et al., 1996), TNF-α and IL-1β (Giulian et al., 1989, 1993) which may be neurotoxic (Kingham et al., 1999). Microglial activation is also likely to be a reactive process, where microglia respond to signals originating from injured neurones (Bruce-Keller, 1999; Streit et al., 1999). Under such circumstances microglia activation may be beneficial as it is thought that the microglia induce the production of trophic factors to support neuronal regeneration. Furthermore, it is postulated that irretrievably damaged neurones release factors which facilitate their rapid removal by microglia, and thus prevents the death of bystanding neurones (Streit et al., 1999).

It would therefore seem likely that it is the level of microglia activation that determines the role of microglia in maintaining the homeostasis of the brain (Keutzberg, 1996).
and there may well be a fine line between microglial generation of survival-promoting and death promoting signals.

6.3. FUTURE WORK

In this thesis the effect of diffusible microglial-derived factors on neuronal survival and proliferation was investigated. Although it was found that a wide variety of signal transduction pathways were likely to involved the identity of the factor(s) was not established. Furthermore it was found that the factor(s) were proteins and had a molecular weight of < 30 kDa, suggesting the possibility of a growth factor or cytokine identity. The characterisation of the factor(s) using 2D gel electrophoresis revealed the presence of in excess of 100 proteins in the conditioned medium it would therefore be of interest to further investigate the nature of these. The premise of these investigations using 2D electrophoresis had been to cross reference the identified proteins on the established proteomic databases however due to unforeseen circumstances this couldn’t be performed and only a single database was assessed. This search did however yield interesting and promising results so would serve further attention. Furthermore, excision of protein spots from the 2D gels would yield samples, which could be further characterised using mass spectroscopy, resulting in sequence information, which would facilitate the identification of the proteins released. It would also be of interest to analyse neuronal gene expression following exposure to microglial derived soluble factors, which may help isolated primary routes of actions of the diffusible factors.

This investigation limited itself to examining the effects of long lasting diffusible factors released by microglia on neurones (figure 6.3. A). An expansion of this project
could also include the investigation of short lasting diffusible factors released by microglia through the use of tissue culture plate well inserts. Such inserts typically contain a barrier that allows unidirectional diffusible communication between two cellular populations. Using this method the effect of neuronal factors on microglial survival could be assessed as well as the affect of microglial factors on neuronal survival (figure 6.3. B). This could in turn be further developed by selectively enriching neuronal cultures with microglia, to create a more in vivo-like situation, and assessing the outcome (figure 6.3. C).

Figure 6.3. Experimental protocols to investigate microglial-neuronal interactions.

A. Effect of long-lasting microglial factors

B. Effect of short lived microglial or neuronal factors

C. Effect of bi-directional communication
6.4. GENERAL CONCLUSIONS

In summary, it is likely that microglia in vitro and in vivo can exert neurotrophic and neurotoxic functions depending on the microenvironment of the brain. The data presented in thesis has identified that diffusible factors released by unstimulated microglia into culture medium have the capacity to enhance neuronal survival and proliferation. A wide number of pathways are likely to be involved in its action including the Notch cascade, Trk receptor signalling via both MAPK and PI3K/Akt, AMPA ionotropic glutamate receptors and a G-protein coupled receptor negatively linked to adenylate cyclase. The factor(s) is a protein and is likely to be a growth factor, with a molecular weight of < 30 kDa. The identification remains unresolved though the actions of PDGF, EGF and bFGF appear important.
7. REFERENCES


208


280


Pocock, J. M., Cousin, M. A., and Nicholls, D. G. (1993). The calcium channel coupled to the exocytosis of L-glutamate from cerebellar granule cells is inhibited by the spider toxin Aga-GI. *Neuropharmacol.* 32, 1185-1194.


